Fluoroquinolone Resistance in

Acinetobacter baumannii

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Abstract

Acinetobacter baumannii is increasingly being isolated in the nosocomial environment where it is a cause of pneumonia, bacteremia, meningitis and urinary tract infections, particularly in immunocompromised patients, although there are reports of it being the causative agent in community-acquired pneumonias. Prior use of antimicrobials is a risk factor for infection and the use of the fluoroquinolone ciprofloxacin has been associated with the selection of fluoroquinolone-resistant strains in the clinical environment.

Ciprofloxacin has been in clinical use since the 1980s and has good activity against Gram negative organisms but it is being superseded by a new generation of fluoroquinolones which retain good potency against Gram negative organisms but also exhibit enhanced activity against Gram positive organisms. The fluoroquinolones are bactericidal drugs that act on the essential cellular enzymes DNA Gyrase and Topoisomerase IV, subunits GyrA and ParC respectively. Resistance to this class of drug is mediated through mutations in the target enzymes. Studies with clinical isolates of *A. baumannii* have shown that a mutation in GyrA, specifically serine 83, is found in strains resistant to ciprofloxacin and in high-level resistant strains with a concurrent serine 80 mutation in ParC.

In this thesis, the anti-*Acinetobacter* activity of the newer fluoroquinolones are investigated by MIC and kill-curve and it is shown that they are 2-16-fold more potent than ciprofloxacin. The target enzymes GyrA and ParC are investigated by polymerase chain reaction, restriction fragment length polymorphism and sequencing. It was found that a serine 83 mutation in GyrA conferred resistance to ciprofloxacin and also to the newer fluoroquinolones except trovafloxacin. Strains exhibiting high-level resistance were found to carry a ParC mutation in serine 80 in addition to the GyrA mutation.

The selection of fluoroquinolone resistance *in vitro* was investigated by step-wise selection in order to investigate the mutational frequency and the development of resistance. It was found that ciprofloxacin selects for a GyrA mutation in one-step at a high frequency. The newer fluoroquinolones however required multiple steps of low frequency selection to acquire this mutation. ParC mutations were found not to be required for high-level resistance suggesting an alternative resistance mechanism.

Mutants were subcultured on antibiotic-free media and their fluoroquinolone MICs were re-tested to look for spontaneous revertants. A revertant was investigated by sequencing of *gyr*A and *par*C but no changes were found. Narrow-range MICs with non-fluoroquinolones showed small differences in MIC between parent, mutant and revertant suggesting changes in permeability. The outer membrane profile was investigated and a porin of 45.5kDa was found to have increased expression in the mutant and this level was lower in the revertant. The porin was extracted and N-terminally sequenced. It was found to have homology to porin D in *Pseudomonas aeruginosa*, a porin associated with imipenem resistance. The narrow-range MICs of imipenem followed this pattern.

These data suggest that the new fluoroquinolones are not only more potent than ciprofloxacin but that the development of resistance will be reduced once they are in clinical use. These data also demonstrate that GyrA is the primary target of the fluoroquinolones and that ParC mutations are not necessary to achieve high-level resistance.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated

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I would also like to thank Dr Richard Paul and Dr David Wallace, my biology teachers from my school days for their inspirational classes and opening my eyes to the world of living things.

Dedication

I would like to dedicate this thesis to the late Gabriele Cortopassi (1964-1998)

Publications and Presentations

1. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 1999. Anti-Acinetobacter activity of gemifloxacin (SB-265805) compared with 11 compounds by MIC. 39th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, USA.

2. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 1999. Bactericidal activity of gemifloxacin (SB-265805) and six comparator fluoroquinolones against *Acinetobacter baumannii.* 39th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, USA.

3. Higgins, P. G., Coleman, K., and Amyes, S. G. B. 2000. High level fluoroquinolone resistance in *Acinetobacter baumannii* requires a mutation in both *gyr*A and *par*C. In Programme and Abstracts of the 3rd European Congress of Chemotherapy, Madrid, Spain , Poster M114.

4. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 2000. High-level fluoroquinolone resistance in *Acinetobacter baumannii* is associated with increased expression of a 45.5kDa porin. 5th International Symposium on the Biology of *Acinetobacter.* Noordwijkerhout, The Netherlands.

5. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 2000. Bactericidal and bacteriostatic activity of gemifloxacin against Acinetobacter spp. in vitro. Journal of Antimicrobial Chemotherapy. 45:71-77.

6. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 2001. High-level fluoroquinolone resistance in *Acinetobacter baumannii* requires a mutation in GyrA but does not require a mutation in ParC with laboratory-bred mutants. 22nd International Congress of Chemotherapy. Amsterdam, The Netherlands.

7. Higgins, P. G., K. Coleman, and S. G. B. Amyes. 2001. Mutation rates of *Acinetobacter baumannii* against gemifloxacin, moxifloxacin and ciprofloxacin. 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. Chicago, USA.

Abbreviations

Acb .... *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex

AFLP .... amplified fragment length polymorphism

bp .... base pair

CCCP...................................... carbonyl cyanide *m*-chlorophenyl-hydrazone

DNA .... deoxyribonucleic acid

DNTP...................................... deoxynucleoside triphosphate

ds DNA................................... double stranded DNA

IST .... isosensitest

kDa .... kilo Daltons

MIC .... minimum inhibitory concentration

MQ... MilliQ pyrogen-free water

MW.. molecular weight

OBC....................................... optimum bactericidal concentration

PCR .... polymerase chain reaction

OMP .... outer membrane protein

PAGE.................................... polyacrylamide gel electrophoresis

PFGE .... pulsed-field gel electrophoresis

QRDR.................................... quinolone resistance determining region

RFLP .... restriction fragment length polymorphism

RNA...................................... ribonucleic acid

SDS....................................... sodium dodecyl sulphate

tRNA .... transfer RNA

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Chapter 1: Introduction

"We must learn to shoot microbes with magic bullets" – Paul Ehrlich

1.1 The Early Years of Drug Discovery

In 1902, Paul Ehrlich was shown how to stain the tubercule microbe by Robert Koch and so began an interest in the use of dyes to visualise cells and the career of a man who would revolutionise the way that chemotherapeutic agents would be discovered. After observing how methylene blue stained only the nerve endings in a live rabbit, he hit upon the idea that there must be one dye that will stain and kill microbes but not tissue from an animal. By chemically altering existing dyes, Ehrlich synthesised and tested hundreds of modified dyes, eventually one worked, preparation 606, Dioxy-diamino-arsenobenzol-dihydro-chloride, a compound which had anti-trypanosome activity and was also active against *Treponema pallidum*, the spirochete that is the causative agent of syphilis (de Kruif, 1926).

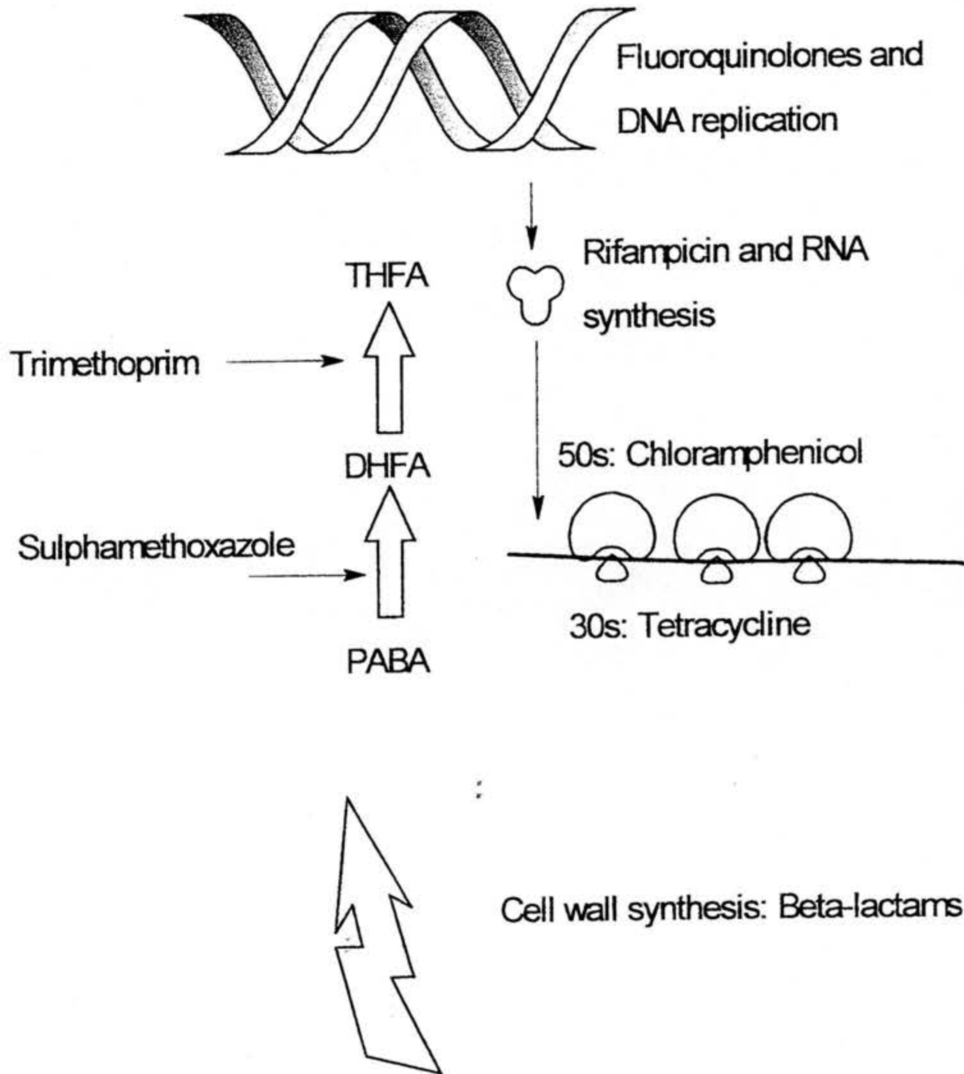
Ehrlich's idea, the structural modification of antimicrobials to increase activity, continues to this day. From the modification of the side-chains surrounding a β-lactam ring with cephalosporins and carbapenems, to the addition of a fluorine atom in the quinolones, billions of pounds are spent developing newer, more potent antimicrobials.

1.2 Classes of antibiotic

Antibiotics should posses certain properties to be of use to mankind: most notably that they should kill, or at the very least prevent microbes from replicating, without harming the host. Most do this by targeting bacterial metabolic pathways that do not exist in man; for example, cell-wall synthesis (Figure 1.1). Others have a higher affinity for the bacterial target such as the quinolones, with an affinity for bacterial topoisomerase that is more than 1000-fold greater than human topoisomerase (Koga *et al*, 1980) and trimethoprim, which inhibits mammalian dihydrofolate reductase 100,000-fold less efficiently than the bacterial enzyme. Bacterial but not mammalian RNA polymerase is targeted by rifampicin, preventing RNA synthesis. In the case of protein synthesis inhibitors, the aminoglycosides and macrolides target the 30S and 50S ribosome subunits of bacteria but not the eukaryotic 40S and 80S ribosomal subunits.

The penicillins, probably the most widely recognised class of antibiotics, are derivatives of the first chemotherapeutically effective drug, penicillin G. Discovered accidentally by Fleming in 1929, it was not until 10 years later that Chain and Florey embarked on purifying the active compound. In doing so, it was discovered that different media would produce variations of the drug of varying potency. Further developments came with the removal of the side-chain and replacing it with a variety of substituents to yield a semi-synthetic compound that was not acid-labile and thus could be administered orally. It was also resistant to some of the early β-lactamases (Stanier *et al*, 1987). This modification of the structure has continued and today we have β-lactam drugs with greater distribution through the body, increased half-life, greater resistance to β-lactamases and a broader spectrum of activity.

Figure 1.1. Drug targets



PABA: Para-aminobenzoic acid, DHFA:Dihydrofolic acid, THFA: Tetrahydrofolic acid

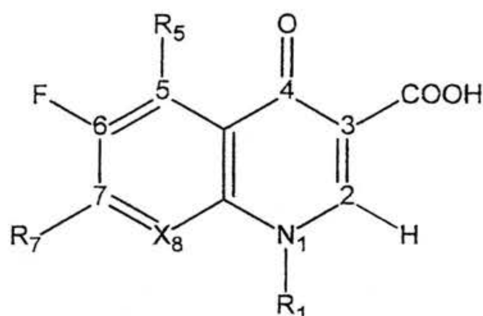
1.3 The Fluoroquinolones

The fluoroquinolones are a synthetic class of antimicrobial whose roots lie in the antimalarial drugs mepacrine and chloroquine (Wise, 2000). The first quinolone, nalidixic acid, was synthesised after it was noted that the by-product distillates in the manufacture of the anti-malarial chloroquine had antimicrobial properties (Lesher *et al*, 1962; Smith, 1984). Figure 1.2 shows the structure of nalidixic acid. It is a naphthyridine derivative that was used primarily for urinary tract infections owing to its poor serum and tissue concentration and narrow-spectrum of activity, which was confined to the Enterobacteriacae (Stamey *et al*, 1967).

Figure 1.2. Structure of Nalidixic acid

Further quinolone development yielded drugs with minor modifications to the nalidixic acid structure but these did not result in any major advance in antimicrobial spectrum or pharmacokintetic activity. It was not until Koga *et al*, (1980) improved absorption and activity with the addition of a fluorine at position 6 of the quinolone pharmacore (Figure 1.3) that the development of the class of drugs known as the fluoroquinolones took off. It should be noted that strictly speaking, C-6 fluorine substituted quinolone drugs with a nitrogen at position 8 are naphthyridones, while those with a carbon at this position are the "true" fluoroquinolones. However, for this thesis, all drugs with a fluorine at position 6 will be termed fluoroquinolones.

Figure 1.3. Fluoroquinolone pharmacore

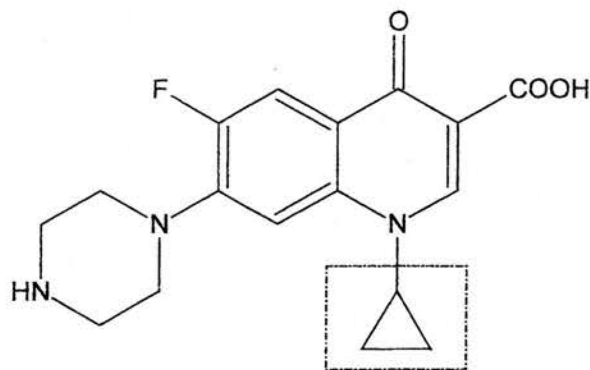


It has been estimated that as many as 10,000 analogues of nalidixic acid have now been investigated and much of this has focused on the fluoroquinolone pharmacore. The addition of various subgroups to this basic design will now be outlined. Some of the subgroups have not been possible to change without loss of basic activity. These are positions 2, 3, 4 and 6. Position 2 must be hydrogen as any larger molecule may create steric hindrance at positions 3 and 4. Position 3 and 4 must be a carboxyl and an oxygen as these are essential for hydrogen bonding to DNA bases (Shen *et al*, 1989a, 1989b) or chelation to DNA phosphates via a Mg^{2+} bridge (Palumbo *et al*, 1993). The fluorine at position 6 is essential for potency as it is 5-100x more active than any other group (Domagala, 1994).

1.3.1 Position 1

A cyclopropyl group at this position has been shown to control potency and have some effect on pharmacokinetics. It has been reported that this confers Gram negative activity and many fluoroquinolones having this substituent are active against Gram negative organisms, for example ciprofloxacin (Figure 1.4).

Figure 1.4. Ciprofloxacin



Other substituents include a 2,4-difluorophenyl moiety found in trovafloxacin and tosufloxacin (Figures 1.5 & 1.6) which are equal, or in some cases more potent than ciprofloxacin against Gram negative organisms. Clearly drug potency is not the result of a single parameter. Another structure at this position is found in levofloxacin which has a fused ring between positions 1 and 8 (Figure 1.7).

Figure 1.5. Trovafloxacin

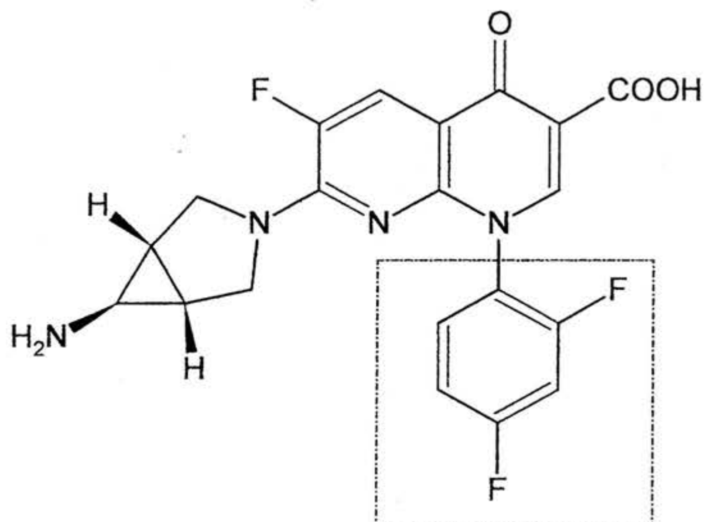


Figure 1.6. Tosufloxacin

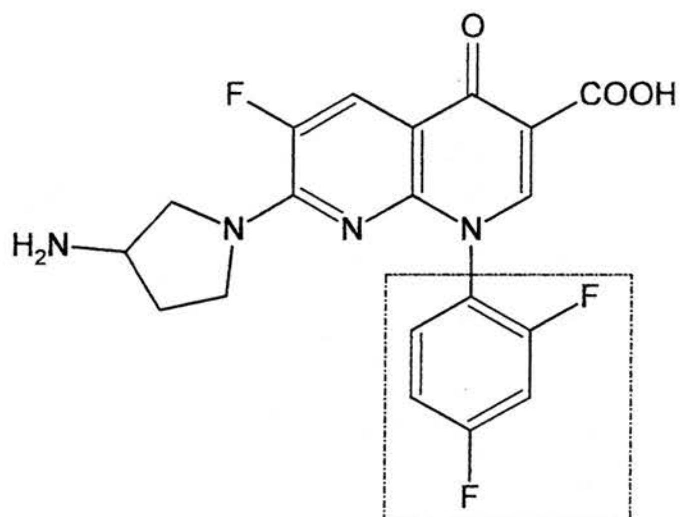
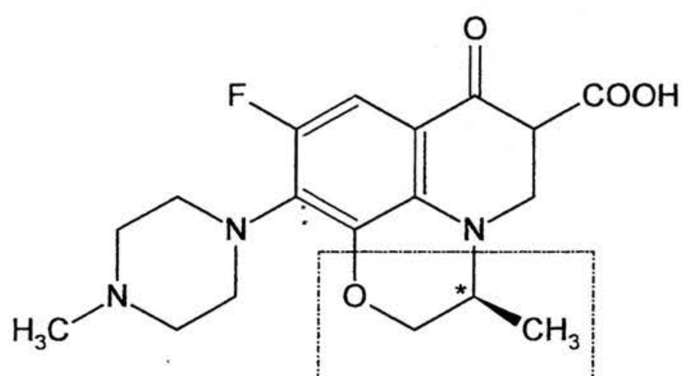


Figure 1.7. Levofloxacin



The rank order of potency is cited as cyclopropyl > 2,4-difluorophenyl > fused ring. An oxygen or nitrogen at this position reduce activity (Domagala, 1994).

1.3.2 Position 5

Substitutions at this position are thought to contribute to potency against Gram positive organisms (Domagala, 1994) for example sparfloxacin and grepafloxacin exhibiting increased activity (Figures 1.8 & 1.9). However there have been side-effects associated with these drugs and their C-5 substituents, leading to their withdrawal.

Figure 1.8. Sparfloxacin

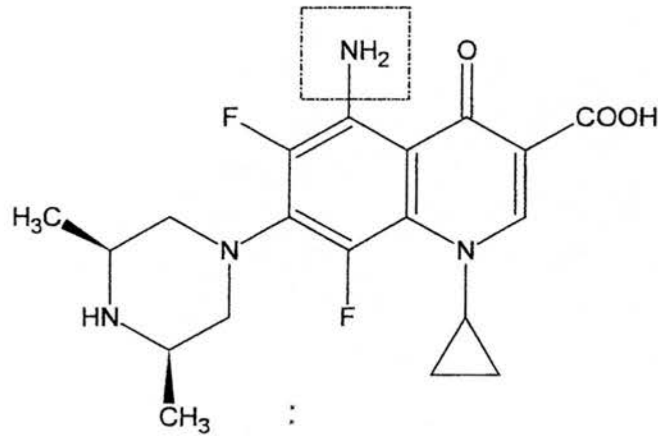
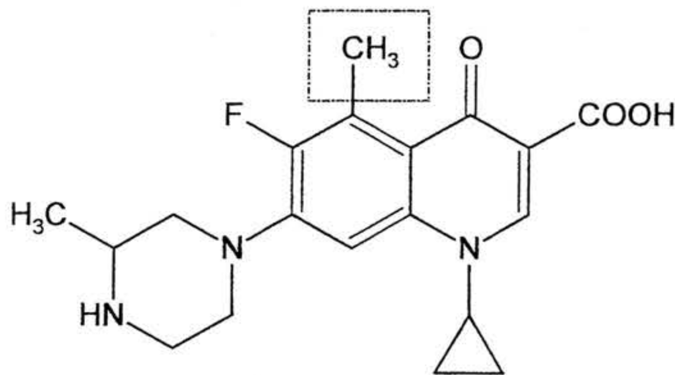


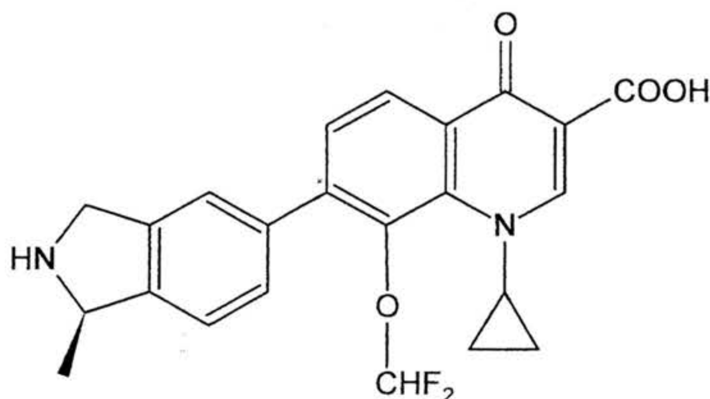
Figure 1.9. Grepafloxacin



1.3.3 Position 6

It was the addition of fluorine at this position giving greater absorption in the body and increased activity that has led to the development of this class of drug. The fluorine atom is responsible for the potency seen against DNA gyrase. However, there has been a recent interest in quinolones without a fluorine at this position (Lawrence *et al*, 2001). Compound BMS-340280 shows greater potency than the newer fluoroquinolone moxifloxacin against both sensitive and resistant Gram positive organisms thus casting doubt on the validity of the necessity of the C6 fluorine. BMS-340280 (Figure 1.10) does however have a di-fluoromethoxy substituent at position 8, although an analogue of this without the C-8 moiety is still as potent as moxifloxacin (Figure 1.12).

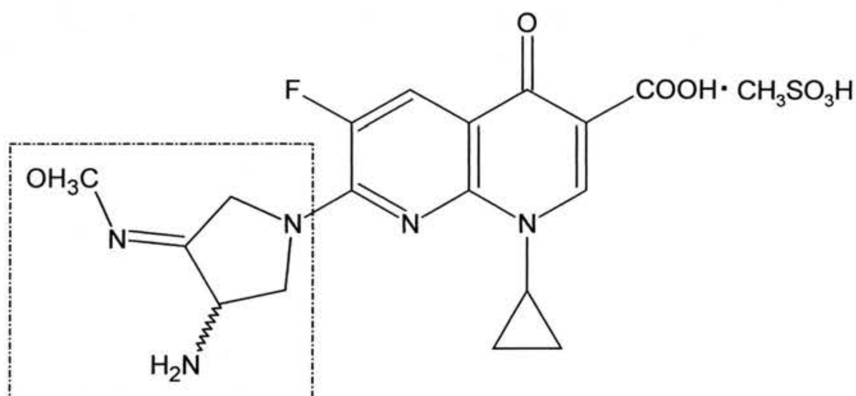
Figure 1.10. BMS-340280



1.3.4 Position 7

The C-7 substituent is the most adaptable site for chemical change. It determines potency and target preference in *Streptococcus pneumoniae* (Alovero *et al*, 2000) and is an area where much intensive research is focused. This area also controls the pharmacokinetic properties of the drugs, with a basic nitrogen required for oral efficacy. A five- or six-membered ring is the most commonly found substitution at position C-7, for example, gemifloxacin (Figure 1.11) and trovafloxacin (Figure 1.5), have an aminopyrrolidine substituent at C-7 compared to a methyl group in nalidixic acid (Figure 1.2). A piperazine substituent as seen with ciprofloxacin (Figure 1.4) confers Gram negative activity. A bulky C-7 substitution is associated with a reduction in active efflux (Pestova *et al*, 2000).

Figure 1.11. Gemifloxacin



1.3.5 Position 8

C-8 substitutions control pharmacokinetics and enhance anaerobe activity. A CF substitution is the most potent, with a methoxy substitution as seen with moxifloxacin and gatifloxacin conferring less activity (Figures 1.12 & 1.13). The naphthyridines are unsubstituted here (Gemifloxacin, Tosufloxacin and Trovafloxacin).

Figure 1.12. Moxifloxacin

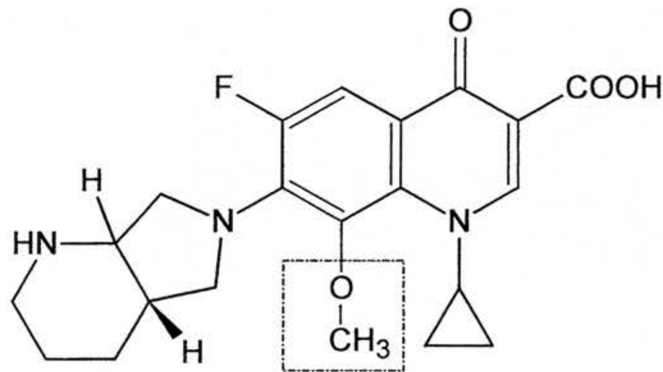
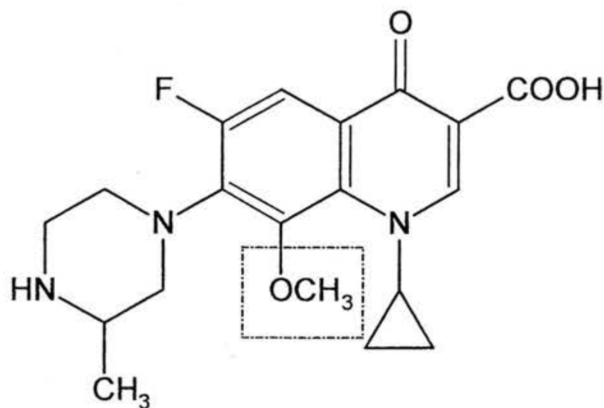


Figure 1.13. Gatifloxacin



Taken together, there are many claims as to what constitutes a potent fluoroquinolone, even the idea that the C-6 fluorine is necessary. Since much of the work done on these drugs is sponsored by pharmaceutical companies trying to market their product as the best, it is easy to see how the data becomes somewhat muddled. However, what is certain, is that positions 2, 3 and 4 will not be altered from the basic pharmacore (Figure 1.3) and position 5 substitutions are associated with toxicity. The remaining positions will continue to be developed within this class of drug. In so doing, the potency, pharmakokinetic and broad-spectrum activity of the fluoroquinolones will improve.

1.4 Bacterial Topoisomerases

A typical bacterium, if one such exists, is in the order of 2 x 1μm in dimension, with a chromosome of double stranded DNA which is 1300μm in length (Smith, 1986). In order for the DNA to fit inside the bacterium, it is negatively supercoiled around an RNA core. During transcription or DNA synthesis, the double stranded DNA is unzipped to allow either a messenger RNA template to be made or a new DNA strand to be synthesised. This unzipping of the DNA causes topological stress upstream of the RNA polymerase or replication fork and induces the formation of positive supercoils which need to be removed. To relieve this stress and remove the positive supercoils, a topoisomerase known as DNA Gyrase, makes double-stranded breaks in the DNA and introduces negative supercoils. After DNA synthesis, the daughter chromosomes are unlinked by another Topoisomerase, Topoisomerase IV, in a process called decatenation. Both these enzymes are essential to bacteria and are the targets of the fluoroquinolones.

1.4.1 DNA Gyrase

DNA Gyrase is a heterotetramer composed of two A subunits and two B subunits, encoded by the *gyr*A and *gyr*B genes. In *E. coli*, GyrA is 97kDa in size and GyrB 90kDa (Reece and Maxwell, 1991). Figure 1.14 shows two 59kDa fragments of GyrA with the active-site tyrosine 122 highlighted in green. The GyrA subunits bind to DNA, the GyrB subunits are ATPases that catalyse the re-setting of the enzyme after the introduction of a negative supercoil. Figures 1.15 and 1.16 show the GyrB subunits and a section through the dimer showing the ATP-binding site. The figures were generated from the proteins' x,y,z coordinates using RasMol. For the purposes of x-ray crystallography, a non-hydrolysable analogue of ATP, ADPMP, was used and is visible in Figure 1.16. The hydrolysis of ATP is not required to introduce the negative supercoiling, instead, upon binding to Gyrase, it induces a conformational shift. (Berger *et al*, 1996). The DNA binds to the C-terminal DNA-binding domain of GyrA and a segment of approximately 130bp wraps around the tetramer (Kampranis and Maxwell, 1996). DNA is cleaved in each DNA strand leaving a 4bp staggered cut (Critchlow and Maxwell, 1996). A covalent DNA-protein intermediate complex is formed between a DNA phosphate and a tyrosine residue (Tyr 122 in *E. coli*) of GyrA. Bond energy is conserved and no energy co-factor is required for breakage or re-joining of DNA. The severed DNA ends come apart and a gate opens to allow the passage of double stranded DNA. Resealing of the DNA reduces the linking number by two. The hydrolysis of ATP re-sets the enzyme for another round of supercoiling.

Figure 1.14. *E. coli* 59kDa dimer of GyrA showing the position of the active-site tyrosine 122 (green), the alpha helices (magenta), beta sheets (yellow) and other residues (white). Figure generated from GenBank accession number 1AJ6.

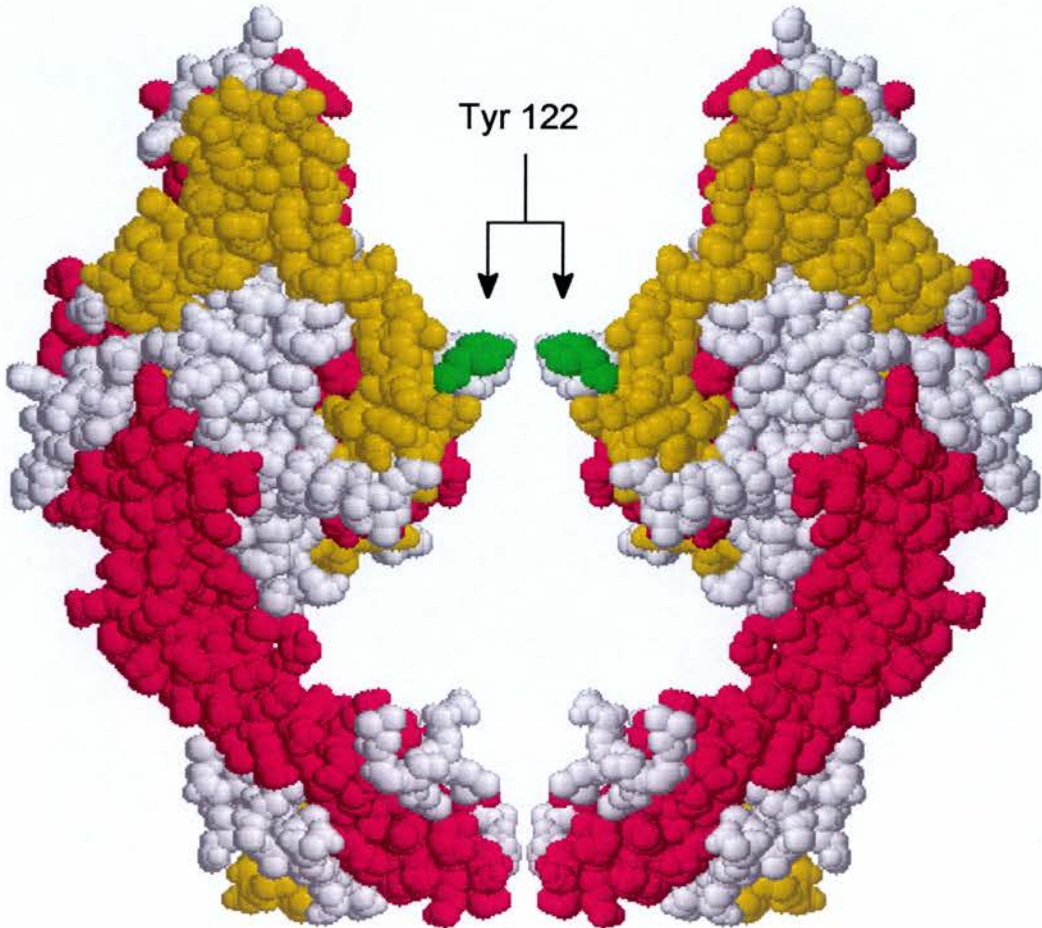


Figure 1.15. The GyrB dimer showing both subunits and the N- and C- domains. The function of the "hole" is unknown and may result from the crystallisation of the protein. Figures generated from GenBank accession number 1EI1.

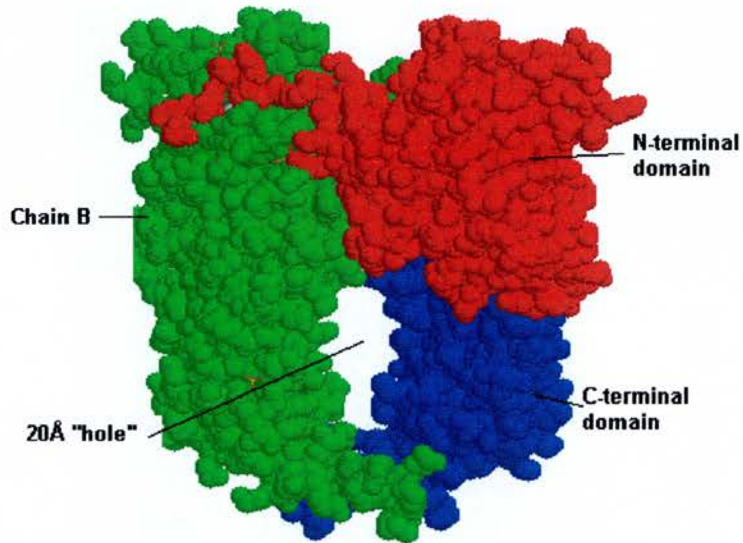
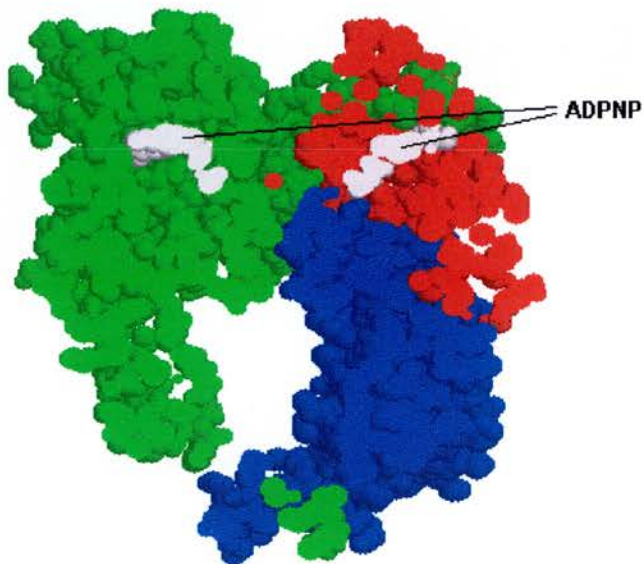


Figure 1.16. A section through GyrB showing the ATP binding sites.



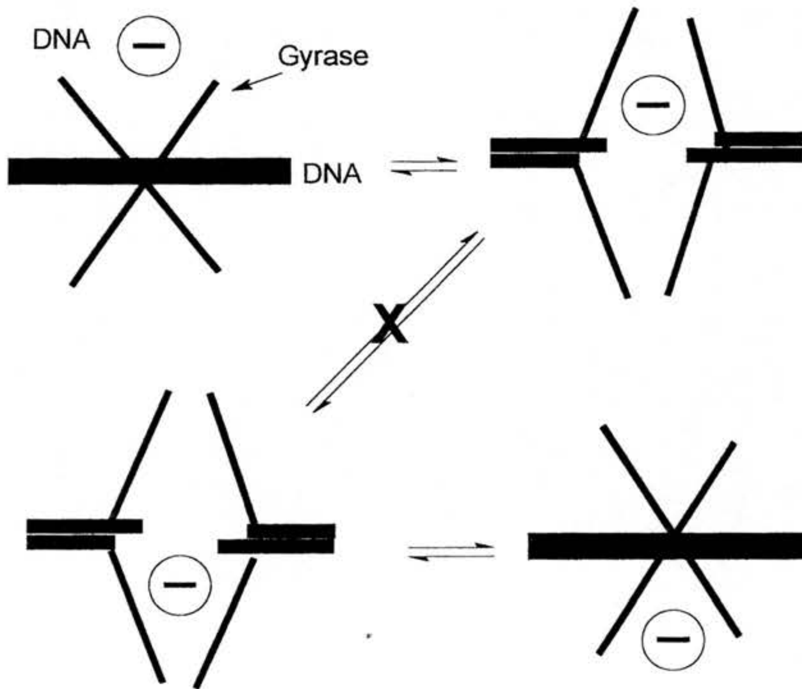
1.4.2 Topoisomerase IV

Like DNA Gyrase, topoisomerase IV is composed of 4 subunits, two each of ParC and ParE. Topoisomerase IV has strong decatenating activity and its mechanism of DNA breakage and reunion is similar to that of DNA Gyrase. The ParC and ParE subunits are homologous to GyrA and GyrB respectively. 36% of the ParC amino acids are identical to those found in GyrA and 40% of ParE amino acids are identical to those found in GyrB (Hooper, 1998). One of the principal differences between DNA Gyrase and Topoisomerase IV is that whilst Gyrase wraps DNA around itself, Topoismerase IV does not. Indeed, removal of the GyrA C-terminal converts Gyrase into an enzyme with strong decatenating activity suggesting that wrapping favours intramolecular strand passage (Kampranis and Maxwell, 1996).

1.5 Fluoroquinolone Action on DNA Gyrase and Topoisomerase IV

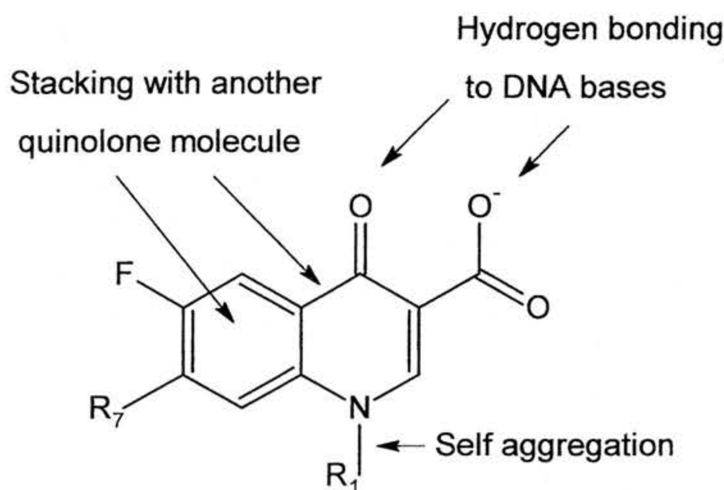
Fluoroquinolones act on DNA Gyrase and topoisomerase IV by preventing strand passage of double stranded DNA on what is termed cleavable complexes. The formation of cleavable complexes is reversible (Drlica and Zhao, 1997). Figure 1.17 illustrates strand passage.

Figure 1.17. Strand passage. DNA is in two-planes, circle and dash coming out of the page, bold horizontal line across the page. DNA Gyrase cleaves one strand of ds DNA and opens a gate to allow the top strand to pass through. Fluoroquinolones block strand passage.



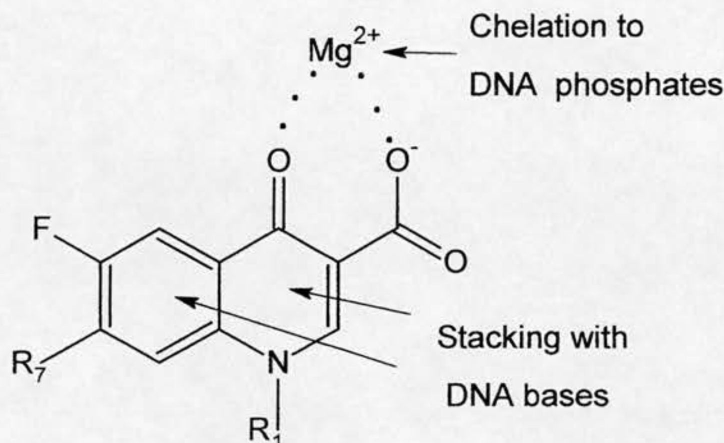
The original model of quinolone action was proposed by Shen *et al*, (1989a,b). It proposed that the drug binds to single-stranded DNA trapping the enzyme. This was based on observations that quinolones bind preferentially to single-stranded DNA but not to duplex DNA. The model suggests that four quinolone molecules bind cooperatively, their C-4 and C-5 substituents hydrogen-bonded to the DNA bases (Figure 1.18). The quinolones were thought to be in two pairs, interacting with each other via π-π stacking and tail-tail hydrophobic interaction between their N-1 and C-8 substituents. However this model relies on the DNA being cut by Gyrase, leaving the 4bp single-stranded DNA, and it has been shown that Tyr122 mutants which cannot cleave DNA can still bind quinolone, a finding also demonstrated with topoisomerase IV (Marians and Hiasa, 1997; Maxwell, 1997).

Figure 1.18. The Shen cooperative binding model of fluoroquinolones



Another model on quinolone action has been proposed, the Mg2^{+} bridge model (Palumbo *et al*, 1993). This model proposes that the C7 and N-1 substituents are not involved in binding to nucleic acid as is thought to happen in the Shen model. The C-4 and C-5 substituents chelate to DNA phosphates (Figure 1.19). The planar ring structures of the quinolones are proposed to stack with DNA bases. This has also been shown with human topoisomerase II where one drug molecule intercalates into DNA while a second molecule is externally bound to the first drug molecule and DNA by two Mg2^{+} bridges (Kwok, Zeng and Hurley, 1999). Support for this model comes from Fan *et al*, however their data suggest that it is gyrase-mediated unwinding of duplex DNA and not single-stranded DNA that is the target (Fan *et al* 1995). In fact, it has been shown that fluoroquinolone binding is followed by a conformational change of the Gyrase-DNA complex and that is responsible for the inhibition of the enzyme and that cleavage of DNA is not necessary for the drug to bind to its target (Kampranis and Maxwell, 1998a; Kampranis and Maxwell, 1998b).

Figure 1.19. Mg_2^+ bridge model



1.6 Antibiotic resistance

Bacteria have been on this world for over 3 billion years. In this time they have had to constantly evolve and adapt themselves to their ever-shifting environment. From the pre-Eukaryotic era when they would have competed amongst their fellow Prokaryotes for nutrients, to the present day where they have still to compete with other Prokaryotes, but also have to compete with the Eukaryotes. Whether they are soil-dwelling, marine, fresh-water, commensal or pathogenic, bacteria are under stress to survive. In order to compete, many microbes produce a variety of antimicrobial compounds such as antibiotics and bacteriocins whose aim is to inhibit non-related species, although some target related species. In this background, it is not surprising that they have the propensity to develop mechanisms of resistance to antimicrobials. However, not only do they have the ability to mutate and acquire resistance to modern antimicrobials, some bacteria have a natural resistance to antibiotics. Resistance is not just a phenomenon of the antibiotic-era. In a study by Dancer on coliforms from glacial ice in the Canadian High Arctic, it was found that some of the organisms they collected were resistant to ampicillin, nalidixic acid, trimethoprim and tetracycline (Dancer, Shears and Platt, 1997). This finding is made

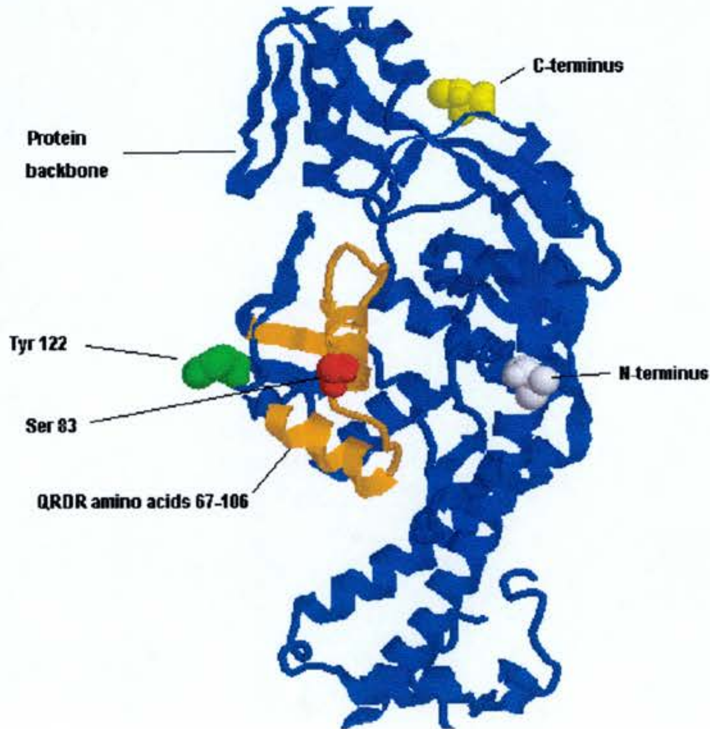
all the more remarkable when they estimate that some of the organisms were over 2000 years old, well before the modern age of antibiotic pressure.

Antimicrobial resistance can occur in a number of ways, but can be summarised in three: destruction of the drug before it reaches the target site, prevention of the drug reaching its target or altering the target so that the drug has reduced affinity. With the natural or semi-synthetic drugs, mechanisms exist for all three strategies. The synthetic drugs on the other hand are believed to be "indestructible" to enzymatic attack because an organism will never have encountered the drug "in the wild". The fluoroquinolones come under the latter category and it is through mutational events in the target site that lead to decreased affinity of the target to the drug combined with changes in the cell envelope, that lead to resistance.

1.6.1 Fluoroquinolone Resistance

Resistance to the fluoroquinolones is mediated chiefly through mutations in the drug targets: DNA Gyrase and Topoisomerase IV. The mutations are found in specific regions of the genes called the quinolone resistance determining region (QRDR) (Figures 1.20 & 1.21). In *E. coli* these are found between amino acids 67 and 106 in GyrA and 426-447 in GyrB (Yoshida *et al*, 1990; Yoshida *et al*, 1991). A new mutation in GyrA has recently been described in *E. coli*, Ala51Val, extending the QRDR (Friedman, Lu and Drlica, 2001). Other mechanisms that contribute to decreased sensitivity to these drugs are active efflux and reduced permeability. DNA Gyrase and Topoisomerase IV are studied by restriction fragment length polymorphism, sequencing of the genes and transformation assays. A mutation in the gene that confers resistance is not dominant and is the basis for transformation assays. If a sensitive gene is introduced into a resistant microbe and then subsequently challenged with a fluoroquinolone, the organism is sensitive. Using these methods, a picture of what causes resistance and how it develops has been built and will be outlined in the next sections.

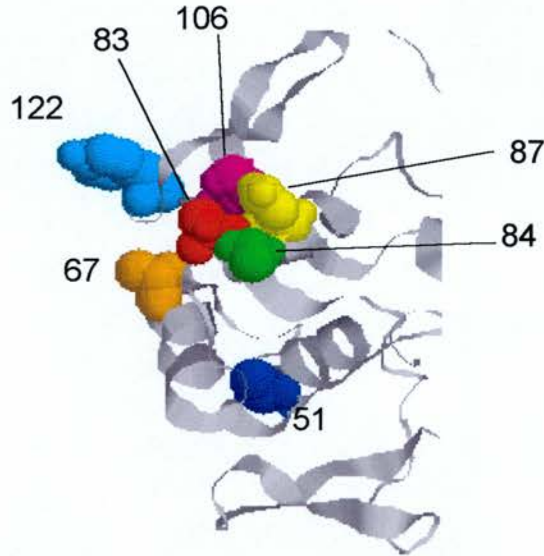
Figure 1.20. QRDR of 59kDa GyrA of *E. coli*. The QRDR of amino acids 67-106 is coloured orange, serine 83 red and the protein backbone in blue. Note the close proximity of the active-site tyrosine (green). Figure generated from GenBank accession number 1AJ6.



1.6.2 DNA Gyrase QRDR

The DNA Gyrase QRDR of both Gram negative and Gram positive organisms have been extensively studied in both clinical isolates and laboratory generated mutants. These data show that GyrA is normally the primary target of the fluoroquinolones in Gram negative bacteria. In the case of Gram positive organisms, the primary target is ParC, with GyrA the secondary target, although not all drugs exhibit this target preference (Alovero *et al*, 2000; Gonzalez *et al*, 1998; Tavio *et al*, 1999).

Figure 1.21. Enlargement of Figure 1.20 showing the QRDR of GyrA. Amino acid residues that are involved in resistance are highlighted in colour. The active site Tyr 122 is shown in cyan. Figure generated from GenBank accession number 1AJ6.



In *E. coli,* Yoshida and coworkers, using laboratory-generated mutants selected on nalidixic acid or pipemidic acid, established GyrA mutations were present by transformation with a wild type *gyr*A gene (Yoshida *et al*, 1990). Sequencing showed mutations that conferred the highest MICs were associated with a serine83-leucine/tryptophan substitution. Using computer analysis on the effect of this mutation, they hypothesised that the mutation causes a conformational change combined with an altered hydrophobic profile of this region. This amino acid substitution at position 83 is the most common mutation found in both Gram negative and Gram positive organisms (Dessus-Babus *et al*, 1998; Mouneimne *et al*, 1999; Pan and Fisher, 1998; Piddock, 1999; Wise *et al*, 1997). The serine mutation in *E. coli* has been found to reduce binding to radiolabelled norfloxacin (Willmott and Maxwell, 1993). Vertical transfer of this mutation has been found to be stable in *S. aureus* after 500 generations in an antibiotic-free environment (Jones *et al*, 2000). Other mutations contribute less to resistance and include in decreasing order for MIC Asp87Asn, Gly81Cys, Ala84Pro, Ala67Ser and Gln106His (Table 1.1).

Not all organisms have a serine residue at position 83 of GyrA. In *Citrobacter freundii* and *P. aeruginosa*, a Thr83Ile mutation in GyrA is found in resistant isolates (Nakano *et al*, 1997; Tavio *et al*, 2000).

Table 1.1. Mutations found in laboratory mutants of *E. coli*, the selecting agent and Ciprofloxacin MIC's

Selecting agent	Ciprofloxacin MIC (mg/L)	Mutation in GyrA
Pipemidic acid	0.05	Ala67Ser
Nalidixic acid	0.05	Gln106His
Nalidixic acid	0.06	Ala51Val
Nalidixic acid	0.1	Gly81Cys
Pipemidic acid	0.1	Ala84Pro
Nalidixic acid	0.2	Asp87Asn
Nalidixic acid	0.39	Ser83Leu
Pipemidic acid	0.39	Ser83Trp

P. aeruginosa is innately more resistant to the fluoroquinolones than *E. coli* and a single mutation in GyrA raises the MIC to above breakpoint values for ciprofloxacin (Nakano *et al*, 1997). Wild type clinical isolates are susceptible to ciprofloxacin with MIC's ranging from 0.05-1.56 mg/L. Nakano and coworkers (1997) found that a Thr83Ile mutation in GyrA gave MICs of 12.5 – 25mg/L and Asp87Gly 6.25mg/L. A GyrA Thr83Ile combined with a ParC Ser80Leu gave MICs of 12.5-50 mg/L. A double GyrA mutation combined with the ParC Ser83Leu gave an MIC of 100 mg/L. Thus the effect of equivalent target-site mutations are not equal and other factors

must be involved. A clue to this is that the most hydrophobic drug of those tested by Nakano, sparfloxacin, showed the greatest increase in MIC. This finding suggests that efflux and/or permeability may have a role to play in sensitivity to these drugs.

In *S. pneumoniae*, the fluoroquinolone C-7 substituent has been found to determine potency and target preference (Alovero *et al*, 2000). Using analogues of ciprofloxacin with a benzenesulfonylamido group added to the C-7 piperazinyl ring, Serine 81 mutations were found in GyrA. This contrasts with ciprofloxacin selecting for a ParC mutation (Alovero *et al*, 2000). In a separate study, Pestova and coworkers (2000) attribute this altered target preference to C-8 substitutions, with C-7 involved in efflux. They found that both moxifloxacin and sparfloxacin target GyrA whereas ciprofloxacin targets ParC . It is difficult to interpret data by using structurally dissimilar fluoroquinolones, as moxifloxacin, ciprofloxacin and sparfloxacin differ not only in C-7 and C-8 but also at position C-5. The use of structural analogues, as in the former study, gives a clearer picture of the development of resistance and the use of dissimilar drugs only muddies the water.

1.6.3 Mutations in GyrB

Mutations in GyrB occur less frequently and are responsible for a modest increase in resistance. Yoshida investigated mutations in GyrB of *E. coli* (Yoshida *et al*, 1991). Using laboratory selected *E. coli* isolated from nalidixic acid or enoxacin plates, it was found that mutants could be divided into two groups. Group 1 were resistant to all the quinolones tested and had an Asp426-Asn mutation in GyrB. Group 2 mutants carried a Lys447-Glu mutation in GyrB and were resistant to acidic quinolones but hypersusceptible to amphoteric quinolones. The wild type parent had a ciprofloxacin MIC of 0.0125 mg/L and nalidixic acid MIC of 3.13 mg/L. Group 1 mutants had MICs of 0.05 and 50mg/L respectively while group 2 had MICs of 0.031 and 50mg/L respectively. These mutations are near hydrophilic regions of the GyrB enzyme as predicted by a hydrophobicity plot of the amino acid sequence.

This area is thought to constitute the surface of the molecule and that the mutations will shift the local conformation of the molecule, reducing its binding affinity.

In *C. freundii*, a His417-Leu substitution in GyrB has been found but its role is unknown, however it did not on its own confer resistance (Tavio *et al*, 2000). A Ser464-Phe mutation in GyrB has been described in *P. aeruginosa* (Mouneimne *et al*, 1999). Complementation with *E. coli* wild-type GyrB showed a 66-fold reduction in ciprofloxacin MIC. However, this mutation is quite rare. In their study it was found in only 2/28 isolates, with a GyrA mutation being found in all isolates tested. In *S. pneumoniae*, GyrB mutations were found to be of no significance (Jones *et al*, 2000). These data suggest that GyrB mutations are quite rare and it is GyrA mutations that are of more importance in the development of fluoroquinolone resistance.

1.6.4 Mutations in Topoisomerase IV

ParC mutations are found in Gram negative organisms most often when there is a concurrent mutation in GyrA. In Gram positive microbes, as we have seen, ParC can be both a primary and secondary target. As a GyrA mutation often confers clinical resistance, a ParC mutation merely raises the MIC higher and in effect are superfluous. However this is not the case with all microbes. In *E. coli*, high-level resistance is mediated through a double GyrA mutation in conjunction with a Ser80 mutation in ParC (Heisig, 1996). With sequential laboratory generated mutants, it has been found that resistance follows a pattern of a Ser83-Leu substitution in GyrA, a Ser80 in ParC and then an Asp87-Asn in GyrA (Bagel *et al*, 1999). The first two mutations have not been found to compromise supercoiling or decatenation in DNA Gyrase or Topoisomerase IV respectively although the Asp87Asn does reduce Gyrase-mediated supercoiling *in vitro*. In *P. aeruginosa*, mutations in ParC have been mapped to regions that are analogous to those found in GyrA, namely Ser80-Leu, although Glu84Lys has been reported (Mouneimne *et al*, 1999). As strains with

a ParC mutation also have a GyrA mutation, it is possible to complement an isolate with sensitive GyrA and it has been shown that the ParC mutation on its own contributes nothing to resistance.

In *Enterococcus faecalis* ParC is the primary target (Kanematsu *et al*, 1998). The *S. aureus* ParC enzyme is called GrlA and the most commonly found mutation is Ser80-Phe which confers ciprofloxacin resistance (Jones *et al*, 2000).

1.6.5 Mutations in ParE

Like GyrB, mutations in ParE are rare and their contribution is minor. In Gram negative organisms, very few studies have looked at mutations in ParE. Everett *et al* (1996) found no amino acid changes in either *E. coli* ParE or GyrB. Just how minor ParE mutations are is illustrated in a study of *S. aureus* GrlB (Tanaka *et al*, 1998). Looking at the inhibitory activity of levofloxacin on purified topoisomerase IV, the IC_{50} for the wild type was 2.3μg/ml, an Asp432-Asn mutation in GrlB was 14μg/ml and 3.4μg/ml with an Asn470-Asp mutant. The authors concluded that position 432 may be responsible for quinolone resistance. However, the IC_{50} of a Phe80 and Lys84 GrlA protein was 180 and 130μg/ml respectively and if combined as a double mutation, an IC_{50} of 1160μg/ml was recorded. Clearly the effect of a GrlB mutation on its own is not sufficient to confer resistance. At best, it will increase the MIC of a strain with a GrlA mutation 2-4 fold.

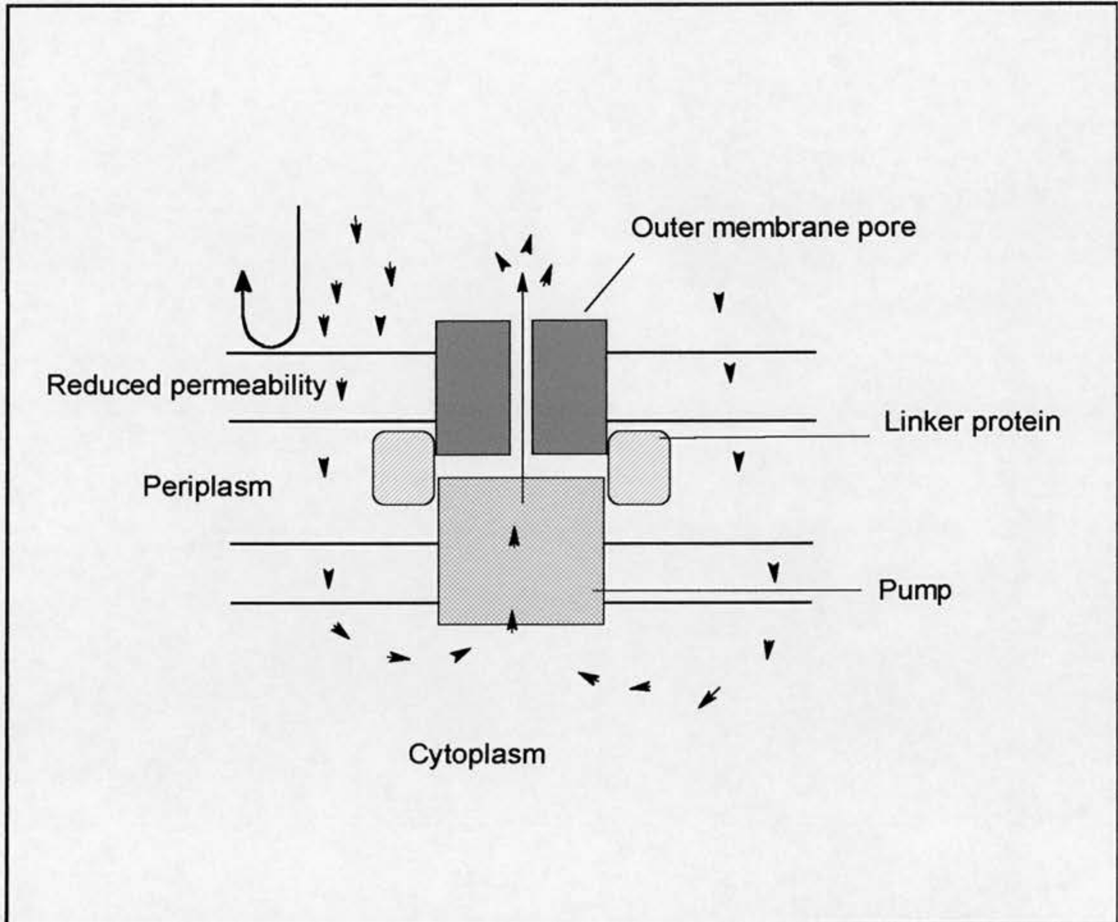
Janoir reports a new mutation in *S. pneumoniae* ParE with an *in vitro* mutant selected on moxifloxacin (Janoir *et al*, 2001). The mutant had a Ser81-Phe mutation in GyrA and a His103-Tyr mutation outside the QRDR of ParE. This double mutation increased the moxifloxacin MIC 4-fold, ciprofloxacin 2-fold and a 2-fold decrease to novobiocin. A mutant with only the GyrA mutation had a 2-fold moxifloxacin increase and no difference in ciprofloxacin or novobiocin MIC.

1.6.6 Efflux-mediated fluoroquinolone resistance

It is not only topoisomerase mutations that confer fluoroquinolone resistance, active efflux of the drug via efflux pumps has been shown to contribute to resistance in both clinical and laboratory mutants. These pumps have been found in both Gram negative and Gram positive organisms and have the ability to pump out a wide range of substrates giving rise to multi-drug resistance (MDR) phenotypes. In Gram negatives, the MDR phenotype has been described in *Stenotrophomonas maltophilia*, *P. aeruginosa E. coli* and *A. baumannii* (Li, Livermore and Nikaido, 1994; Mazzariol *et al*, 2001; Zhang, Li and Poole, 2000; Magnet *et al*, 2001).

There are two types of efflux pump that differ in their construction among Gram negative bacteria. One, the EmrE pump of *E. coli* is a member of the Smr family (Staphylococcal multidrug resistance) in which drugs are pumped into the periplasmic space (Nikaido, 1998). It is composed of a single cytoplasmic membrane-spanning H^+ antiport protein. This type of pump is not known to contribute significantly to clinical resistance. The second type of pump, known as the RND type (resistance-nodulation-division) contains at least three components, a transporter protein in the cytoplasmic membrane, an outer membrane pore and a linker protein that is found within the periplasm (Paulsen, Brown and Skurray, 1996) (Figure 1.22). Examples of RND pumps are the AcrAB system of *E. coli,* AdeABC of *A. baumannii* and MexAB-OprM of *P. aeruginosa*. These pumps can efflux drugs directly out of the cell and into the medium and if coupled with a permeability barrier, can lead to significant resistance levels. In a laboratory *E. coli* mutant that had GyrA mutations, knocking out the AcrAB efflux pump converted it from resistant to ciprofloxacin and ofloxacin to hypersusceptibility (Oethinger *et al*, 2000). One criticism of laboratory mutants is that their phenotypes may not be found *in vivo*. However, overexpression of AcrAB has been reported in 9/10 high-level fluoroquinolone resistant *E. coli* clinical isolates (Mazzariol *et al*, 2001).

Figure 1.22. Schematic representation of an RND efflux pump



Fluoroquinolone resistance attributed to a transferable plasmid has been reported (Martinez-Martinez, Pascual and Jacoby, 1998). Upon transformation with the plasmid from *K. pneumoniae*, the MIC of fluoroquinolones and non-fluoroquinolones were raised, suggesting that the plasmid encoded a multiresistance efflux pump. The fluoroquinolone MICs of susceptible organisms rose 4-30 fold, but not to a concentration conferring resistance. Therefore the authors failed to demonstrate fluoroquinolone resistance. However, if this were to happen in the clinical setting, the results could prove to be disastrous. Possession of this efflux system could allow an organism the opportunity to accumulate a Gyrase mutation and thus become resistant. By also carrying the plasmid, the organism would be at a

selective advantage in areas with high drug usage and thus may be extremely difficult to eradicate.

In Gram positive organisms, efflux pumps are investigated using the pump inhibitor reserpine to block its action and show increased drug accumulation and sensitivity (Beyer *et al*, 2000). Reserpine has no established effect on Gram negative pumps although Miyamae *et al* (1998) and Vila *et al* (2002) have recently claimed limited success with reserpine on *Bacteroides fragilis* and *A. baumannii*. A more effective method to illustrate active efflux is with CCCP. CCCP is an uncoupler that is used to break the proton motive force and thus inhibit the pump. These methods have shown that resistant organisms efflux more drug than sensitive strains (Denis and Moreau, 1993; Li, Livermore and Nikaido, 1994). Drug potency has been shown not to correlate with accumulation i.e. the most potent drugs do not necessarily accumulate the most (Bazile *et al*, 1992).

Other studies have shown there is a correlation between hydrophobicity and accumulation in *S. aureus* and an inverse correlation in *E. coli* and *P. aeruginosa* (Piddock, Jin and Griggs, 2001). Table 1.2 shows drug accumulation and corresponding MIC in *P. aeruginosa*. Ciprofloxacin is more potent and hydrophilic than sparfloxacin yet accumulates less drug. In the same study, DNA Gyrase inhibition assays found a correlation between potency and MIC. Thus it is the inhibitory activity of the drugs that are important and not how much they accumulate. However, if less drug reaches the target, their inhibitory effect will be less. *P. aeruginosa* intrinsically accumulates less fluoroquinolone than *E. coli* and this is attributed to a low number of porins that lowers its permeability. High-molecular weight drugs appear to also limit their accumulation in *P. aeruginosa*.

Table 1.2. Drug accumulation and MIC of *P. aeruginosa*

Drug	MIC (mg/L)	Accumulation (ng drug/ mg dry cell)
Ciprofloxacin	0.5	36
Sparfloxacin	1	48

1.6.7 Outer Membrane Permeability

One other factor found to contribute to fluoroquinolone resistance in Gram negatives are changes in the outer membrane leading to a decrease in permeability. This mechanism has also been linked to resistance with other classes of antibiotic; for example imipenem resistance in *A. baumannii* associated with reduced expression of a 33-36kDa OMP (Clark, 1996). In *P. aeruginosa* a reduction in protein D2 has been linked to a decrease in both sparfloxacin and imipenem sensitivity (Micheahamzehpour, Furet and Pechere, 1991). Outer membrane protein expression is not the sole parameter in permeability as lipopolysaccharide has been linked to reduced fluoroquinolone diffusion into *P. aeruginosa* and *S. typhimurium* (Micheahamzehpour, Furet and Pechere, 1991; Hirai, Aoyama *et al*, 1986a). However not all OMP mutants exhibit reduced fluoroquinolone sensitivity so their contribution alone is not clear-cut. In a study on *Klebsiella pneumoniae*, Hernandez found an association with an altered OMP profile and resistance to β-lactams but not with fluoroquinolones, suggesting porin specificity (Hernandez-Alles *et al*, 2000). However, in an efflux strain, an altered OMP profile does show fluoroquinolone resistance (Chevalier *et al*, 2000). Given that diffusion of drug reaches its maximum concentration within 3 minutes of challenge, it seems unlikely that a reduction in permeability will contribute to resistance unless it is coupled with efflux (Li,

Livermore and Nikaido, 1994). Therefore the role of OMPs is secondary to active drug efflux.

1.6.8 Fluoroquinolone Resistance in *A. baumannii*

The *gyr*A QRDR of *A. baumannii* was first investigated by Vila using primers designed from conserved amino acid motifs from diverse GyrA proteins (Vila *et al*, 1995). Analysis of the *gyr*A QRDR showed 71% identity to *E. coli*, and 69% with *P. aeruginosa* at the nucleotide level. The protein sequence has 82% identity with *E. coli*, 74% with *S. aureus*, 75% with *Campylobacter jejuni* and 83% with *P. aeruginosa*.

It was found that the same GyrA amino acids were mutated in resistant strains of *A. baumannii* as are found in other Gram negative organisms, namely a Ser83-Leu substitution. The major difference was that whereas in *E. coli* the serine 83 mutation still leaves the organism susceptible to ciprofloxacin, in *A. baumannii* it conferred resistance. In *E. coli* this mutation raises the wild type ciprofloxacin MIC from 0.0125mg/L to 0.39mg/L (Yoshida *et al*, 1990). In *A. baumannii* the wild type ciprofloxacin MIC's are ≤ 1mg/L and the serine mutation confers an MIC of 4-16mg/L. Thus it seems that the effect of equivalent mutations are not equal. One explanation of this could be that *A. baumannii* Gyrase is innately less sensitive than that of *E. coli*. Results of DNA gyrase supercoiling inhibition assays demonstrated that sensitive *A. baumannii* Gyrase had a minimal effective dose that was 5-10 times less sensitive to ciprofloxacin than wild type *E. coli* Gyrase (Moreau *et al*, 1996). Another explanation is that *A. baumannii* exhibits low outer membrane permeability coupled with efflux (Hancock, 1998). Other mutations in *A. baumannii* GyrA were found but these appear to have little effect on MIC sensitivity. These mutations have also been found by Seward and Towner (1998).

Moreau measured drug accumulation in *A. baumannii* and found no significant difference in accumulation with regard to MIC, although the more hydrophobic drug pefloxacin accumulated less than ciprofloxacin (Moreau *et al*, 1996).

The ParC gene was investigated by Vila (Vila *et al*, 1997). At the nucleotide level, *A. baumannii par*C has 72% identity with *E. coli*, 71% with that of *Salmonella typhimurium and 72%* with *Haemophilus influenzae* and at the protein level, 89% identity with *E. coli*, 88% with *S. typhimurium* and 84% with *H. influenzae*. The QRDR showed a Ser80-Leu mutation in those strains already carrying a GyrA Ser83-Leu mutation. These mutations recorded ciprofloxacin MICs of 32-128mg/L. One strain had a Glu84-Lys mutation in conjunction with a GyrA Ser83Leu mutation, recording an MIC of 64mg/L. Seward also investigated the ParC gene and found that the double Ser83/Ser80 mutation gave ciprofloxacin MICs of 64 mg/L. One strain had a second mutation in ParC, Tyr74-Cys, but this appears to have no net effect as it recorded an MIC that is equal to the single mutation (Seward and Towner, 1998).

Thus in *A. baumannii* ciprofloxacin resistance is mediated through a single mutation in GyrA, at serine 83. High-level resistance is found in strains with a GyrA mutation and a concurrent serine 80 mutation in ParC.

1.7 The Genus *Acinetobacter*

The genus *Acinetobacter* are Gram negative cocco-bacilli that are strict aerobes and do not ferment glucose. They are non-motile, oxidase negative and catalase positive (Vila, 1998). The organisms are widely distributed in the environment and are sometimes part of the human skin flora. Although not strictly pathogenic, they are becoming increasingly important nosocomial pathogens in intensive care units (ICU), although they have been associated with community acquired pneumonia (CAP) in the tropics (Anstey, Currie and Withnall, 1992).

The acinetobacters are often cited as being commensal organisms and a study by Seifert *et al* on the distribution of *Acinetobacter* spp on human skin confirms this (Seifert *et al*, 1997). While it was found that members of the genus were recovered from humans, *A. baumannii* was only found in 1% of samples compared with 47% for the less clinically relevant *A. lwoffii*. In a contrasting study by Adams, 21% of health care workers were found to be carrying *A. calcoaceticus* (Adams and Marrie, 1982a).

One question that has been puzzling researchers is where does *A. baumannii* come from? The organism has been isolated from many surfaces in hospitals and is hard to eradicate once it has established itself. Experiments have shown that the organism can survive on dry menstra for up to 30 days (Jawad *et al*, 1996). It has been recovered from computer keyboards (Neely, Maley and Warden, 1999) where it was found that gloved medical staff would go from patient to computer keyboard without removing gloves and that other staff not touching patients would enter and retrieve data from the same computer. Thus a reservoir for the organism was established leading to a rise in the number of colonisations in their burns unit. It has been recovered from bed rails in an ICU outbreak where patients were moved between different sectors of the ICU (Catalano *et al*, 1999). Feather pillows have been found to harbour *A. baumannii* (Weernink *et al*, 1995) and even vegetables (Berlau *et al*, 1999) where genospecies 1, 2, 3 & 13TU were significantly more resistant to ciprofloxacin and gentamicin than the other genospecies isolated. It is not only *A. baumannii* that has been isolated from inanimate objects. Kappstein *et al* describes tap aerators as being the reservoir for an *A. junii* outbreak in a childrens oncology ward. Disturbingly, the organism was found only in the tap aerators of the staff room (Kappstein *et al*, 2000).

Given that the organism survives well in the hospital environment, one can conclude that effective control measures to prevent spread of the organism need to be implemented and strictly adhered to. In Holland, guidelines are in force to prevent the spread of MRSA from patients transferred from abroad. They are placed in strict isolation upon admission to a Dutch hospital. In a retrospective study by Bernards, 3

patients were colonised with MRSA and multi-resistant *Acinetobacter* and placed in 3 separate hospitals. The MRSA did not spread but two of the *Acinetobacters* did. The "clean" hospital used airborne precautions while the other two didn't (A. T. Bernards, PhD thesis).

The distribution of the organism on skin may be transient. It has been found that the colonisation rates of patients increased with their length of stay (Seifert *et al*, 1997). Patients carried more *Acinetobacter* than healthy controls although 7/40 of the controls were laboratory workers and may well have picked up the organisms in their laboratories. A quantitative count of the organisms showed that they were present at 2 $\log_2$ less than Gram positive skin organisms. Previous work looking at skin colonisation was done with old taxonomy and thus may be unreliable. Of the body sites tested, the throat and perineum was the least colonised and that the rest of the body was equally distributed with the organisms.

1.7.1 Speciation of the Acinetobacters

Speciation of acinetobacter has been a problematic area for some considerable time. Traditionally, biochemical tests have been employed to speciate bacteria and clinical laboratories routinely use biochemical test strips such as the API-20NE system. However this has a success rate reported to be as low as 45% and cannot differentiate between genospecies 1, 2, 3, & 13TU (Bernards *et al*, 1996). Because of the difficulty in differentiating between these genospecies by phenotypic methods, Tjernberg and coworkers suggested naming them the *A. calcoaceticus-baumannii* complex (Acb) (I. Tjernberg PhD thesis). Recently, more automated systems like Vitek have been used as they are less labour intensive and species identification is done by machine. However with the acinetobacters, the use of such techniques does not guarantee successful identification. In a study by O'Hara and co-workers to evaluate Vitek GNI+ and Crystal E/NF identification systems, it was found that the overall accuracy for correctly identifying Gram negative bacilli was less than 90%

(OHara, Westbrook and Miller, 1997). The accuracy for non-*Enterobacteriacae* was 60%, with the Vitek identifying 1/5 of *A. lwoffii* correctly.

A Vitek system has also given false positive results for imipenem sensitivity with 32 out of 35 isolates testing sensitive using more traditional sensitivity tests (Tsakris *et al,* 2000). It was not until the advent of molecular methods to speciate the organism did a true picture of the genus became known. Bouvet and Grimont (1986) used DNA-DNA hybridisation to identify 12 *Acinetobacter* genospecies. They named *A. baumannii, A. haemolyticus, A. johnsonii, A. junii,* and emended *A. calcoaceticus* and *A. lwoffiii.* Prior to this only *A. calcoaceticus* and *A. lwoffii* were approved names and *A. calcoaceticus* was the only one in Bergeys Manual. Tjernberg used DNA-DNA hybridisation to further identify 3 other genospecies, 13TU, 14TU and 15TU (I. Tjernberg PhD thesis). This technique has been further used in conjunction with AFLP and 16S rRNA to speciate the clinically isolated phenoms I and II and name them *A. ursingii* and *A. schindleri* (Nemec *et al*, 2001). An environmental isolate, *A. venetianus,* has also been described (di Cello *et al*, 1997). Since then, 6 more genospecies have been identified but are as yet unnamed with the exception of species 12, *A. radioresistens.*

Other methods used to speciate have included cell envelope profiles (Dijkshoorn, Michel and Degener, 1987), ribotyping (Koeleman *et al*, 1997; Seifert *et al*, 1997; Vaneechoutte *et al*, 1999), carbon source utilisation (Bouvet and Grimont, 1987) and *gyr*B sequences (Yamamoto, Bouvet and Harayama, 1999). These methods have good discrimination although have the disadvantage of being tedious and require many hours of work. A rapid method exists which is PCR based and relies on species specific conserved regions of the spacer regions of tRNA (Ehrenstein *et al*, 1996). It has been proven to be quick and reliable for identification of most *Acinetobacter* spp although it cannot differentiate between *A. baumannii* & 13TU and *A. calcoaceticus* & group 3 (Ehrenstein *et al*, 1996). For routine use it is probably the best option however DNA-DNA hybridisation remains the only method that will differentiate with certainty.

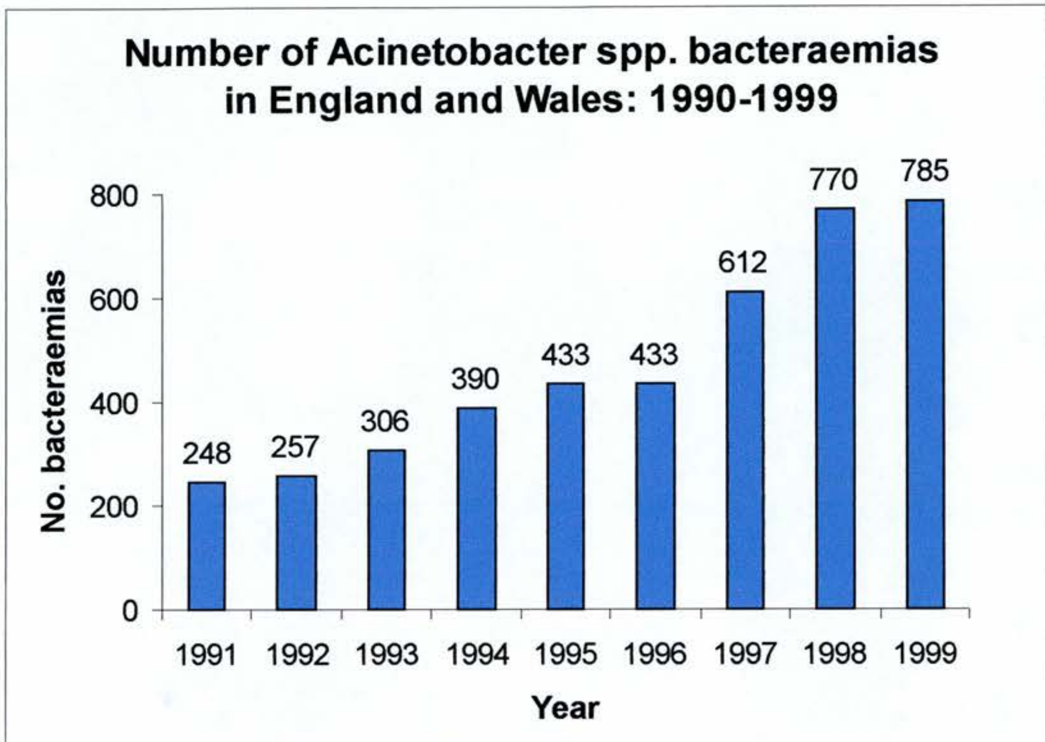
1.7.2 Acinetobacter in the clinical setting

Acinetobacter spp cause a range of nosocomial illnesses including pneumonia, bacteraemia, secondary meningitis, urinary tract infections and surgical wound infections. Speciation has revealed that 73-90% of *Acinetobacter* infections are associated with *A. baumannii* or genospecies 13TU (Seifert *et al*, 1997) although a 10-year study by Traub and Bauer (2000) found that genospecies 3 was the most prevalent species isolated, accounting for almost 50% of 2359 isolates. *A. baumannii* accounted for 15%. Traub only employed phenotypic tests and these are not adequate to differentiate between genospecies 1, 2, 3 and 13TU, therefore the true total for *A. baumannii* may be higher.

Acinetobacter is a significant coloniser of materials such as ventillator tubings, endotracheal tube, intravascular access devices, surgical drains and urinary catheters. Outbreaks and infections associated with contaminated suction bottles and catheters in neonatal units, arterial lines, central venous catheters and ventilators are well documented (Minnaganti and Cunha, 2000; Pillay *et al*, 1999; Richards *et al*, 1999; Lesseva, 1998; Seifert *et al*, 1995).

The incidence of bacteraemias caused by *Acinetobacter* spp. increased year on year in England and Wales over the 1990s. Data from the Public Health Laboratory Service are shown graphically in Figure 1.23. These data show that the numbers of bacteraemias has more than tripled in the nine-year period. To put this into perspective, during this same period, *Salmonella typhi* bacteraemias decreased from 182-181 cases, *E. coli* rose from 7780-11329 cases and *P. aeruginosa* from 1084-1367. Although acinetobacter is not the most prevalent Gram negative pathogen causing bacteraemia, it has shown the greatest rise in this period. Gram positive bacteraemias caused by *S. aureus* doubled from 4890 in 1990 to 10235 in 1999 while *S. pneumoniae* went from 3507-4001. These data do not include other infections.

Figure 1.23. Incidence of Acinetobacter spp. bacteraemia



1.7.3 Risk Factors Associated with Infection

The biggest risk factor for infection (as opposed to colonisation) is an immunocompromised status. Even the reports of CAP linked with an *Acinetobacter* infection was associated with a compromised host: smoking, alcoholism, an underlying chronic obstructive airway disease and a history of pneumonia are cited by Anstey, Currie and Withnall (1992). There are other factors like survival on innate objects previously discussed and transmission through inadequate infection controls. However, prior-use of broad-spectrum antibiotics has a large role to play in the dissemination of this organism. The use of ceftazidime to treat *Pseudomonas* infections and inadvertently selecting for *Acinetobacter* has been well-documented (Koeleman *et al*, 1997; Yoo *et al*, 1999) as has the use of a fluoroquinolone (Cendrero *et al*, 1999; Husni *et al*, 1999). Villers *et al* (1998) found a relationship between intravenous ciprofloxacin and *Acinetobacter* infection and hypothesised that

oral ciprofloxacin exerts less selective pressure in the gut. Length of stay has also been linked to infections (Koeleman *et al*, 1997).

The selective pressure of ciprofloxacin also results in an increase in fluoroquinolone resistance. Muder *et al* (1991) looked at the association between prior fluoroquinolone use and ciprofloxacin resistance among Gram negative bacteria. They found a significant relationship between prior drug use and resistant bacteria, with a stronger association seen with those in long-term care as opposed to acute care. This finding is not seen in all hospitals. Pieroni looked at the incidence of ciprofloxacin resistance in blood-culture isolates before and after the introduction of ciprofloxacin in 1989. He found that there was no significant increase in resistance to this drug in either *Acinetobacter* spp or *Pseudomonas*. Resistance rates to other drugs in this hospital remained low over the 16-year study period suggesting a prudent antibiotic policy (Pieroni *et al*, 1997).

A study by Fagon *et al* (1996) looked at the mortality relationship between colonisation of *Acinetobacter* or *Pseudomonas* spp in patients undergoing mechanical ventilation and those with pneumonia due to these pathogens ($<10^3$ cfu/ml, $\geq10^3$ cfu/ml respectively). As patients undergoing this procedure are usually the most severely ill and have the most severe nosocomial infections, it is sometimes hard to establish if mortality is due to the infection or if the patient would have died as a result of their underlying illness. They found that there was a relationship, with 29% mortality of those colonised (28% for non-colonised patients) and 73% of those infected.

There has been speculation as to seasonal variation in *Acinetobacter* infections, with one report from Germany stating no difference was found between seasons (Seifert *et al*, 1997) and another from the USA contrary to this which found a higher incidence between July and October (McDonald, Banerjee and Jarvis, 1999). However given the fact that most infections occur in ICU wards and that in the USA these will be air-conditioned and thus climatically-stable, it seems unlikely that warm summer days will lead to a higher incidence of infection. An alternative explanation for

McDonald and co-workers could be that during summer holidays, experienced staff are temporarily replaced with part-time staff and this combined with an influx of freshly-graduated junior doctors, leads to an increase in patient-to-patient transmission mediated through a breakdown in infection control measures. Hand carriage of Gram negative organisms on hospital workers is well-documented, with colonisation rates of 31% and 21% of healthcare workers carrying *A. calcoaceticus* on their hands. This is unsurprising given that Husni reports that only 10% of healthcare workers wash their hands between patients (Adams and Marrie, 1982a; Adams and Marrie, 1982b; Husni *et al*, 1999).

1.7.4 Typing of *Acinetobacter*

As with speciation, many methods have been employed in the study of the spread of this organism through the nosocomial environment. It is of paramount importance during an outbreak to determine if the organism responsible is either a clone and thus spreading between patients, or unrelated strains that are endogenous to the patient. Another reason why this is important is to discover the source of an outbreak, so that adequate measures to eradicate the organism can commence. Early attempts to type *Acinetobacter* relied upon phenotypic characteristics such as biotypes and antibiotypes. For example, carbon utilisation at different temperatures (Bouvet and Grimont, 1987). However, as we will see, these methods are unreliable. Once again, it was the development of molecular techniques such as pulsed-field gel electrophoresis (PFGE) that have led to a better understanding of the epidemiology of the organism and also its reservoirs.

In a comparative study, perhaps the easiest to interpret, Seifert and coworkers compared 4 different methods for epidemiologic typing of *A. baumannii*: plasmid profile, antimicrobial susceptibility, PFGE with the restriction enzyme *Apa*I, and biotyping (Seifert *et al*, 1994). Plasmid profiles were found to be good and may be used as a cost-effective first-step in epidemiologic typing. However, one of their

outbreak strains lacked a plasmid and so was untypable with this method. Antibiotic testing put some strains in the same group but with PFGE they were not identical. Biotyping has poor discrimination and classified as the same biotype isolates that had different PFGE patterns. In conclusion to this, it was found that PFGE is reproducible and easily readable, a finding that has also been reported by Gouby (Gouby *et al*, 1992).

Another more recent study looked at an outbreak of *A. baumannii* and typed them by antibiotyping and ribotyping (Biendo *et al*, 1999). These methods appeared to correlate but they had to combine results as they had 4 antibiotypes and 7 ribotypes. Biotyping did not discriminate well as it grouped different DNA groups together. Seifert compared ribotyping and PFGE and found that PFGE was more discriminatory as it identified 49 distinct patterns and 7 variants while ribotyping only identified 8 patterns with *Cla*I and 31 patterns with *Eco*RI (Seifert and GernerSmidt, 1995). However they conclude that while PFGE is a more discriminatory method for typing, ribotyping may be used to speciate within the Acb complex as it generates bands that are unique to each DNA group.

Another method used for typing is infrequent restriction site PCR (IRS-PCR). This has been reported to be as discriminatory as PFGE but is less laborious and time-consuming (Riffard *et al*, 1998). Yoo *et al* (1999) used IRS-PCR to investigate an outbreak and found that the outbreak strain was isolated from the Y-piece of a mechanical ventilator and also from the hands of two health care workers. However no comparative studies of this technique has been done to date on *Acinetobacter* spp. Comparisons have been conducted between PFGE and IRS-PCR on other Gram negatives with Garaizar *et al* (2000) stating that interassay and intercentre IRS-PCR has low reproducibility and discrimination compared to PFGE, a finding that is contradicted by others (Riffard *et al*, 1998; Su *et al*, 2000).

Epidemiological studies suggest that Northern Europe is experiencing the spread of epidemic *Acinetobacter* clones (Dijkshoorn *et al*, 1996). A survey of 31 *A. baumannii* isolates comprising 14 strains from 14 outbreaks and 17 sporadic strains

from north-western European cities were typed using numerous methods, both phenotypic and genotypic. It was shown that 12 strains from unrelated outbreaks were linked into two clusters. The sporadic strains were more heterogeneous. This suggests that the outbreak strains have a common clonal origin. Interestingly, at both the genomic and outer membrane protein profile typing level, the strains were homogeneous, but their biotype and antibiograms showed more variability, illustrating how undiscriminating some of these methods can be. Not all outbreak strains could be grouped in this way therefore outbreaks are not exclusively caused by the two clones. In a follow-up to this, the development of ciprofloxacin resistance was investigated during a 20 month period (Horrevorts *et al*, 1997). It was found that two strains had the same OMP profile but had different carbon source growth requirements. There was a shift in these requirements with the conversion of strains to being ciprofloxacin resistant. The switch coincided with the introduction of ciprofloxacin in the ICU. The protein profiles were found to resemble clones from group 1 (described above). The data suggest ciprofloxacin exerted selection pressure.

Given these facts, one can ask the question if the same *Acinetobacter* clones are found throughout the world. Seward looked at the epidemiology of fluoroquinolone resistant strains and found that the strains were genotypically distinct and conclude that resistance is occurring independently worldwide (Seward and Towner, 1998).

Taking all this together, molecular methods and in particular PFGE, are the most discriminating and reproducible technique for epidemiological studies; however they are time consuming and often need sophisticated software to read the banding patterns. There is a need for simple tests that can be used in routine clinical laboratories for surveillance purposes.

1.7.5 Treatment of *Acinetobacter baumannii* infections

With the organism being innately resistant to many antimicrobials, what treatments are available? The fluoroquinolones are still prescribed and with prudent use and a knowledge of the indigenous strains in a hospital, they are very effective as seen in the survey undertaken by Pieroni *et al* (1997). If fluoroquinolone use is not an option, then a carbapenem such as imipenem can be effective. Resistance to this drug is on the increase. A world-wide survey undertaken by the SENTRY Antimicrobial Surveillance Programme found nearly 11% of isolates were resistant to imipenem (Gales *et al*, 2001). Another option is ampicillin/sulbactam (Cisneros *et al*, 1996). However, perhaps one of the best treatments for *Acinetobacter* infections is prevention through adequate infection control procedures and disinfection of shared equipment.

1.8 Conclusions

- *A. baumannii* is a serious nosocomial pathogen
- Incidence of bacteraemia is increasing
- Acinetobacter is multiresistant
- Clonal spread of resistant strains threatens hospital patients
- Fluoroquinolone resistance is on the increase
- Infection is associated with this class of drug as is selection of resistant phenotypes
- There is a reliance on carbapenems but this class of antibiotic for therapy is under threat
- There is a need for newer, more active drugs
- Little is known about how the new fluoroquinolones compare to the older members of this drug class against *A. baumannii*

1.9 Aims of This Thesis

- Speciate a cohort of *Acinetobacter* spp. using a molecular method
- Compare the potency of the old and newer fluoroquinolones against *Acinetobacter* spp. by kill-curve and agar dilution MIC
- Determine the mechanism of resistance to the newer fluoroquinolones with *A. baumannii*
- Investigate the *in vitro* development of fluoroquinolone resistance in *A. baumannii* and compare the mutation rates of ciprofloxacin to gemifloxacin and moxifloxacin
- Examine other factors that may contribute to fluoroquinolone resistance

Chapter 2: Materials and Methods

2.1 Bacterial strains

The standard laboratory strains *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aureus* NCTC 6571 and *Acinetobacter baumannii* ATCC 19606 were used as controls for the determination of MICs. One hundred and one isolates of *Acinetobacter* spp. (47 *A. baumannii*, 18 *A. anitratus*, 18 *A. lwoffii*, 13 *A. calcoaceticus* and 4 unspeciated *Acinetobacter* spp.) were bloodstream isolates obtained from the University of Iowa College of Medicine, Iowa City, USA. They had been previously speciated in a Vitek machine.

The standard Acinetobacter DNA groups listed in Table 2.1 were used for molecular speciation and were a gift from Dr K. Towner except Species 2, which was gift from Scott Cameron. Strains were stored at -70°C in Nutrient Broth (Oxoid, Basingstoke, UK) containing 10% glycerol and subcultured overnight on MacConkey agar (Oxoid) at 37°C except for *Acinetobacter* spp. 6, 5 & 12 which were subcultured on Nutrient Agar (Oxoid) at 30°C.

2.2 Media, Buffers and Reagents

All buffers were made with chemicals purchased from Sigma (Poole, UK) unless otherwise stated. Acetic acid and Hydrochloric acid were purchased from BDH (Poole, UK). Saline was made with 0.8% NaCl and sterilised before use. Media was purchased in powder form from Oxoid (Basingstoke, UK) and made with distilled water before sterilisation.

Table 2.1. Standard *Acinetobacter* strains

Genospecies No.*	Species Name	Strain No.
Species 1.	*A. calcoaceticus*	ATCC 23055
Species 2.	*A. baumannii*	ATCC 19606
Species 3.	unnamed	ATCC 19004
Species 4.	*A. haemolyticus*	ATCC 17906
Species 5.	*A. junii*	ATCC 17908
Species 6.	unnamed	ATCC 17979
Species 7.	*A. johnsonii*	ATCC 17909
Species 8.	*A. lwoffii*	ATCC 5866
Species 12.	*A. radioresistens*	

* (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989)

2.3 Antimicrobial Agents

The following antibacterial agents were used: Gemifloxacin (GlaxoSmithKline, Collegeville, PA, USA), Ciprofloxacin (Bayer A. G., Leverkusen, Germany), Trovafloxacin (Pfizer, Sandwich, UK), Grepafloxacin (GlaxoSmithKline, Stevenage, UK), Azithromycin (Mast Diagnostics, Merseyside, UK), Imipenem (Merck Sharpe Dohme, Rahway, NJ, USA), Sparfloxacin (Dainippon, Japan), Cefuroxime (Sigma, Poole, UK), Moxifloxacin (Bayer A. G., Leverkusen, Germany), Levofloxacin (Hoechst Marion Roussel, Romainville, France), Ofloxacin (Sigma), Gatifloxacin (Grünethal, Aachen, Germany), Sulbactam (Pfizer, Sandwich, UK), Tetracycline & Chloramphenicol (Sigma).

2.4 MIC determination and Breakpoints

The agar dilution method was used to determine MICs on IST agar using the British Society for Antimicrobial Chemotherapy guidelines for susceptibility testing (British Society for Antimicrobial Chemotherapy, 1991). Briefly, an overnight culture in IST broth was diluted in sterile 0.8% saline and approximately 10^4 organisms inoculated onto the antibiotic-containing media. Breakpoint values were taken from the BSAC guidelines unless otherwise indicated and are as follows: gemifloxacin, 0.5mg/L (Wise and Andrews, 1999), moxifloxacin, 1mg/L (Andrews *et al*, 1999), sparfloxacin 2mg/L (Phillips *et al*, 1994), cefuroxime 2mg/L, ciprofloxacin 1mg/L, imipenem 4mg/L, ofloxacin 4mg/L, azithromycin 0.5mg/L, levofloxacin 4mg/L/ml, gatifloxacin 2mg/L, trovafloxacin 2mg/L (Heinemann *et al*, 2000).

2.5 Optimum Bactericidal Concentration

To determine the optimum bactericidal concentration (OBC) of gemifloxacin, ciprofloxacin, moxifloxacin, levofloxacin, grepafloxacin, trovafloxacin and sparfloxacin, nutrient broth was inoculated with *A. baumannii* ATCC 19606 and incubated overnight at 37°C. Doubling dilutions of the test agent (0.03–256 mg/L) in nutrient broth were inoculated with 0.2 ml overnight culture (final volume 10 ml) and incubated for a further 3 h at 37°C. The viable count of the resulting cultures was determined by serial dilution and plating onto nutrient agar (Lewin *et al*, 1989). The results were plotted on a graph to determine the OBC, the OBC being the lowest point on the graph.

2.6 Time-Kill Kinetics

The killing kinetics were performed at the OBC and at four times the MIC (MIC values were previously determined for strain ATCC 19606 as: gemifloxacin 0.12 μg/ml: trovafloxacin 0.06 μg/ml: moxifloxacin 0.25 μg/ml: levofloxacin 0.5 μg/ml: ciprofloxacin 0.5 μg/ml: grepafloxacin 0.12 μg/ml & sparfloxacin 0.12 μg/ml). Briefly, 5 ml sterile double-strength nutrient broth was dispensed into 20 ml

universal tubes and sterile distilled water was added to give a final volume of 9.8 ml. A starting inoculum of 10^7 organisms (0.2ml overnight culture) was added to the tubes and incubated for 2 hours until in log-phase. The antibiotic was added and an initial count was determined by serial dilution and plating onto nutrient agar. Samples were taken every 30 minutes for 3 hours and these were serially diluted and plated onto nutrient agar. A final count was taken after 24 hours. All plates were incubated overnight in air at 37°C (Lewin *et al*, 1989).

2.7 PCR Reagents

All PCR reactions were performed with Promega reagents (Promega, Southampton, UK). *Taq* polymerase (5u/µl) in buffer A (10 x buffer: 500mM KCl, 100mM Tris-HCl pH 8.0, 1% TritonX-100), $MgCl_2$ (25mM) and deoxynucleoside triphosphates (100mM). Ultra pure pyrogen-free water (MilliQPF, Millipore) was used as the diluent.

2.7.1 PCR Primers

Table 2.2 shows the sequences for the primers for the QRDR of GyrA (*gyr*A), ParC (*par*C), genotypic speciation (T3A/T5B), a 45.5kDa porin (ABOMP1& 2), inverse PCR of the porin (ABINV1 & 2) and M13.

Table 2.2. PCR primers

Gene	PCR Primer Sequence	Reference
*gyr*A[a]	5'AAATCTGCCCGTGTCGTTGGT3'	(Vila *et al*, 1995)
	5'GCCATACCTACGGCGATACC3'	
*par*C[a]	5'AAAAATCAGCGCGTACAGTG3'	Derived from (Vila *et al*, 1997)
	5'CGAGAGTTTGGCTTCGGTAT3'	
T3A[b]	5'GGGGGTTCGAATTCCCGCCGGCCCCA3'	(Ehrenstein *et al*, 1996)
T5B[b]	5'AATGCTCTACCAACTGAACT3'	
ABOMP1[b]	5'GA $^{A}/_{G}$ GCI*AA $^{A}/_{G}$ GGITT $^{C}/_{T}$ GTIGA $^{A}/_{G}$ G3'	Degenerate primers derived from OMP
ABOMP2[b]	5'GCICC $^{C}/_{T}$ TG $^{C}/_{T}$ TT $^{C}/_{T}$ TT A/G TCIC $^{G}/_{C}$ IG3'	
ABINV1[b]	5'GTTCGCATCCTCCACAAACC3'	Inverse PCR primers
ABINV2[b]	5'ACCGGCGACAAAAAACAAGG3'	
M13 1[c]	5'GTAAAACGACGGCCAGT3'	
M13 2[c]	5'GTTTTCCCAGTCACGAC3'	

*I: inosine, [a] Oswel DNA Service (Southampton, UK), [b]MWG-Biotech, [c]Invitrogen (Groningen, The Netherlands)

2.7.2 Preparation of DNA for PCR

A 1µl loopful of overnight culture on solid media was suspended in 50µl sterile pyrogen-free water and boiled for 10 minutes. The suspension was centrifuged briefly at 13,000g and the supernatant used as template.

2.7.3 Phenol:chloroform:isoamyl alcohol DNA extraction

Phenol:chloroform:isoamyl alcohol (25:24:1) (PCI) was used to cleanup DNA for inverse PCR. Sample containing DNA was mixed with an equal quantity of PCI and vortexed for 3 minutes. After centrifugation at 13,000g for 5 minutes at room temperature, the supernatant was removed to a new tube and another equal quantity of PCI was added, vortexed and centrifuged as previously described. The supernatant was removed and an equal quantity of chloroform added, vortexed and centrifuged as before. The supernatant was transferred to a new eppendorf and 10% its volume of 3M NaOAc and 2 x volume of 100% EtOH added (EtOH precipitation). The mixture was placed at -20°C for 30 minutes and then centrifuged at 13,000g for 20 minutes at 4°C. The supernatant was discarded and the pellet washed with 500µl 70% EtOH. The pellet was dried in a heating block and resuspended in distilled water (Laboratory of Molecular Biology protocol).

2.7.4 Visualisation of PCR products by Agarose Gel Electrophoresis

PCR products were visualised on a 1.5% electrophoresis grade agarose gel (Gibco BRL, Paisley, UK) dissolved in 40mM Tris-acetic acid 20mM disodium EDTA (TAE buffer) pH8. Ten μl of PCR product was added to 1μl PCR loading buffer (Promega, Southampton, UK) and run in TAE buffer at 100 volts. A 100bp DNA Ladder or PCR Marker (Promega) was used as molecular weight markers. The 100bp marker has bands from 100bp up to 1.5kb, 100bp apart. The PCR marker has bands at 50, 150, 300, 500, 150 and 1000kb. The gel was stained with 0.5μg/ml Ethidium bromide and visualised on a UV transilluminator or in a Bio-Rad GelDoc 2000 (Bio-Rad, Hemel Hemstead, UK).

2.7.5 Speciation of *Acinetobacter* spp using tDNA fingerprinting

The 101 *Acinetobacter* isolates were speciated by a genotypic fingerprinting method using universal primers T3A and T5B that anneal to the spacer regions of tRNA as described by Ehrenstein *et al* (1996) (Table 2.2). The standard *Acinetobacter* strains listed in Table 2.1 were subjected to the same PCR and their banding pattern was used to define each species and the clinical isolates were compared to this. Template DNA was prepared as described earlier and 2μl of the lysate was used in each reaction containing 0.2mM each DNTP, 1μM each primer, 1 x PCR buffer, 1.5mM $MgCl_2$, 2 units *Taq* polymerase, MQ water to final volume 50μl. Cycling parameters were as follows: 2 minutes at 94°C initial denaturation followed by 45 cycles of 40 seconds at 94°C, 40 seconds at 50°C and 2 minutes at 72°C. A final extension of 72°C for 3 minutes. PCR products were visualised as previously described.

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2.7.6 PCR of the *gyr*A QRDR

The primers used for amplifying *gyr*A are shown in Table 2.2. Isolation of template DNA has been previously described. A PCR reaction (50µl) of 1.25mM $MgCl_2$, 0.1mM DNTPs, 1 x reaction buffer, 0.05mM each primer, 2.5µl template and 0.2 units of *Taq* in pyrogen-free water were cycled at the following parameters: 2 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 57.5°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 5 minutes producing a product of 342bp.

2.7.7 PCR of the *par*C QRDR

The *par*C primers were derived from the sequence published by Vila *et al* (1997) using Primer 3 software (http: and waldo.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi, 2001) by Dr Ahmed Hamouda (Table 2.2). A PCR reaction (50µl) of 1.25mM $MgCl_2$, 0.1mM DNTPs, 1 x reaction buffer, 0.05mM each primer, 2.5µl template and 0.2 units of *Taq* in pyrogen-free water were cycled at the following parameters: 2 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 5 minutes, producing a product of 197bp.

2.7.8 PCR of 45.5kDa Porin using Degenerate Primers

A 40 amino acid fragment of the 45.5kDa porin from mutant strain BE1, a resistant mutant dervied from SB13, was used to design degenerate primers that gave an 87bp PCR product (Table 2.2). Design of the primers is shown in Appendix I. Template DNA was extracted as previously described from mutant BE1. A PCR reaction (50µl) of 8mM $MgCl_2$, 0.1mM DNTPs, 1 x reaction buffer, 0.05mM each primer, 2.5µl template and 0.2 units of *Taq* in pyrogen-free water were cycled at the

following parameters: 2 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 5 minutes. The PCR product was directly sequenced and analysed as previously described.

2.7.9 Inverse PCR

Inverse PCR was used to try to determine the sequence of the 45.5kDa OMP both upstream and downstream of the partial amino acid sequence. The region of interest containing a known sequence is circularised and primers derived from the known sequence are used to amplify DNA in opposite directions (Appendix II). Whole genomic DNA (2.5μg) was digested with the restriction enzymes *Hae*III, *Alu*I, *Rsa*I, or double digested with *Hae*III & *Alu*I, *Hae*III & *Rsa*I and *Alu*I & *Rsa*I for two hours at 37°C. The DNA was re-extracted using PCI. Assuming that 2.5μg of DNA remained, the sample was diluted to 40ng/μl. Five μl was added to ligation solution (Promega) at a final concentration of 1-10ng/μl DNA and the ligation was performed at 16°C overnight. A control ligation of λ ladder cut with *Hind*III was performed to determine if a ligation reaction was successful. The mixture was then EtOH precipitated as previously described. The PCR reaction was then carried out using 1μl of ligated DNA solution, 10μM of each primer, 0.25mM DNTPs, 2.5mM $MgCl_2$. The PCR cycles were as follows: 1 cycle of 96°C for 4 minutes followed by 30 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute and a final extension of 72°C 5 minutes (Laboratory of Molecular Biology protocol).

2.7.10 Cloning of the Porin

The 87 base pair PCR product was cloned into a pCR®4-TOPO® vector (Invitrogen) by Mr Richard Gibbs and the plasmid was extracted. The vector carries M13 priming sites 73bp upstream and 60bp downstream of the insertion point. Using

these primers, the OMP sequence was amplified by PCR using Promega reagents previously described. The 255bp product was run on a gel, excised and purified with a Gel Extraction Kit (Qiagen) and sequenced using the M13 primers.

2.7.11 Sequencing of PCR products

The PCR products of *gyr*A and *par*C were purified using a PCR cleanup kit (Qiagen) and run on an automatic sequencer. DNA sequences were analysed using Chromas software and the *gyr*A and *par*C sequences compared to the published sequences of *A. baumannii* ATCC 19606, GenBank accession numbers AF100557 and AF108132 respectively, using the online search facility BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The amino acid sequences were translated using the EXPASY translate website (http://www.expasy.ch/tools/dna.html). Multi-alignment of DNA or amino acid sequences was performed with the online website multalin (http://www.toulouse.inra.fr/multalin.html). The PCR products of the 45.5kDa porin was sequenced directly and also after cloning.

2.7.12 *Hinf*I Restriction Fragment Length Polymorphism of *gyr*A and *par*C

The loss of the *Hinf*I restriction site in the *gyr*A and *par*C PCR products of *A. baumannii* is associated with a serine mutation at position 83 and 80 in GyrA and ParC respectively (Vila *et al*, 1997; 1995). A 10µl aliquot of PCR product was added to 2µl restriction enzyme buffer (10mM Tris-HCl pH 7.3, 50mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol), 0.2µl bovine serum albumin (0.5 mg/ml) and 5u *Hinf*I (Promega), final volume 20µl, and incubated for 2 hours at 37°C. The resulting products were run on a 1.5% agarose gel and visualised as previously described. If the restriction site is cut by the enzyme, *gyr*A gives two products of

291 and 52bp and *parC* 144 and 53bp. ATCC 19606 was used as a wild-type control (both genes cut), SB13 as a *gyr*A control (not cut) and SB21 as a *par*C control (uncut).

2.8 Fluoroquinolone selection of mutants

Mutants were selected from *A. baumannii* ATCC 19606 and the clinical isolate SB13, which carries a GyrA Serine 83 mutation, at 2 x MIC on gemifloxacin, ciprofloxacin or moxifloxacin-containing IST agar (Oxoid, Basingstoke, UK). An overnight culture of ATCC 19606 in IST broth was used to inoculate the selection plates. This was sufficient for mutation rates $\geq 10^{-8}$. For mutation rates lower than this, 10ml of overnight culture was centrifuged at 15,000g and concentrated 10-fold before plating onto selection plates. All selection was done on triplicate plates. A viable count was performed to determine the inoculum to calculate the mutation-frequency. Selection plates were incubated at 37°C in air for 48 hours before mutant colonies were subcultured and tested for increased fluoroquinolone MICs. Gemifloxacin-selected mutants were subcultured onto MacConkey agar (Oxoid) for 10 x passage to determine mutant stability. Further selection of 2nd, 3rd and 4th generation mutants was performed at 2 x MIC.

2.9 Isolation and Analysis of the Outer Membrane Proteins (OMPs)

The OMPs were extracted from exponentially growing cells using previously described methods (Masuda, Sakagawa and Ohya, 1995). A 10ml aliquot of an overnight culture was used to inoculate 2L of pre-warmed LB broth (20g Tryptone (Difco), 10g Yeast Extract (Difco), 10g NaCl). The broth was incubated at 35°C with vigorous shaking in an orbital incubator until log-phase (3-4 hours). The cells were harvested by centrifugation at 8000g for 15 minutes. The supernatant was

discarded and the cells resuspended in 40ml 30mM Tris-Cl pH 7. The cell suspension was sonicated on ice at an intensity of 7 microns for 30 seconds on and 30 seconds off for 5 minutes and the unbroken cells pelleted at 8,000g for 10 minutes. The supernatant was removed and centrifuged at 100,000g for 1 hour to pellet the membranes. The supernatant was removed and the pellet resuspended in 2ml 1.5% sarkosyl to solubilise the inner membrane and incubated at room temperature for 30 minutes. A final centrifugation at 100,000g pelleted the sarkosyl-insoluble outer membranes, which were resuspended in 0.5ml Tris-Cl pH 7 or pyrogen-free water and frozen at -70°C until needed. To increase the yield of OMPs, a modification to the method was as follows: after sonication, sarkosyl was added and incubated as described previously. The cell suspension was centrifuged at 8,000g for 10 minutes to remove unbroken cells and then ultracentrifuged as described. This did not alter OMP profiles.

2.9.1 SDS-PAGE of Outer Membrane Proteins

The outer membrane proteins were separated by SDS-PAGE (Laemmli, 1970). OMPs were standardised to 0.6mg protein/ml in sample buffer (62.5mM Tris-HCl pH 6.8, 0.2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 3 minutes before loading onto a 4% stacking gel and 10% separating gel (acrylamide/bisacrylamide 37:1) in electrode buffer (25mM Tris-HCl pH 8.8, 0.2% SDS). A constant current of 16mA was applied for the loading phase and the current was increased to 24mA for separating. Molecular weights were determined from standard bands using Precision Protein Standards (Bio-Rad) and were detected with Coomassie brilliant blue. All gel analysis was carried out using Quantity One software (Bio-Rad).

2.9.2 Visualisation of Protein Bands: Coomassie Stain

Gels were stained with Coomassie brilliant blue in a 1-step stain, 4-step destain method (Table 2.3) or silver stained.

Table 2.3. Coomassie stain reagents and protocol

Step	Coomassie brilliant blue R-250	Propan-2-ol	Methanol	Acetic acid	Distilled H_2O
1(overnight)	1g	500ml	-	200ml	1300ml
2 (1 hour)	100mg	200ml	-	200ml	1600ml
3 (1 hour)	48mg	-	-	200ml	1800ml
4 (1 hour)	-	-	800ml	200ml	1000ml
5 (leave)	-	-	-	200ml	1800ml

2.9.3 Visualisation of Protein Bands: Silver Stain

The samples were treated and run on SDS-PAGE as previously described. After running the gel, it was placed in prefix A for 30 minutes (50% methanol, 10% acetic acid), prefix B for 30 minutes (5% methanol, 7% acetic acid), fixed in 10% glutaraldehyde (BDH) before being washed twice in distilled water and left in distilled water overnight. The water was discarded and the gel was treated with 100ml 5mg/L dithiothreitol for 30 minutes. An ammoniacal silver nitrate solution was added to the gel (0.6% $AgNo_3$, 0.076% NaOH, 1.4ml ammonia solution (BDH

analar, SG 0.88) for 15 minutes and then washed in 4 x 200ml distilled water. 200ml developer was added (0.005% citric acid, 0.019% formaldehyde solution (BDH analar, 38-40%) and left until protein bands were visible (Microbial Pathogenicity Research Laboratories protocol).

2.9.4 Ion-Exchange, Electroelution and Amino Acid Sequencing of a 45.5kDa Porin

In order to purify an OMP of 45.5kDa from strain BE1, a sample of total OMPs was solubilised in the non-ionic detergents $C_{12}E_8$ (2% w/v) or Triton-X100 (1% v/v) and separated on a GradiFrac FPLC system using an ion-exchange Hi-Trap Q Sepharose (anion exchanger) or Hi-Trap SP (cation exchanger) 1ml column (Pharmacia). A pH range of 4-8 in 1 pH intervals of sodium acetate was used for the SP column and a pH range of 5-8 in 1 pH intervals of 20mM Tris-HCl for the Q column and the proteins were eluted with a NaCl solution (1M) concentration gradient of 0-100% for 30 ml with a flow-rate of 1ml/min. The recovered fractions were separated by SDS-PAGE as previously described. The ion-exchange column diluted the protein to a level where it would not be visualised with a coomassie stain therefore the more sensitive silver stain was used. As this did not purify the OMP of interest, the proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (PVDF) (Matsudaira, 1987). A pre-wetted PVDF membrane was applied to an SDS gel containing the separated proteins and a constant voltage of 50V applied for 30 minutes at room temperature to transfer the proteins. The transfer buffer was 90mM CAPS 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol pH11. The membrane was stained with 0.1% Coomassie Blue R-250 in 40% methanol, 1% acetic acid for 1 minute and destained with 50% methanol before air-drying. The 45.5kDa band of interest was excised from the membrane and N-terminally sequenced at GlaxoSmithKline laboratories, Collegeville, PA, USA.

2.10 Pulsed-field gel electrophoresis (PFGE)

Typing of *A. baumannii* was performed by PFGE using a modification of previously described methods (Seifert *et al*, 1994). Cells were grown overnight in LB broth (Gibco BRL) at 35°C and harvested by centrifugation at 10,000g for 20 minutes. After washing twice in SE buffer (75mM NaCl, 25mM EDTA, pH 7.4), the cells were adjusted to 10^9 cfu/ml in SE buffer. 500µl of cells were added to 700µl of 2% low-melting agarose (Sea-plaque) in SE buffer and kept at 55°C until put into plugs. The plugs were incubated in lysis buffer (50mM Tris, 50mM EDTA, 1% sarkosyl, 1mg/ml proteinase K, pH 8.0) for 24 hours at 55°C in a water bath. Cell lysis was complete when the plugs change from opaque to clear. After washing in TE buffer 5 times, the DNA plugs were restricted with 20 units of *Apa*I overnight at 37°C. The plugs were placed into a 1.2% PFGE-grade agarose gel and run in 0.5xTBE buffer (45mM Tris, 45mM boric acid, 1mM EDTA, pH 8.0) for 24 hours at 4.5 volts/cm with a pulse time of 5-20 seconds at 14°C. Finished gels were stained in ethidium bromide and visualised on a u/v transilluminator.

Chapter 3: Speciation of Acinetobacter Isolates using tRNA fingerprinting

3.1 Introduction

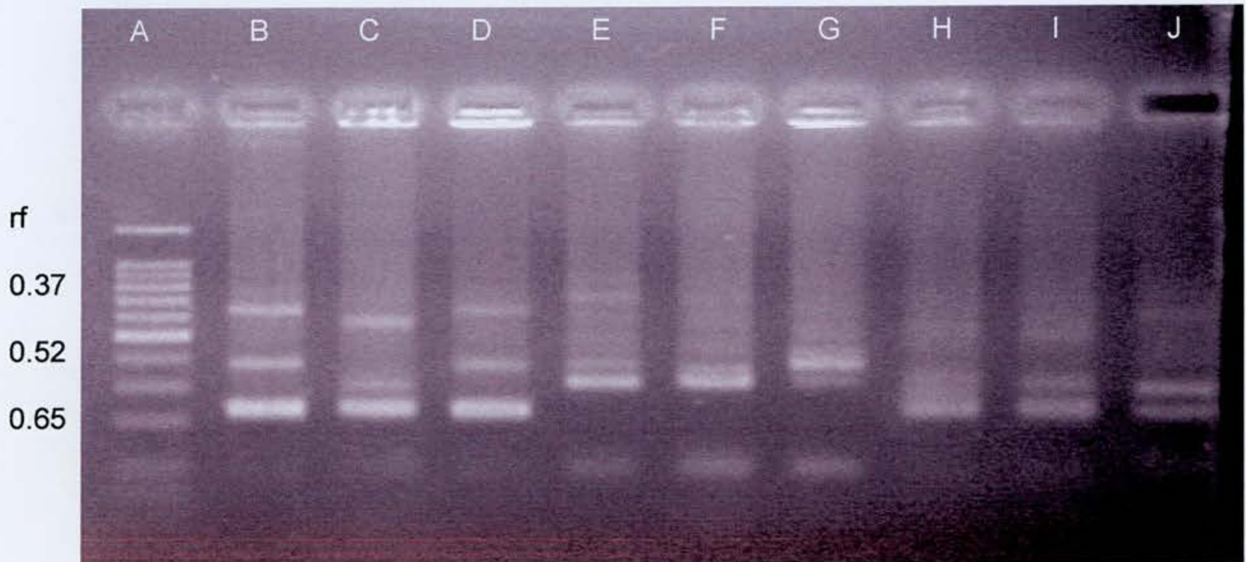
The molecular method of tRNA fingerprinting was used to speciate a collection of *Acinetobacter* bloodstream isolates from the University of Iowa College of Medicine. They were originally speciated by Vitek as: 48 *A. baumannii*, 18 *A. anitratus*, 18 *A. lwoffii*, 13 *A. calcoaceticus* and 4 *Acinetobacter* spp. The method of tRNA fingerprinting relies on species-conserved spacer regions of tRNA (Lu *et al*, 2000). PCR of isolates using universal primers yields unique species-specific banding patterns that are compared to the profiles of known DNA groups. However, this method cannot differentiate between genospecies 1 (*A. calcoaceticus*) and genospecies 3, therefore they will be grouped together as species 1/3 (Ehrenstein *et al*, 1996). Differentiation between species 2 (*A. baumannii*) and 13TU is also not possible, and though there was no standard 13TU used in this study, any isolate with the species banding pattern of *A. baumannii* will be grouped as species 2.

3.2 Results

One of the strains was immediately discarded as it fermented glucose and acinetobacter is a non-fermenter. The colony morphology of this strain was very different from acinetobacter; turning MacConkey agar purple and having a very distinct smell, akin to brewing. Of the remaining 100 strains, tRNA fingerprinting was carried out at least twice per strain. The first round was done numerically according to strain number; i.e. SB1, SB2... etc. The second round of speciation grouped like-species together to ensure that the correct primary identification had been made. Each round of PCR was conducted in tandem with the standard strains. Figure 3.1 shows the fingerprinting pattern of the standard strains. The gels did not

always photograph clearly therefore species identification was determined directly on a u/v transilluminator and by using BioRad Quantity One software to compare banding patterns.

Figure 3.1. Fingerprint of the standard strains. Lane A: 100bp Marker, B-I: genospecies 1-8, J: genospecies 12, rf: relative front



Lanes B and D (species 1 & 3) have identical patterns and therefore cannot be distinguished using this method. The other strains have different patterns, with at least 3 bands per strain that are distinguishable with ethidium bromide staining. The faint bands were not always clear on a gel and the molecular weights of the bright bands were used for visual matching. For example, species 1 & 3 have two bright bands corresponding to approximately 380bp and 250bp, while species 2 has two bands at 300bp and 250bp.

Figure 3.2 shows the banding profiles of lanes B, C & D using Quantity One software. This reiterates that species 1 and 3 have the same pattern and that using the software one can discriminate between the other species. The lane profiles of the other species are shown in Appendix III. In determining species identity using the

software, the intensity of the band is unimportant as this is related to the concentration of PCR product on the gel. It is the pattern of peaks, corresponding to the bands, that was used to determine species identity.

Using this method, the *Acinetobacters* were reclassified as 9 genospecies 1/3, 71 genospecies 2 (*A. baumannii*), 7 genospecies 4 (*A. haemolyticus*), 2 genospecies 5 (*A. junii*), 2 genospecies 7 (*A. johnsonnii*), 1 genospecies 8 (*A. lwoffii*) and 2 genospecies 12 (*A. radioresistens*). The remaining 6 isolates either did not amplify by PCR or their banding pattern did not match against the 9 standard DNA groups used in this study, therefore they remain unspeciated. Appendix IV lists all strains with their original species identification alongside the results of this study.

Figure 3.2. Banding pattern of Figure 3.1

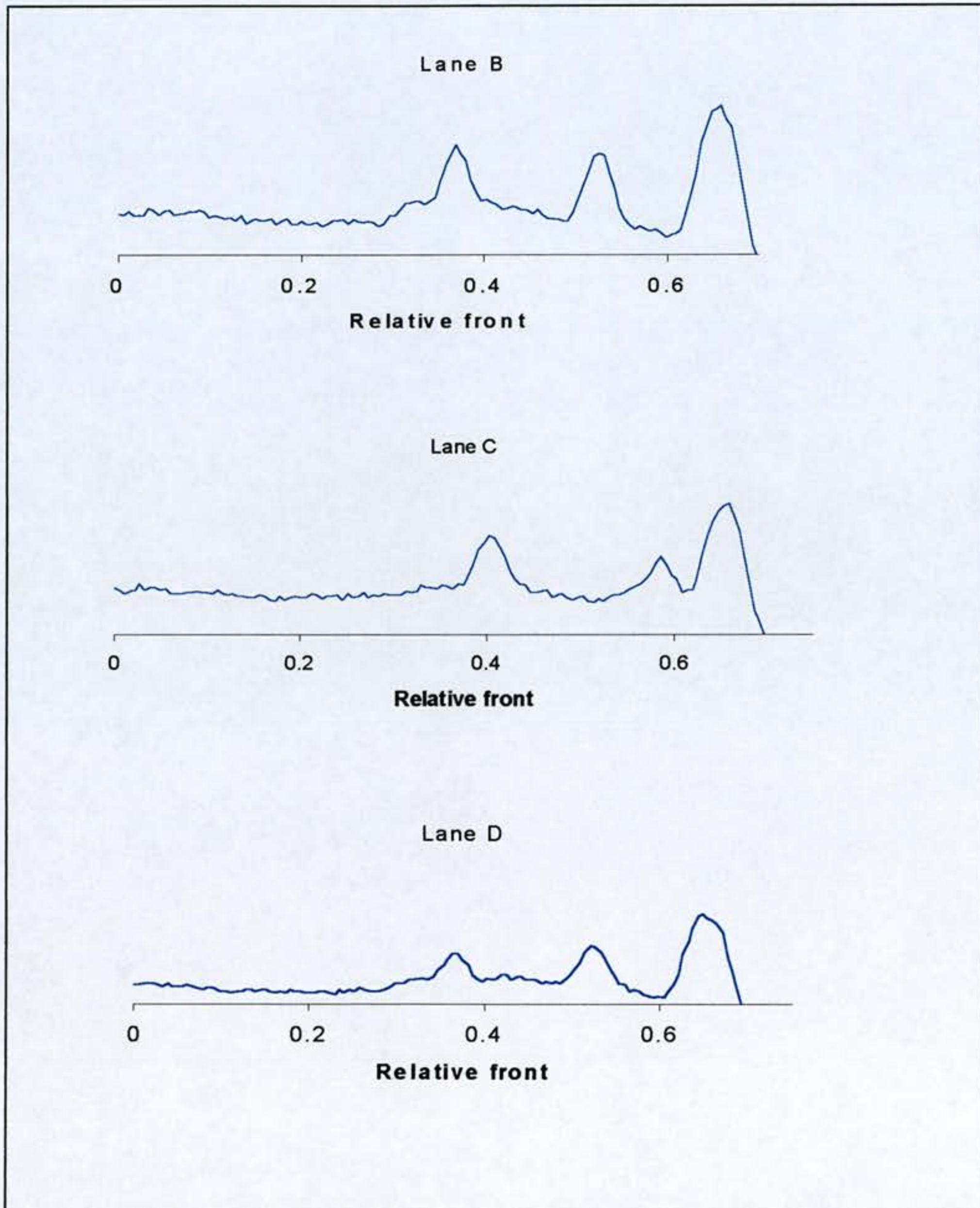
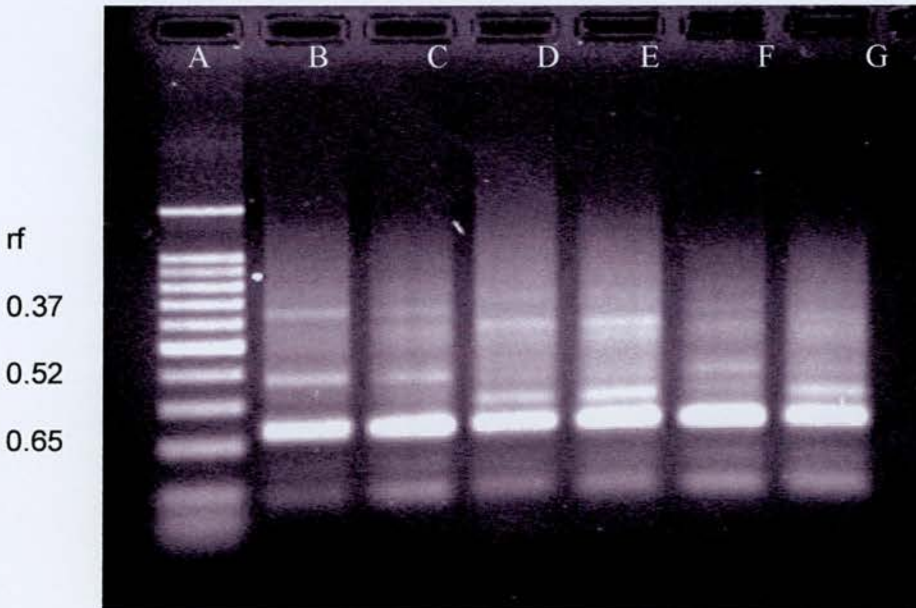


Figure 3.3 shows a typical gel with species 1, 2 & 3 and unspeciated isolates. A visual match to speciate the isolates show that SB91 & SB101 are species 2 and that SB94 is species 1/3. This is confirmed in Figure 3.4 with the intensity profiles of two standard strains and two of the unknowns. The peaks to right of the graph

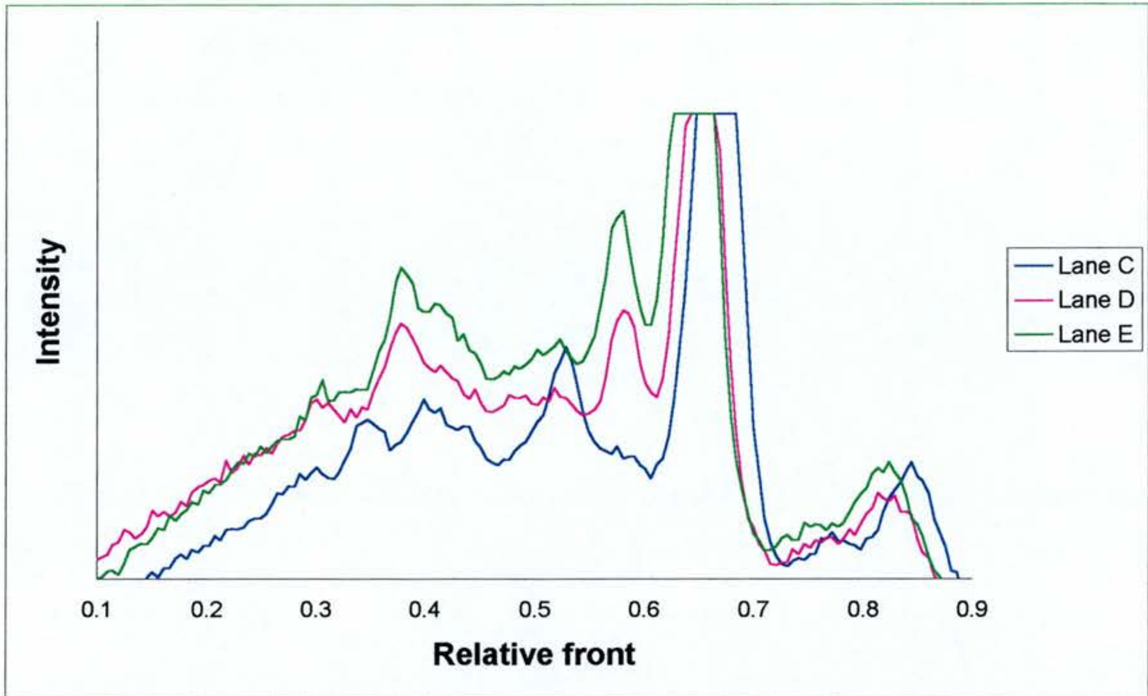
correspond to primer dimers and are disregarded. The presence of primer dimers was not found in all gels (Figure 3.8).

Figure 3.3. Lane A: 100bp marker, B: sp1, C: sp3, D: sp2, E: SB91, F: SB94, G: SB101, rf: relative front



Quantity One software allows for the direct comparison of lanes within a gel as shown in Figure 3.4. This figure shows that the blue line, corresponding to species 3 (Lane C), has a different profile to that of lanes D and E (species 2).

Figure 3.4. Band intensity of Figure 3.3



This PCR fingerprinting technique does not guarantee success as an isolate does not always amplify by PCR, illustrated with SB95 (Figure 3.5 lane M). Indeed, some of the isolates proved very difficult to speciate because of this failure, needing multiple PCR before any product was seen. The banding pattern of lane C in Figure 3.5 is not clear and speciation is difficult. By comparing the lane banding patterns using Quantity One software as shown in Figure 3.6, its pattern closely matches that of lane L (species 7) but is different from lane I (species 8). The background smearing and faint bands of some isolates make species identification difficult which is why the procedure was performed more than once. In Figure 3.5, lanes D, E and F match genospecies 4 (lane B) and their intensity profiles confirm this (Figure 3.7).

Figure 3.5. Lane A: 100bp ladder, B: sp4, C: SB31, D: SB56, E: SB58, F: SB92, G: sp5, H: SB85, I: sp8, J: SB100, K: sp6, L: sp7, M: SB95, N: sp1, O:sp2, rf: relative front

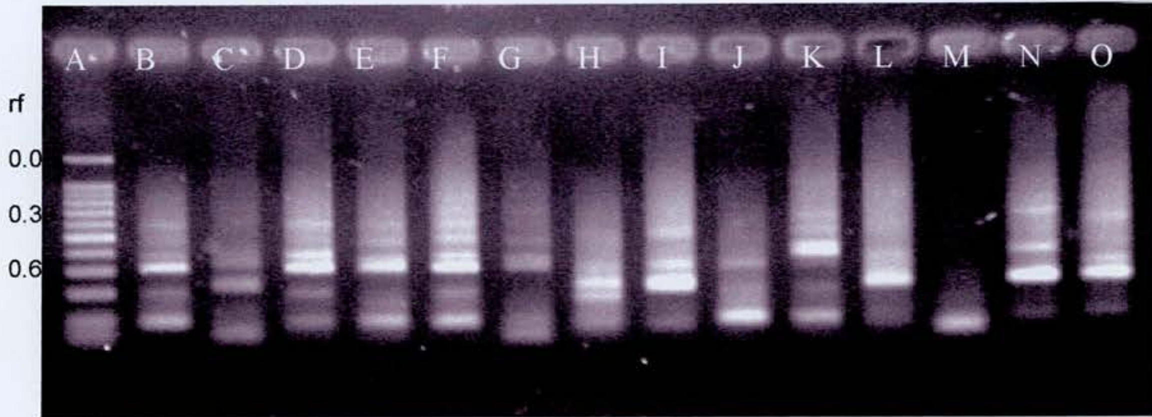


Figure 3.6. Banding pattern of lanes C, I & L from Figure 3.5

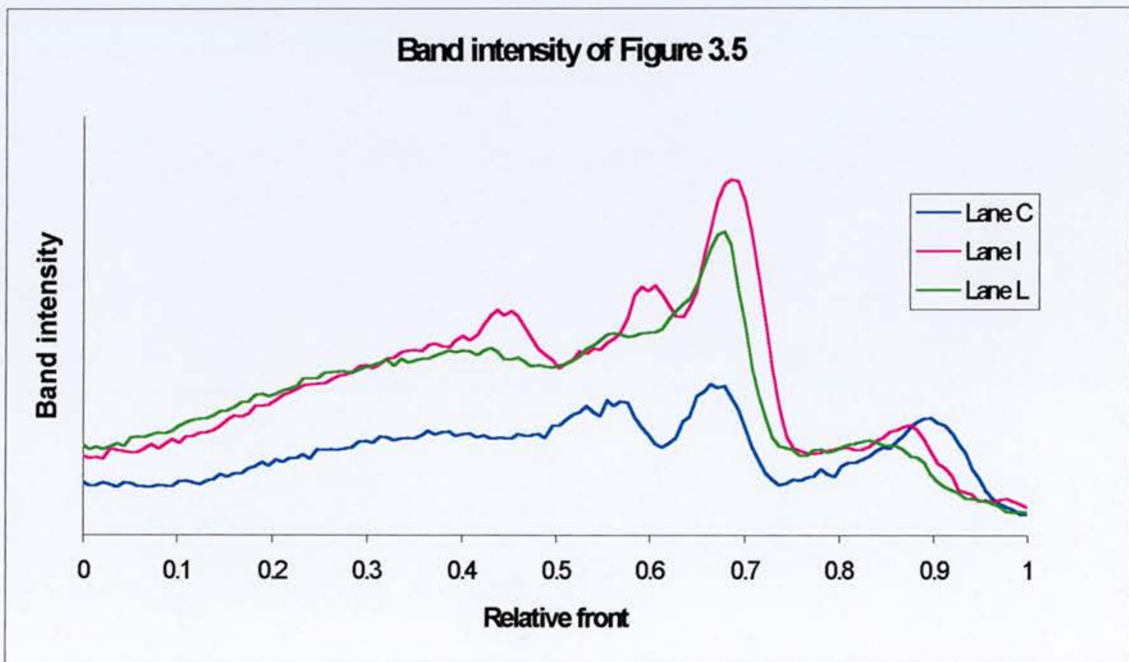


Figure 3.7. Banding patterns of lanes B, D, E & F

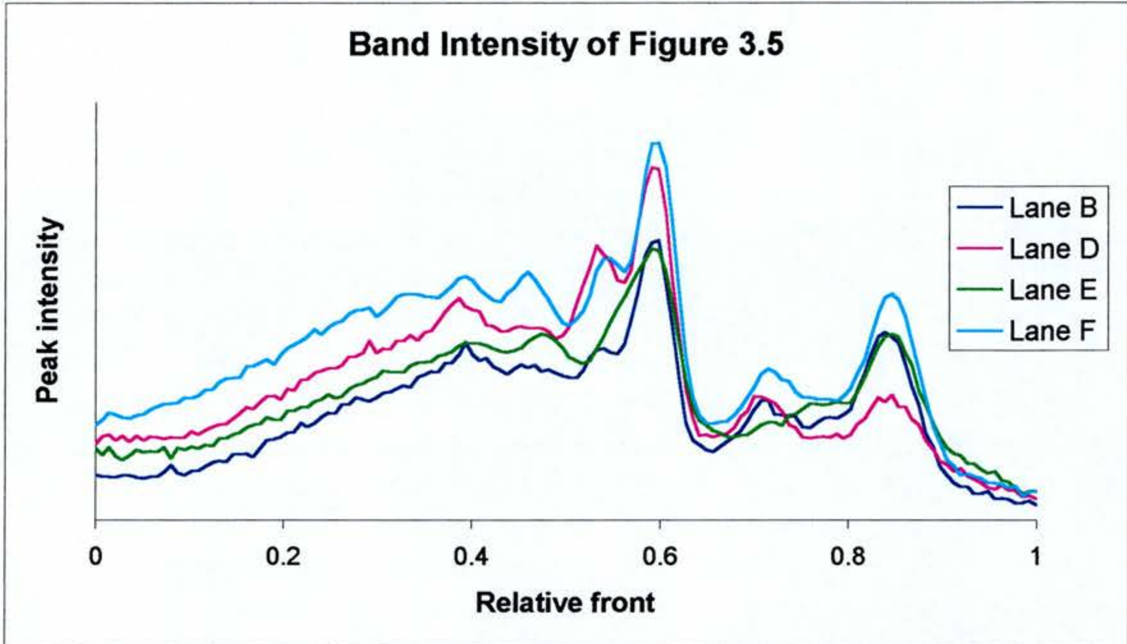
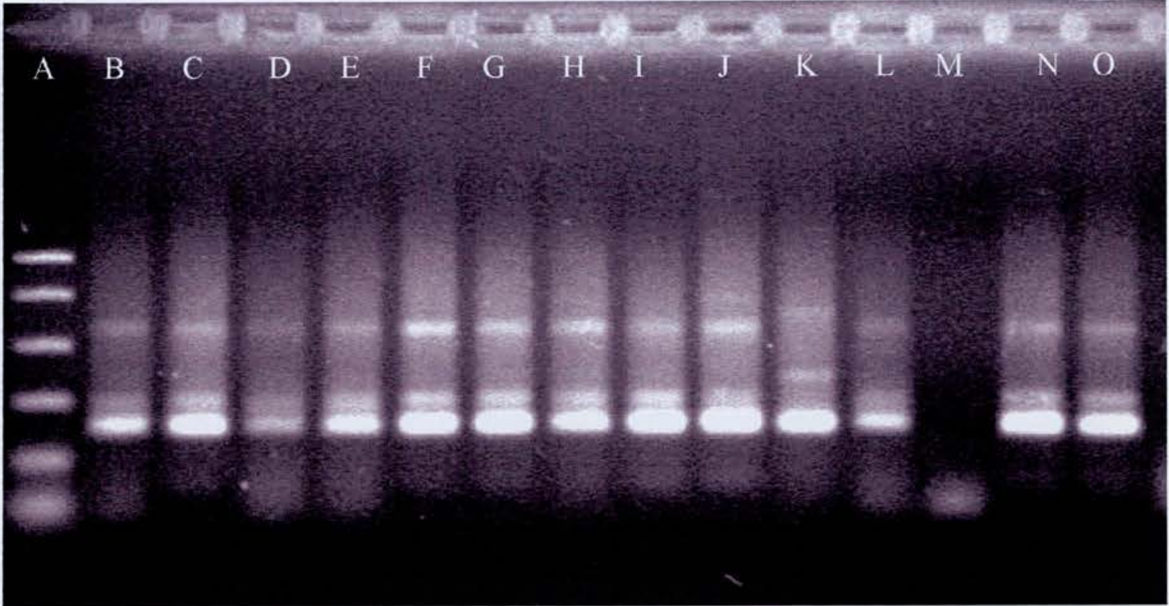


Figure 3.8 shows a second round of speciation. In this gel, all the isolates were identified as belonging to species 2 with the exception of lane K which has the fingerprint characteristic of species 1/3. This gel is easier to interpret when compared to Figure 3.5 as there are fewer patterns.

Figure 3.8. Lane A: PCR Ladder, B: sp2, C: SB41, D: SB43, E: SB46, F: SB45, G: SB47, H: SB49, I: SB52, J: SB53, K: SB57, L: SB62, M: negative control, N: SB64, O: SB67



3.3 Discussion

The use of universal primers to speciate organisms was described by Lu *et al* (2000). Their method included the digestion of the PCR products with a variety of restriction enzymes. Ehrenstein *et al* (1996) omitted this step and instead used fluorescene-labelled primers. In the present study, restriction of PCR products and the use of labelled primers were deemed unnecessary as the PCR yielded at least three species specific bands that were visible upon ethidium bromide staining. The use of Quantity One software gave similar profiles as those published by Ehrenstein *et al* (1996). These were sufficient to speciate with a high degree of certainty. The

technique did not always yield clear banding patterns and strains had to be analysed more than once to be certain of species identification.

The speciation of *Acinetobacter* spp. has been the subject of many studies and it was not until the advent of molecular techniques that the taxonomy began to be resolved. In particular, DNA-DNA hybridisation has been used extensively to speciate the Acinetobacters into 19 DNA-groups (I. Tjernberg, PhD thesis). Prior to this, taxonomy was based on phenotypical parameters. However these do not adequately differentiate between DNA groups 13, 14 & 15TU and 16 & 17BJ. Even in the present study, differentiation between genospecies 1, 2, 3 & 13TU was not possible owing to the limitations of the technique.

The BioLog system was tested by Bernards *et al* (1995) on 129 *Acinetobacter* strains belonging to 13 DNA groups. This system clustered species 1, 2, 3 & 13TU together as well as 4 & 6 and 10& 11. Only species 7 and 14 were unclustered. However, six strains of four different groups did not cluster with their respective DNA groups, showing the systems limitations. The commonly used API20NE also cannot distinguish between species 1, 2, 3 & 13TU, the Acb complex (Bernards *et al*, 1996). This may in part be caused by limitations of the database that holds species information. As *Acinetobacter* is not considered to be a major pathogen, there may be little commercial interest in developing the database and chemical tests to cover all the DNA groups. Perhaps clinicians are happy with the loose term *Acinetobacter*? In any event, as most commercial tests are used by routine clinical laboratories it is probably more important to determine a source of infection than to get bogged down in taxonomy (I. Tjernberg, personal correspondence). Thus typing of isolates may be given higher priority.

Automated machines not only speciate organisms, they also generate antibiotic sensitivity data. This must prove useful to clinicians; being able to name an organism and what drugs may be used for treatment. However the acinetobacters not only suffer from mis-speciation with automated machines, but also false sensitivity testing. This is described in a study by Tsakris *et al* (2000). A Vitek GNS-506 had

recorded a high-prevalence of imipenem resistant *A. baumannii*. However, upon disc diffusion, agar dilution and broth microdilution, it was found that all 35 imipenem resistant strains were sensitive.

In the present study, the finding that out of an original 18 *A. lwoffii* there was only 1 speciated by a molecular technique is not surprising. O'Hara and coworkers (1997) evaluated Vitek and found that it gave a 1 in 5 positive result for *A. lwoffii*. However the authors were basing the accuracy of the machine against strains that had previously been biochemically tested. If in their study they had used characterised DNA groups then the accuracy of their study would be more valid. In a more recent study, Joyanes *et al* (2001) found that a Vitek 2 correctly identified only 76% of *A. baumannii*.

Not all biochemical testing to speciate the acinetobacters has such a low rate of success. The phenotypic identification scheme of Bouvet and Grimont has been shown to correlate well with DNA-DNA hybridisation (Bouvet and Grimont, 1987; Seifert *et al*, 1997). The method of Bouvet and Grimont utilises 14 carbon sources, only two of which are used in Vitek and three in the Crystal E/NF identification systems. The growth of an organism over a temperature range is also used by Bouvet and Grimont, something which neither of the automated systems can do, which is the crux of the matter; they are useful for speciating "regular" bacteria but not for atypical nosocomial pathogens like the acinetobacters.

However, one cannot criticise diagnostic laboratories for using biochemical testing and automated machines, as they need rapid identification of samples and do not have the resources for more involved study. The Vitek 2 system in the study by Joyans gave a result in only 3 hours, something which tRNA fingerprinting cannot achieve. The problem with the machines is more to do with the fact that they have to differentiate a broad range of organisms. A species can only be given a name when it is phenotypically distinguishable from other members of its genus. Only seven of the 19 *Acinetobacter* DNA groups fit this criteria, the most obvious being the Acb complex. However the tRNA of *A. baumannii* and 13TU are very similar and

explains why they cannot be speciated by this technique. Even by DNA-DNA hybridisation they have a similarity of level of 70%, which is the cut-off value for new DNA groups (A. T. Bernards, PhD thesis).

There exists other PCR-based methods which can be used to identify *Acinetobacter* such as ARDRA which relies on amplified ribosomal DNA which is then restricted with a variety of restriction enzymes (Berlau *et al*, 1999; Koeleman *et al*, 1997; Lu *et al*, 2000). However this method may need up to 6 different restriction enzymes making it considerably more laborious and expensive, both in materials and manpower, than tRNA fingerprinting.

In conclusion, tRNA fingerprinting, even with its limitations, is a more accurate technique for species identification than a commercial system. Owing to its lack of discrimination between the Acb complex, it is not as discriminatory as DNA-DNA hybridisation. However tRNA species identification is rapid, reliable, cheap and easy to interpret, therefore is a useful tool that could easily be used in a routine laboratory.

Chapter 4: Killing Kinetics of Seven Fluoroquinolones against *A. baumannii* ATCC 19606

4.1 Introduction

The action of the fluoroquinolones *in vivo* is bactericidal, but unusually these drugs exhibit a biphasic dose-response. This is measurable *in vitro,* where the lethality of the drug increases proportionally to its concentration until an optimum bactericidal concentration (OBC) is reached. Above this point, the bactericidal activity steadily decreases with further increases in concentration. The newer fluoroquinolones have been predominately tested against Gram positive species and the biphasic response has seldom been demonstrated. In this chapter, the fixed-time killing activity of increasing concentrations of fluoroquinolone on *A. baumannii* ATCC 19606 was compared against ciprofloxacin and levofloxacin and five new fluoroquinolones, and the time-dependant killing was examined at the OBC and at 4xMIC.

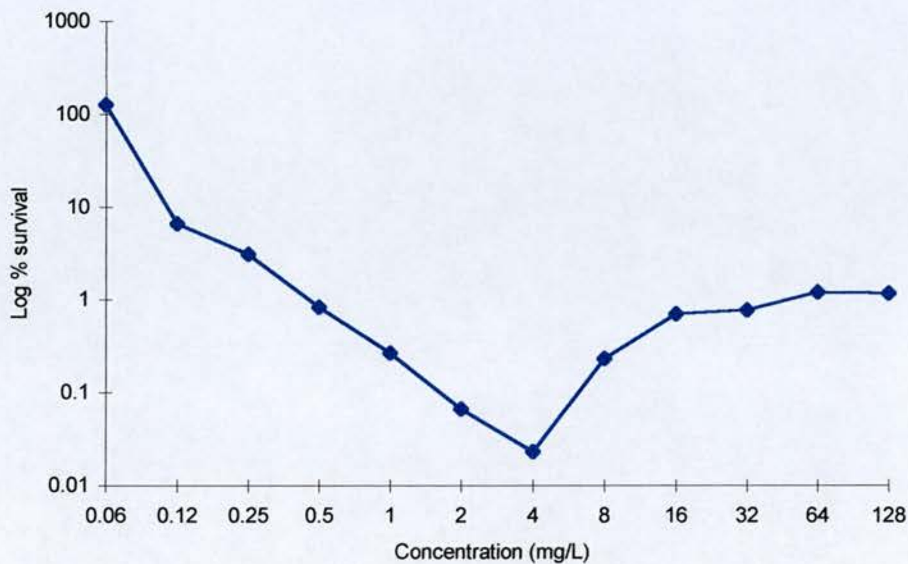
4.2.1 Results: Dose Response

Table 4.1 shows the MIC and OBC values for the antimicrobials used against *A. baumannii* ATCC 19606. A representative graph to determine the OBC of gemifloxacin is shown in Figure 4.1.

Table 4.1. MIC and OBC values for the fluoroquinolones tested against *A. baumannii* ATCC 19606

Antimicrobial	OBC (mg/L)	MIC (mg/L)	4 x MIC (mg/L)
Ciprofloxacin	4	0.5	2
Gemifloxacin	4	0.12	0.5
Grepafloxacin	8	0.12	0.5
Levofloxacin	4	0.5	2
Moxifloxacin	4	0.25	1
Sparfloxacin	8	0.12	0.5
Trovafloxacin	4	0.06	0.25

Figure 4.1. Dose response of *A. baumannii* ATCC 19606 to gemifloxacin.



All the drugs tested exhibited a biphasic dose-response, similar to that shown in Figure 4.1, with the lowest point of the graph corresponding to the OBC. The MIC of the drug is also seen in the graph where the percentage survival drops below 100%. In the case of gemifloxacin, the MIC by agar dilution is 0.12mg/L. The graph cuts below 100% survival at a concentration just above 0.06mg/L, thus the MIC is 0.12mg/L. The killing rate decreases with concentrations above the OBC.

4.2.2 Results: Kill-Curves

The killing kinetics for the fluoroquinolones at both the OBC and four-times the MIC are shown in Figures 4.2–4.8. The most rapid killing is within the first hour of drug challenge, with the greatest rate seen at the OBC. However, with both gemifloxacin and levofloxacin, there is little difference in killing between the concentrations. The rate of kill decreases after 1 hour. One of the most striking results is that after 24 hours, none of the drugs had killed 100% of the challenge organism.

There is little difference between killing rates at the OBC and 4xMIC with gemifloxacin (Figure 4.2). A reduction in viable organisms of $>2_{log10}$ is seen in the first hour at both concentrations, and after 24 hours the number of organisms has been reduced by 5_{log10}. With trovafloxacin (Figure 4.3), a greater difference is seen between the two concentrations. At the OBC, there is $>2_{log10}$ reduction in viable cells compared with $<2_{log10}$ at 4xMIC in the first hour. The difference is less pronounced after 24 hours, with both concentrations reducing cell numbers by 6_{log10} from the starting inoculum.

Moxifloxacin reduces cell numbers by 3_{log10} in the first hour at the OBC and 2_{log10} at 4xMIC (Figure 4.4). After 24 hours, both concentrations reduce cell numbers by $>5_{log10}$. Levofloxacin sees the greatest initial kill-rate in the first half-hour, reducing cell counts by 3_{log10} at both OBC and 4xMIC (Figure 4.5). Thereafter the drug remains almost bacteriostatic for a further 2.5 hours. After 24 hours there is a reduction in viable cells that is 6_{log10} less than the starting inoculum. Ciprofloxacin and grepafloxacin (Figures 4.6 and 4.7) exhibit similar killing rates in the first hour, reducing viable cell counts by 3_{log10} at the OBC and $<2_{log10}$ at 4xMIC and 5_{log10} after 24 hours. Sparfloxacin is the most potent of the drugs tested. In the first hour at the OBC, it reduced cell numbers by 4_{log10} (Figure 4.8). At 4xMIC, it is less potent, reducing cell numbers by 3_{log10}. After 24 hours the difference between the concentrations is still visible, with cell numbers reduced by 6_{log10} at the OBC and 4_{log10} at 4xMIC.

Figure 4.2. Bactericidal activity of gemifloxacin against *A. baumannii* ATCC 19606.

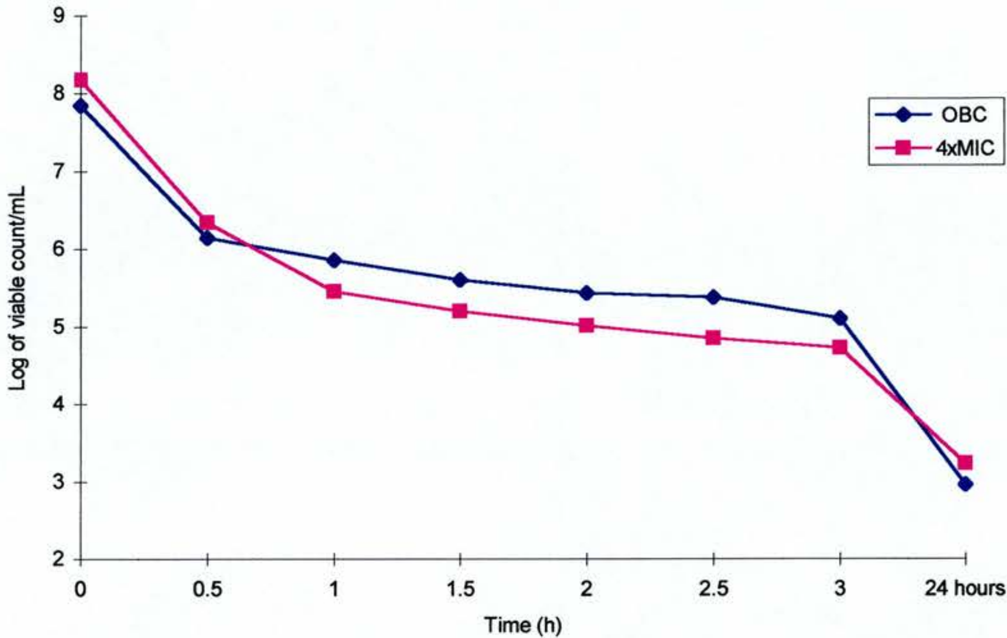


Figure 4.3. Bactericidal activity of trovafloxacin against *A. baumannii* ATCC 19606.

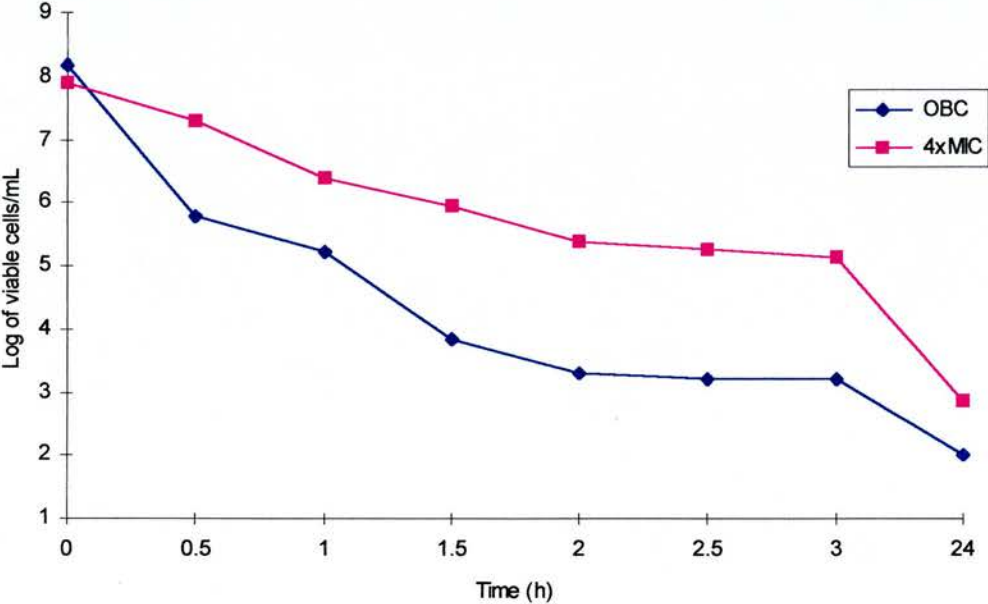


Figure 4.4. Bactericidal activity of moxifloxacin against *A. baumannii* ATCC 19606.

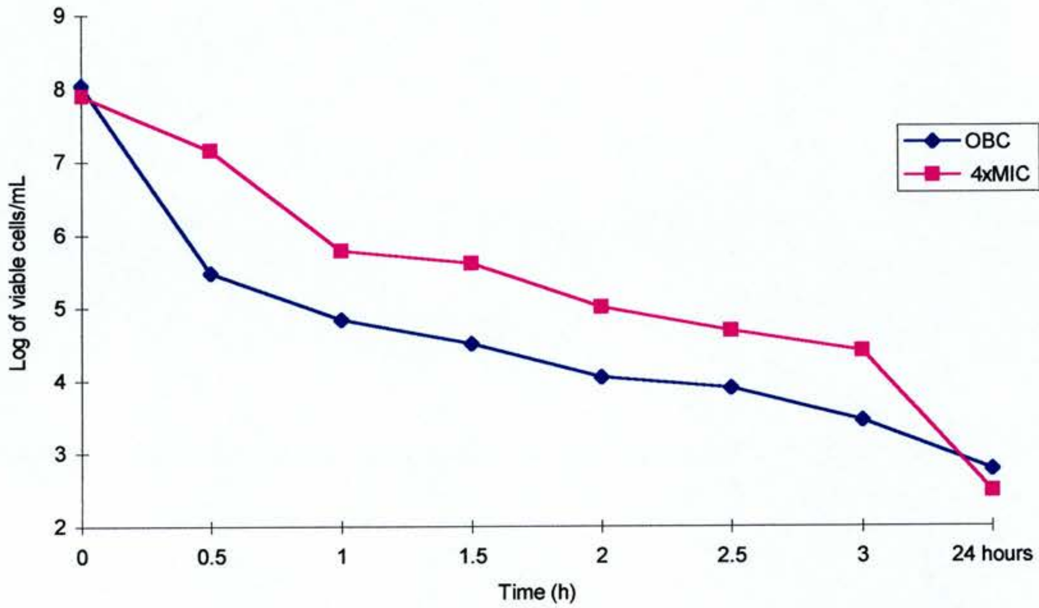


Figure 4.5. Bactericidal activity of levofloxacin against *A. baumannii* ATCC 19606.

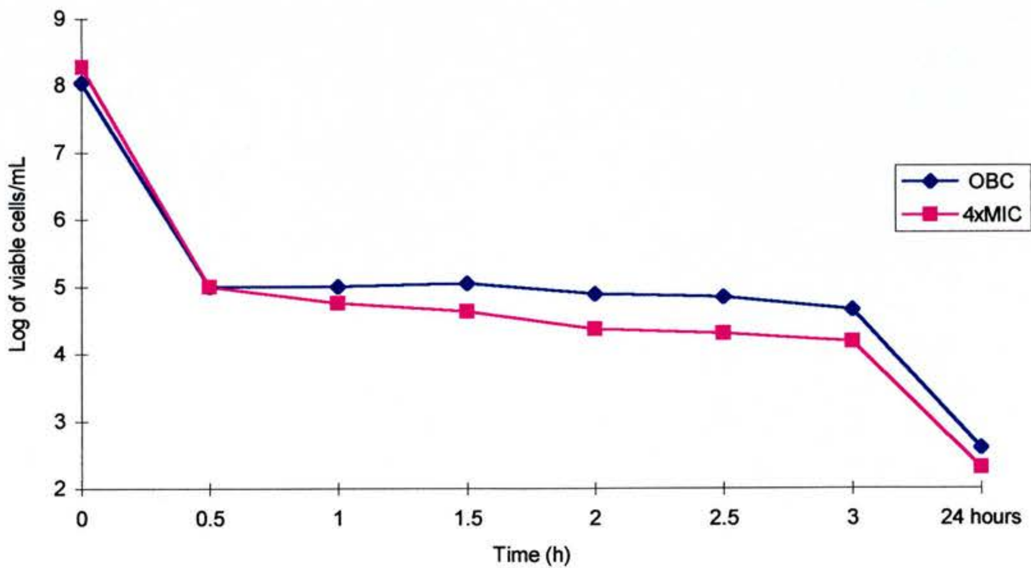


Figure 4.6. Bactericidal activity of ciprofloxacin against *A. baumannii* ATCC 19606.

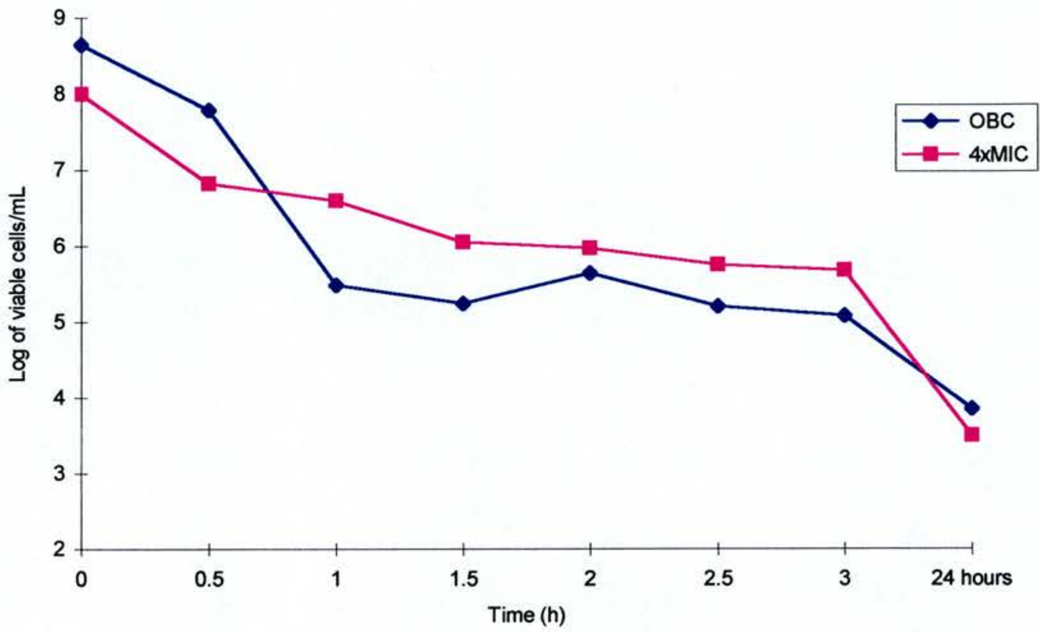


Figure 4.7. Bactericidal activity of grepafloxacin against *A. baumannii* ATCC 19606.

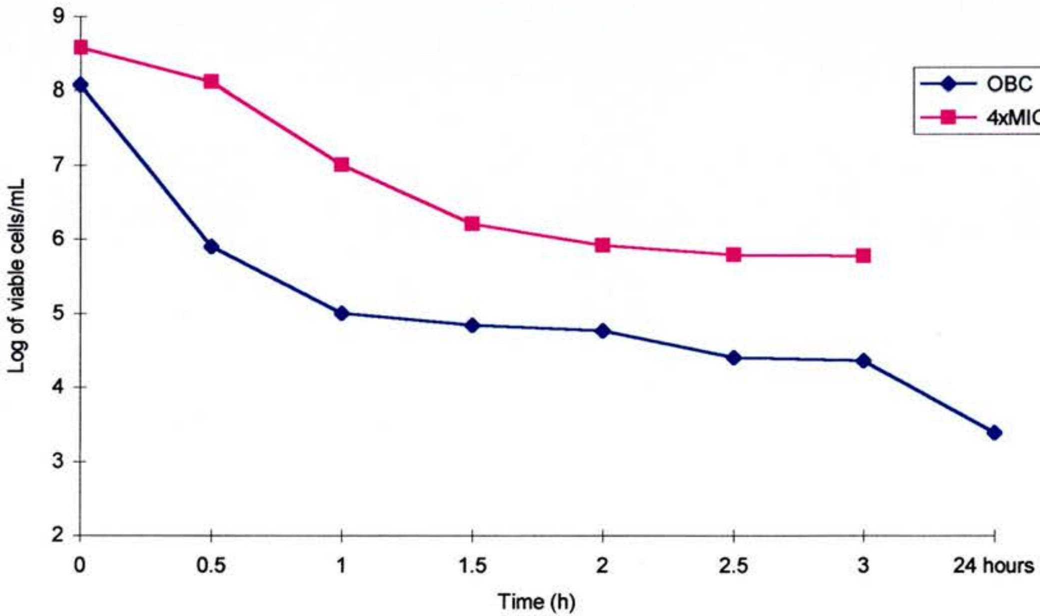
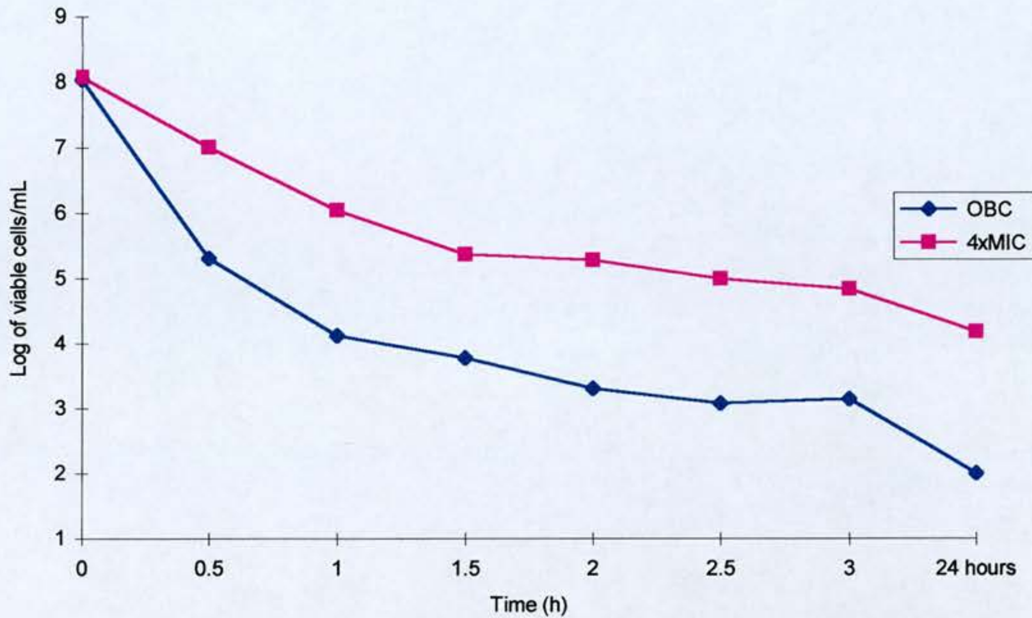


Figure 4.8. Bactericidal activity of sparfloxacin against *A. baumannii* ATCC 19606.



4.3 Discussion

In this study all test fluoroquinolones exhibited the typical biphasic response of this drug class. The OBCs of these agents were found to be within one dilution of each other, indicating that the killing mechanism for each drug is similar even though their respective MICs vary greatly. A similarity in OBC values has been reported by Lewin *et al* (1992) who found that the *E. coli, S. aureus* and *S. epidermidis* OBCs of sparfloxacin were identical at 0.9mg/L. In the same study, the OBCs for ciprofloxacin were 3mg/L for *S. aureus* and *S. epidermidis* and 1.5mg/L for *E. coli*. In the present study, the OBCs for these drugs were 8mg/L and 4mg/L respectively, which may reflect the innate resistance of *A. baumannii* to these drugs.

The biphasic-dose response is poorly understood. Crumplin and Smith (1975) have shown that at the most bactericidal concentration, nalidixic acid inhibits DNA synthesis but not RNA or protein synthesis. At higher concentrations where it is less

bactericidal, nalidixic acid appears to inhibit both RNA and DNA synthesis. The OBC also correlates with the maximum SOS response (Lewin *et al.*, 1989). This is not surprising as quinolones cause the formation of cleaved complexes of DNA and this would be expected to elicit an SOS response. However, this does not mean that the SOS response is responsible for cell-death. SOS mutants of *E. coli* are not any less susceptible to quinolones than their SOS wild-type. However, it has been shown that SOS-independent recombination-repair aids survival and that recombination-repair mutants are more susceptible to nalidixic acid (Lewin *et al* 1989).

Although bacterial killing is optimal at the OBC, this concentration is not necessarily found *in vivo,* as the peak-serum levels (C_{max}) of the fluoroquinolones do not reach these concentrations. For example, Morrissey (1997) cites C_{max} values for ciprofloxacin and trovafloxacin as 2.6mg/L and 3.0mg/L respectively. Their OBCs against *A. baumannii* ATCC 19606 are greater than these values, therefore outside therapeutic levels. This has led Morrissey (1997) to propose the bactericidal index which links bacterial killing over a drug concentration range that is achievable *in vivo*. The bactericidal index gives a single value that represents the bactericidal activity for an antibiotic against a strain. Using this method, Morrissey demonstrated that trovafloxacin had greater bactericidal activity than ciprofloxacin against *E. faecalis.*

In the present study, the bactericidal index was not calculated but results achieved at four times the MIC are a much better indicator of a drug's *in vivo* efficacy than the OBC. These data show that gemifloxacin exhibited superior killing kinetics at four times the MIC compared with ciprofloxacin. Furthermore, its killing kinetics at four times the MIC were almost equal to those at the OBC even though the concentration was much lower (0.5 mg/L *versus* 4 mg/L, respectively). Only levofloxacin showed equal killing kinetics at the OBC and four times the MIC, though at a concentration four-times higher than that of gemifloxacin (2 mg/L *versus* 0.5 mg/L, respectively).

Trovafloxacin was found to be bactericidal against *A. baumannii* ATCC 19606, reducing viable cell counts by $6_{\log10}$ over 24 hours. Appleman *et al* (2000) reports

that trovafloxacin was bacteriostatic against multiresistant *A. baumannii*. The strains Appleman used were moderately susceptible to trovafloxacin (no MIC data given) suggesting that a GyrA mutation is present (Chapter 6). With a resistant Gyrase enzyme, the drug will be targeting topoisomerase IV. Given that the role of topoisomerase IV is to decatenate double-stranded DNA after DNA synthesis but before cell division, inhibition of this enzyme will lead to bacteriostasis and not cell-death. This suggests that cell-death may be cell-cycle dependant.

A working hypothesis of fluoroquinolone action is that upon drug challenge, cells that are undergoing DNA synthesis will be killed by the drug. Death is mediated through the action of DNA Gyrase. Cells that have completed DNA synthesis but have not yet divided will not be killed as the lethal cleavable complexes will not be formed. The fluoroquinolone will bind to topoisomerase IV and will thus be unable to decatenate their DNA and will filament. Filamentation has been described in fluoroquinolone-treated cells (Smith, 1984; Tanaka *et al*, 1996). This effect is also seen in cells with perturbed cell cycles (Domian *et al*, 1997; Holland, 1998).

The kill-curve assay is based on viable cell counts because of the filamentation phenomena and thus spectroscopic methods cannot be used to monitor cell-death by a reduction in turbidity. As the effect of fluoroquinolones is reversible, when the drug is removed from the cells and plated on drug-free media, the bacteria will be able to divide and thus be viable. Because time-kill assays are performed on log-phase bacteria, one will assume that most of the cells are actively synthesising DNA and thus are susceptible to drug challenge. However, a proportion of the cells will be in their G2 phase and upon drug challenge, bacteriostasis will occur. This hypothesis explains why there is such a rapid kill-rate in the first hour of fluoroquinolone challenge and thereafter kill-rates slow down. To put this to the test, cells will have to be synchronised to be at the same part of the cell-cycle before drug-challenge.

In conclusion, these data show that the newer fluoroquinolones are bactericidal against *A. baumannii* ATCC 19606. The initial one hour period shows the greatest kill-rate, thereafter the kill-rate slows down. However, none of the drugs tested

killed 100% of the initial inoculum after 24 hours. The OBC drug concentration is not achievable *in vivo* therefore a concentration of four times the MIC was also used. This showed that the newer fluoroquinolones are more potent than ciprofloxacin but not more so than levofloxacin.

Chapter 5: Clinical MICs

5.1 Introduction

Bacteria of the genus *Acinetobacter*, particularly *Acinetobacter baumannii*, are increasingly being isolated from hospitalised patients. In this setting, these pathogens are associated with bacteraemia, secondary meningitis, urinary tract infection and pneumonia, particularly in immunocompromised individuals. *Acinetobacter* spp. have become resistant to almost all currently available antimicrobial agents, including the aminoglycosides, older fluoroquinolones and broad-spectrum β-lactams. The majority of strains are resistant to cephalosporins and some demonstrate resistance to the carbapenems, including imipenem (Lyytikinen *et al*, 1995; Paton *et al*, 1993). The inability to eradicate these bacteria has allowed them to colonise those niches left vacant when more susceptible microbes are eradicated. Resistance to ciprofloxacin has been found in *Acinetobacter* spp. within the hospital setting (Rodriguez Bano, 1999; Villers *et al*, 1998), and is more pronounced in areas of high fluoroquinolone use, particularly where there is a history of prior ciprofloxacin use (Muder *et al*, 1991). This study compared the in-vitro activity by MIC of ciprofloxacin, gemifloxacin, grepafloxacin, gatifloxacin, levofloxacin, ofloxacin, moxifloxacin, sparfloxacin, trovafloxacin, imipenem, azithromycin and cefuroxime against the clinical isolates of Acinetobacter outlined in chapter 3.

5.2 Results

The range of MICs of the 12 antibiotics against the isolates are shown in Table 5.1. As there was a small number of isolates except for *A. baumannii*, MIC_{50}/MIC_{90} values were not calculated. These values have been calculated against species 2 and are shown in Table 5.1. The MICs of all the strains are shown in Appendix VI.

All the non-*A. baumannii* isolates tested were sensitive to imipenem, and with two exceptions, resistant to cefuroxime. Resistance to azithromycin was seen in all but three isolates. Only one isolate was sensitive to both azithromycin and cefuroxime. There was no correlation between strains in the higher MIC range against imipenem being in the high range against cefuroxime. Resistance to the fluoroquinolones was found primarily in *A. baumannii* although it was also found in one *A. calcoaceticus*/species 3 and one *A. haemolyticus* isolate.

Table 5.1 MIC range of *Acinetobacter* spp

Species	Antimicrobial	Range (mg/L)
1/3	Cip	0.12-128
(n = 9)	Gat	0.03-8
	Gem	0.03-16
	Gre	0.06-32
	Lev	0.12-16
	Mox	0.03-8
	Ofl	0.25-32
	Spa	0.03-8
	Tro	0.03-8
	Imi	0.06-0.25
	Cef	32-128
	Azi	1-4

Species	Antimicrobial	Range (mg/L)
2	Cip	0.015->128
(n = 71)	Gat	0.008-128
	Gem	0.008->128
	Gre	0.015-64
	Lev	0.008-64
	Mox	0.008-64
	Ofl	0.03->128
	Spa	0.008-32
	Tro	0.008-32
	Imi	0.008-2
	Cef	2->256
	Azi	0.12->128

Cip: ciprofloxacin, Gat: gatifloxacin, Gem: gemifloxacin, Gre: grepafloxacin, Lev, levofloxacin, Mox: moxifloxacin, Ofl: ofloxacin, Spa: sparfloxacin, Tro: trovafloxacin, Imi: imipenem, Cef: cefuroxime, Azi: azithromycin

Species	Antimicrobial	Range (mg/L)
4	Cip	0.06-0.25
(n = 7)	Gat	0.06-0.25
	Gem	0.03-0.12
	Gre	0.03-0.12
	Lev	0.12-0.5
	Mox	0.06-0.12
	Ofl	0.25-1
	Spa	0.015-0.06
	Tro	0.015-0.06
	Imi	0.06-0.25
	Cef	2-128
	Azi	0.5-2

Species	Antimicrobial	Range (mg/L)
5	Cip	0.12-0.5
(n = 2)	Gat	0.12-0.25
	Gem	0.06-0.12
	Gre	0.06
	Lev	0.12-0.25
	Mox	0.06-0.12
	Ofl	0.25-0.5
	Spa	0.03-0.06
	Tro	0.03
	Imi	0.12
	Cef	8-32
	Azi	0.5-2

Species	Antimicrobial	Range (mg/L)
7	Cip	0.12-16
(n = 2)	Gat	0.06-4
	Gem	0.06-1
	Gre	0.03-0.5
	Lev	0.25-4
	Mox	0.06-0.5
	Ofl	0.5-8
	Spa	0.015-0.5
	Tro	0.015-0.5
	Imi	0.008-0.25
	Cef	64
	Azi	0.25

Species	Antimicrobial	MIC (mg/L)
8	Cip	0.12
(n = 1)	Gat	0.03
	Gem	0.015
	Gre	0.015
	Lev	0.12
	Mox	0.06
	Ofl	0.25
	Spa	0.008
	Tro	0.008
	Imi	0.25
	Cef	32
	Azi	0.5

Species	Antimicrobial	Range (mg/L)
12	Cip	0.06-0.25
(n = 2)	Gat	0.06-0.12
	Gem	0.06
	Gre	0.06-0.12
	Lev	0.12-0.25
	Mox	0.06-0.25
	Ofl	0.25-0.5
	Spa	0.03-0.12
	Tro	0.03-0.12
	Imi	0.12-0.5
	Cef	32->128
	Azi	0.5-16

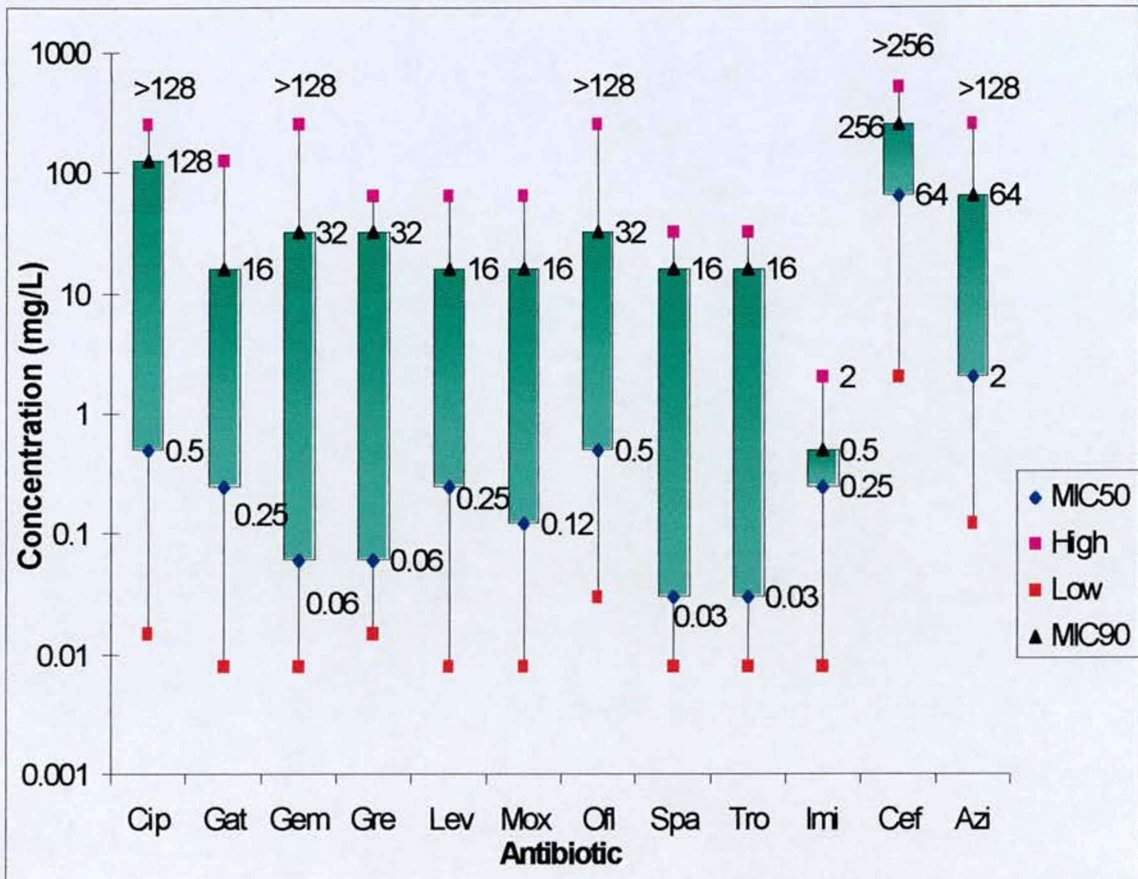
Trovafloxacin and sparfloxacin were the most potent of the fluoroquinolones against all the acinetobacters and ofloxacin was the least potent. Gatifloxacin and moxifloxacin were equipotent against species 1/3. Levofloxacin, the L-isomer of the racemic ofloxacin is one dilution more potent than ofloxacin. As the number of non-*A. baumannii* isolates is too low to draw conclusions, the remainder of this chapter will focus on the activity of the fluoroquinolones against *A. baumannii*.

A. baumannii MIC_{50}, MIC_{90} and the range of MICs are shown graphically in Figure 5.1. These data show that the MIC_{50}s for all the fluoroquinolones tested are below breakpoint levels. Trovafloxacin and sparfloxacin were 16-fold more potent than ciprofloxacin. Gemifloxacin and grepafloxacin were 8-fold more potent than

ciprofloxacin and 4-fold more potent than levofloxacin and gatifloxacin. However at least 10% of the isolates are resistant to all the fluoroquinolones as seen with the MIC_{90} values. All the fluoroquinolones with the exception of ciprofloxacin had MIC_{90}s of 16 or 32 mg/L, well above therapeutic levels. Some isolates recorded MICs to ciprofloxacin, gemifloxacin and ofloxacin of >128mg/L.

Cefuroxime was the least potent of all the drugs tested, with an MIC_{50} of 64mg/L. Azithromycin had slightly higher activity with an MIC50 of 2mg/L but its MIC90 was 64mg/L. The most potent of the drugs tested was imipenem with even the highest MIC recorded (2mg/L) within therapeutic levels.

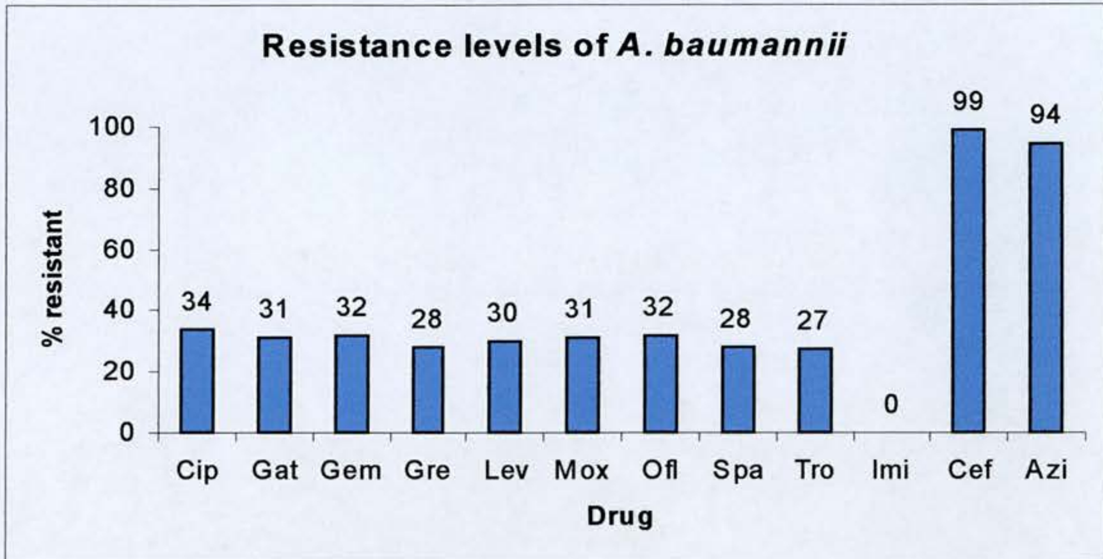
Figure 5.1. Range, MIC_{50} and MIC_{90} of *A. baumannii* against 12 antibiotics. Those drugs with an MIC range higher than that tested have the values above their data series. For other values, refer to Table 5.1.



An MIC_{50} or MIC_{90} value does not indicate resistance levels to the antibiotic and are used to monitor a population of bacterial isolates over time to determine changes in sensitivity. What is of paramount importance is the percentage of isolates that are resistant to each antibiotic; this relates MIC to breakpoint values. For example, both gemifloxacin and grepafloxacin record the same MIC_{50} and MIC_{90} which is just one dilution either side of trovafloxacin and sparfloxacin. However, Figure 5.2 illustrates the resistance levels seen within the *A. baumannii* isolates. These data show that resistance to gemifloxacin is higher than that seen with grepafloxacin and that grepafloxacin now equals potency with sparfloxacin. Ciprofloxacin when viewed

this way becomes the least potent with 34% of isolates resistant. Trovafloxacin retains its potency in both figures.

Figure 5.2. Resistance levels of 71 *A. baumannii* isolates to 12 antimicrobials



What is also of interest is that the newer fluoroquinolones, such as sparfloxacin and trovafloxacin, which were developed as anti-Gram positive drugs, are markedly superior to both ciprofloxacin and levofloxacin, both of which are recognised as anti-Gram negative drugs. Imipenem, the drug of choice to treat *A. baumannii* infections, retains its potency with all isolates susceptible. Only one strain showed an MIC below breakpoint for cefuroxime and four were sensitive to azithromycin.

5.3 Discussion

Fluoroquinolones are not marketed by drug companies to treat acinetobacter infections although they are sometimes used against them. Risk factors for acinetobacter infection also includes the use of a fluoroquinolone (Cendrero *et al*, 1999; Friedrich, White and Bosso, 1999; Villers *et al*, 1998) and thus it is of interest to know the activity of the newer drugs of this class in order to determine if the

organism may be selected for in the nosocomial environment. Resistance to ciprofloxacin in *A. baumannii* has been reported frequently (Bajaksouzian *et al*, 1997; Berlau *et al*, 1999; Horrevorts *et al*, 1997; Moreau *et al*, 1996; Sahm *et al*, 2001). The question is "Does resistance to ciprofloxacin also have a corresponding effect on the newer fluoroquinolones?"

In the present study, it has been shown that cross-resistance does occur irrespective of the fluoroquinolone tested. However, some strains that are ciprofloxacin resistant are susceptible to the newer drugs. Some of the newer fluoroquinolones such as gemifloxacin and moxifloxacin have poor serum distribution, evidenced by their low breakpoint values of 0.5mg/L and 1mg/L respectively (Andrews *et al*, 1999; Wise and Andrews, 1999). In spite of this, they perform marginally better than ciprofloxacin with a breakpoint of 2mg/L. If improvements in their pharmakokinetics can be achieved, then the effects of cross-resistance will be minimised. Trovafloxacin and sparfloxacin although being the most potent of the drugs are of limited use in the clinical setting as they have been withdrawn because of toxic side-effects and are reserved as a drug of last choice.

Cross-resistance to sparfloxacin, grepafloxacin, gemifloxacin, moxifloxacin, trovafloxacin and gatifloxacin is not found in *S. pneumoniae* isolates with low-level resistance to ciprofloxacin (8mg/L) (Jones *et al*, 2000; Marchese, Debbia and Schito, 2000; Ogasawara, Okamoto and Inoue, 1999; Pan and Fisher, 1998; Pestova *et al*, 2000), *Streptococcus mitis* (Gonzalez *et al*, 1998) or *E. coli* against sparfloxacin (Mouneimne *et al*, 1999; Tavio *et al*, 1999). However, *P. aeruginosa* does exhibit resistance to trovafloxacin and sparfloxacin but not ciprofloxacin (Wise *et al*, 1997).

Limited data have been published on the newer fluoroquinolones and the acinetobacters and this study was the first to encompass a large number of *A. baumannii* isolates and fluoroquinolones. Heinemann *et al* (2000) investigated the comparative activities of new and old fluoroquinolones against *A. baumannii*. Their data support that found in this study, that against fluoroquinolone susceptible isolates, ciprofloxacin is the least potent drug and trovafloxacin the most potent.

Most comparative studies on antibiotics that are published only show MIC data in the form of MIC_{50} and MIC_{90}. While this format is useful as it condenses many data into a single graph or figure, it does not give the whole picture (Jones, 2000). As the fluoroquinolones are not topical agents and their efficacy is linked to serum concentration, MIC data should also be linked to breakpoint levels. Even the use of breakpoints is fraught with difficulty. BSAC and NCCLS have different breakpoints and it has been argued that susceptibility testing should be standardised worldwide (Gould, 2000). Indeed, in the case of azithromycin, two breakpoints have been recommenced, with the NCCLS reporting a value of 4mg/L and the BSAC 0.5mg/L.

Using the breakpoint of 0.5 mg/L set by Wise and Andrews (Wise and Andrews, 1999), gemifloxacin inhibited 68% of the *Acinetobacter* spp. investigated in this study. The rank order of inhibitory activity of the quinolones against *A. baumannii* using MIC_{50}/MIC_{90} was: trovafloxacin = sparfloxacin > gemifloxacin = grepafloxacin > moxifloxacin > levofloxacin = gatifloxacin > ofloxacin > ciprofloxacin. This is similar to previously published data, (Bergogne-Berezin, 1996). The MIC_{50} and MIC_{90} values for gemifloxacin, trovafloxacin, grepafloxacin, sparfloxacin, ciprofloxacin and ofloxacin reported in this study against *A. calcoaceticus* are in agreement with the findings of Paek et al. (Paek, Kim and Choo, 1998), but are slightly different from those recorded by Oh *et al*, (1996), who reported MIC_{90}s that were one dilution higher. This order of activity changes somewhat if we apply the published breakpoint values and view the data through resistance levels. Thus, trovafloxacin > sparfloxacin = grepafloxacin > levofloxacin > moxifloxacin = gatifloxacin > gemifloxacin = ofloxacin > ciprofloxacin. These data show that the perceived potency of a drug can change depending upon how the data is viewed.

A previous study has noted resistance levels to ciprofloxacin, trovafloxacin and sparfloxacin of 93.3%, 56.7% and 66.7%, respectively, in multidrug-resistant *A. baumannii* (breakpoints: ciprofloxacin ≥ 2 mg/L; and sparfloxacin and trovafloxacin ≥ 4mg/L) (Pascual *et al*, 1997). In the present study, a breakpoint value of 2mg/L was used for trovafloxacin. Heinemann *et al* (2000) used a

breakpoint value of 2mg/L and found trovafloxacin resistance in 14.7% of isolates. In contrast, using the same breakpoints this study reported resistance rates of 34% for ciprofloxacin, 27% for trovafloxacin and 28% for sparfloxacin.

A decrease in sensitivity to one quinolone was mirrored by a decrease to all the others. However, this reduction in sensitivity did not impair sensitivity to the non-quinolone agents, as all strains were sensitive to imipenem and most were resistant to cefuroxime and azithromycin. Previous studies of the activity of azithromycin against *Acinetobacter* spp. have shown that a concentration of 4 mg/L is sufficient to inhibit most isolates (Fass, 1993). In the present study, MIC_{50} values for azithromycin was below 4 mg/L. However, this value is above the breakpoint of 0.5 mg/L set by the British Society for Antimicrobial Chemotherapy, and thus these isolates are recorded as being resistant to azithromycin (British Society for Antimicrobial Chemotherapy, 1991). The high MIC_{50} value (64 mg/L) recorded for cefuroxime against these Acinetobacter test strains is in agreement with a survey by Traub and Spohr (1989). These researchers recorded similarly high MICs against the cephalosporins, with little species difference being noted. The 100% susceptibility of test strains to imipenem reported in the present study also reflects the findings of previous investigators (Pieroni *et al*, 1997; Traub and Spohr, 1989). This may reflect the collection of strains used for their study. Data from the worldwide SENTRY survey show imipenem resistance rates to be at 11% (Gales *et al*, 2001).

In conclusion, the newer fluoroquinolones exhibit greater potency against *Acinetobacter* spp. than ciprofloxacin. However, a reduction in ciprofloxacin sensitivity is mirrored by a reduction in sensitivity to the newer fluoroquinolones. This reduction does not necessarily translate into resistance therefore there is potential for these drugs to be useful in the treatment of *Acinetobacter* spp. infections. The molecular mechanism of resistance to these drugs will be investigated in chapter 6.

Chapter 6: Mutations leading to fluoroquinolone resistance in clinical isolates of *A. baumannii*

6.1 Introduction

Acinetobacter baumannii is increasingly isolated in the nosocomial environment where it is recognised as a serious threat to the immunosuppressed (Cisneros *et al*, 1996; Towner, 1997). With the increasing use of fluoroquinolones within the hospital environment, selection of resistant phenotypes is not uncommon (Horrevorts *et al*, 1997). In this study, the susceptibility of one clinical *A. johnsonii isolate*, 16 clinical isolates of *A. baumannii* and the standard *A. baumannii* ATCC 19606 was determined against eight fluoroquinolones. The *gyr*A and *par*C genes were amplified by PCR and restricted with *Hinf*I. This restriction enzyme cuts the PCR products within the codon encoding a serine residue at positions 83 and 80 in GyrA and ParC respectively (Vila *et al*, 1997). Loss of this site is associated with an increase in MIC and thus is a useful genotypic marker of reduced sensitivity. Six of the strains were further analysed by sequencing *gyr*A and *par*C. PFGE was used to investigate the relationship between strains and their outer membrane profiles were examined to determine if there was any significant differences in protein expression that may contribute to fluoroquinolone resistance.

6.2 Results

6.2.1 Antibiotic susceptibility

The MIC data and results of RFLP are summarised in Table 6.1. If we apply the criteria of MIC range to those organisms that have MICs below breakpoint values,

trovafloxacin is the most potent of the drugs, followed by sparfloxacin and gemifloxacin. The older fluoroquinolones, ciprofloxacin and ofloxacin, are the least potent. Levofloxacin, the L-isomer of ofloxacin, is 2-fold more potent than ofloxacin. Against the resistant strains, trovafloxacin is still the most potent drug, followed by sparfloxacin and levofloxacin. Gemifloxacin loses its potency against resistant strains, recording an MIC as high as >128 mg/L.

6.2.2 Restriction of *gyr*A and *parC*

The strains in Table 6.1 can be divided into three groups: group A, with *Hinf*I-restricted *gyr*A and *par*C PCR products, group B with *gyr*A that is uncut but *par*C is cut, and group C with both genes uncut. No strain had lost the *par*C restriction site without a loss of *gyr*A. The MIC data show that group A strains are susceptible to all the fluoroquinolones used in the study. Ciprofloxacin is the least potent drug in this group and trovafloxacin exhibits the greatest potency. There is a wide variation of MIC amongst group A to each drug; for example 0.03-2 mg/L (66-fold) against ciprofloxacin and 0.008-1 mg/L (125-fold) with moxifloxacin. The least sensitive of these strains (SB4) has MICs that are within 1 dilution of ciprofloxacin, moxifloxacin and gemifloxacin breakpoints.

Group B strains have lost the *gyr*A restriction site but their *par*C gene is still cut by *Hinf*I. This group are resistant to all the fluoroquinolones with the exception of trovafloxacin, with a breakpoint value of 1mg/L. Strain SB31 is also resistant to both moxifloxacin and sparfloxacin. The range of MICs are less pronounced in this group compared to group A, the greatest range being 8-fold against sparfloxacin. The four strains within group B show no MIC range against gemifloxacin and trovafloxacin.

Table 6.1. MIC's and RFLP results of the strains tested

		MIC mg/L								HinfI restriction	
Group	**Strain**	**Cip**	**Gem**	**Tro**	**Spa**	**Mox**	**Ofl**	**Lev**	**Gre**	**gyrA**	**parC**
A	SB59	0.03	0.008	0.008	0.008	0.008	0.03	0.008	0.015	+	+
	SB5	0.25	0.06	0.06	0.03	0.12	1	0.25	0.06	+	+
	SB32	1	0.06	0.06	0.03	0.12	1	0.5	0.06	+	+
	SB14	0.5	0.12	0.03	0.03	0.12	0.5	0.25	0.06	+	+
	19606	1	0.12	0.06	0.12	0.5	1	0.5	0.12	+	+
	SB10	1	0.12	0.06	0.06	0.25	1	0.5	0.12	+	+
	SB4	2	0.25	0.12	0.25	1	1	0.5	0.25	+	+
B	SB13	4	1	0.5	4	2	4	1	2	-	+
	SB22	8	1	0.5	1	2	8	4	1	-	+
	SB24	16	1	0.5	1	4	8	4	1	-	+
	SB31*	16	1	0.5	0.5	0.5	8	4	0.5	-	+
C	SB75	64	32	8	16	16	16	8	64	-	-
	SB36	64	32	16	16	16	32	16	32	-	-
	SB67	128	16	8	8	16	16	16	16	-	-
	SB47	>128	16	16	16	16	32	16	64	-	-
	SB1	>128	32	8	16	16	32	16	64	-	-
	SB87	>128	64	32	32	64	>128	64	32	-	-
	SB21	>128	>128	32	32	64	64	32	64	-	-

Cip: ciprofloxacin, Gem: gemifloxacin, Tro: trovafloxacin, Spa: sparfloxacin, Mox: moxifloxacin, Ofl: ofloxacin, Lev: levofloxacin, Gre: grepafloxacin, + wild type PCR fragment (cut by *Hinf*I), - mutant PCR fragment (*Hinf*I site lost), *species 7 (*A. johnsonii*)

Those strains in group C are resistant to all the fluoroquinolones, with some recording MICs >128mg/L. The highest MICs are recorded against ciprofloxacin. Trovafloxacin was still the most potent drug closely followed by sparfloxacin. One strain, SB21, was highly resistant to ciprofloxacin and gemifloxacin, with an MIC >128mg/L but the MICs to the other drugs were 4-8 fold less. The MIC range in this group has risen from those seen in group B, with at least a 16-fold increase against gemifloxacin and levofloxacin.

Figure 6.1 shows a representative agarose gel of *gyr*A *Hinf*I RFLP. Uncut *gyr*A has a single band of 343bp. When cut, two fragments are produced of 291bp and 52bp. The smallest of these bands is unresolved in the gel but restriction can be seen to have taken place by the reduction in size of the larger band.

Figure 6.1. *HinfI* restriction of *gyr*A PCR products. Lane a: 100bp ladder, b: control – no restriction enzyme, c: SB5, d: SB22, e:SB24, f: SB10, g: SB4, h: SB67

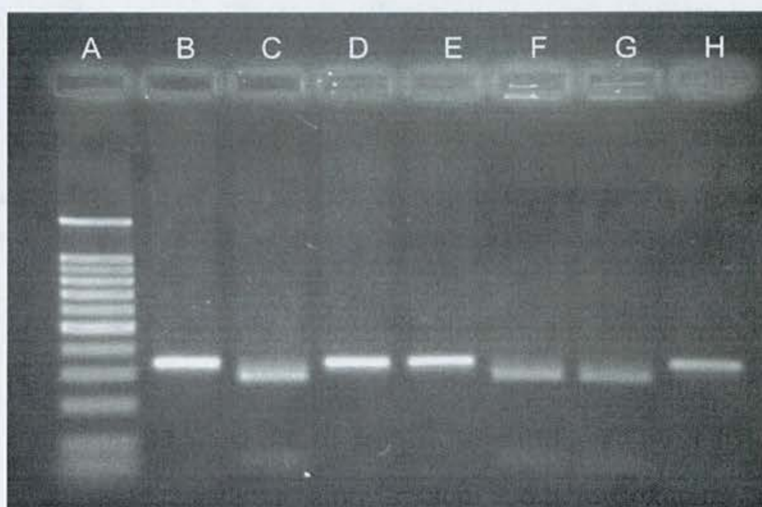
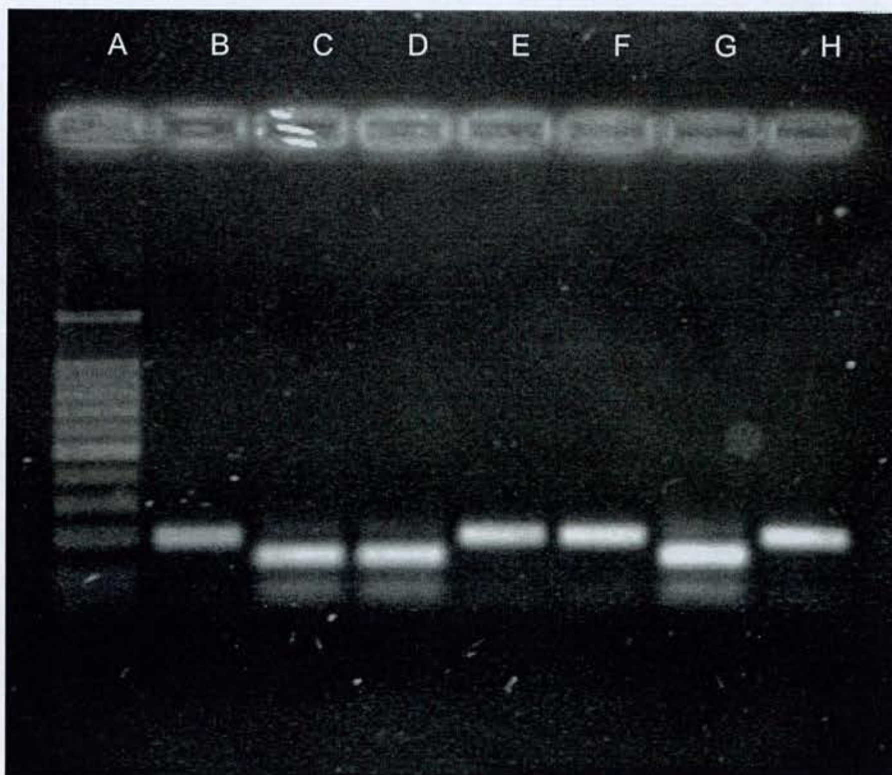


Figure 6.2 shows a representative *par*C *Hinf*I restriction digest. The uncut PCR product retains its size of 197bp. When cut, two fragments are seen, of 114bp and 53bp.

Figure 6.2. *HinfI* restriction of *par*C PCR products. Lane A: 100bp ladder, B: control – no restriction enzyme, C: SB10, D: SB5, E:SB36, F: SB1, G: SB24, H: SB75



6.2.3 Sequencing of *gyr*A

Seven of the strains had the QRDR of *gyr*A and *par*C sequenced, representing 2 each from groups A & B and 3 from group C. Table 6.2 shows the sequence alignment of *gyr*A and Table 6.3 the corresponding amino acid substitutions. At the nucleotide level, there is a point mutation in the *gyr*A gene; a C→ T transversion (highlighted in red) in the *HinfI* restriction site (yellow). Three single nucleotide polymorphisms are found in ATCC19606 (green) one of which is also found in SB1, SB21 and SB36. The mutation in the restriction site of *gyr*A changed the codon from TCA (serine) to TTA (leucine) at codon 83. No other amino acid substitutions were found in the strains.

Table 6.2. *gyrA* alignment. The *HinfI* restriction site is highlighted in yellow, the C→T transversion of the serine residue is in red and single nucleotide polymorphisms are in green.

```
Group A 19606 GTTGGTGACG TAATCGGTAA ATATCACCCG CATGGTGACT CAGCTGTTTA
         SB10 .......... .......... ..ATCACCCG CATGGTGACT CAGCTGTTTA
Group B  SB13 .......... ...TCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA
         SB24 .......... ...TCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA
Group C  SB36 ........CG TAATCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA
          SB1 .......... ...TCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA
         SB21 ........CG TAATCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA

        19606 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
         SB10 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
         SB13 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
         SB24 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
         SB36 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
          SB1 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG
         SB21 TGAAACCATT GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG

        19606 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
         SB10 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
         SB13 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
         SB24 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
         SB36 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
          SB1 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA
         SB21 TTGATGGTCA GGGTAACTTC GGTTCGATCG ATGGTGATAG CGCTGCGGCA

        19606 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
         SB10 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
         SB13 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
         SB24 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
         SB36 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
          SB1 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC
         SB21 ATGCGTTATA CCGAAGTCCG TATGACTAAG CTGGCACATG AGCTTCTTGC

        19606 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
         SB10 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
         SB13 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
         SB24 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
         SB36 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
          SB1 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG
         SB21 AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC GACGGTTCGG

        19606 AACGTATCCC TGAAGTACTT CCGACACGAG TTCCAAACTT GTTAATCAAC
         SB10 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAAC.. ..........
         SB13 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAACTT ATTAA.....
         SB24 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAACTT ATTAA.....
         SB36 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAACTT GTTAATTAAC
          SB1 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAACTT GTTAA.....
         SB21 AACGTATCCC TGAAGTACTT CCGACACGTG TTCCAAACTT GTTAATTAAC
```

Table 6.3. Amino acid substitutions in GyrA and ParC in sequenced strains

Group	Strain	GyrA	ParC
A	ATCC 19606	Wild type	Wild type
	SB10	None	None
B	SB13	83 Ser → Leu	None
	SB24	83 Ser → Leu	None
C	SB36	83 Ser → Leu	80 Ser → Leu
	SB1	83 Ser → Leu	80 Ser → Leu
	SB21	83 Ser → Leu	80 Ser → Leu

6.2.4 Sequencing of *parC*

Table 6.4 shows the *par*C sequence alignment. The published gene sequence of ATCC19606 (GenBank accession number AF108132) was different from that found in this study (data not shown), a finding confirmed by Scott Cameron (personal correspondence). Interestingly, the difference was only at the nucleotide level and did not affect the translated sequence. This difference was not found with *gyr*A. There is more heterogeneity within this gene compared to that of *gyr*A. A total of eight point mutations were found, two of which were found in other strains. The mutation in the restriction site TCG (serine) to TTG (leucine) was in codon 80. No other amino acid substitutions were found.

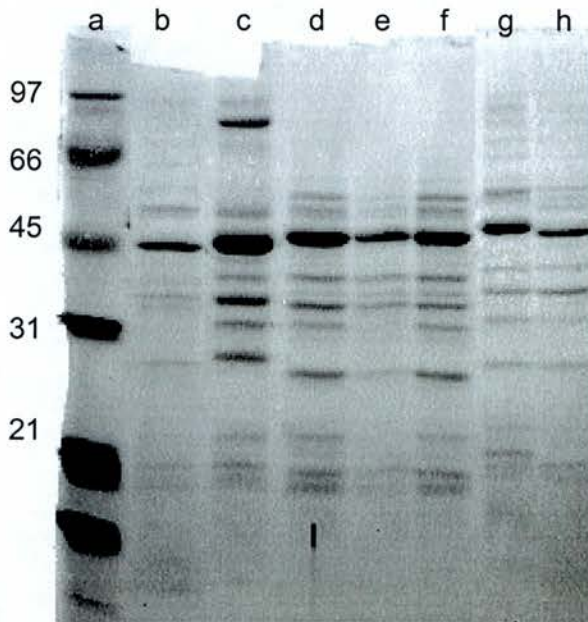
Table 6.4. *par*C alignment. The *Hinf*I restriction site is highlighted in yellow, the C→T transversion of the serine residue is in red and single nucleotide polymorphisms are in green.

```
Group A 19606 ....CTTGGT AAATACCACC CACATGGTGA CTCGGCATGT TATGAAGCCA
         SB10 TGTACTTGGT AAATACCACC CACATGGTGA CTCGGCATGT TATGAAGCCA
Group B  SB13 .GTACTTGGT AAATACCACC CACATGGTGA CTCGGCATGT TATGAAGCCA
         SB24 .......GGT AAATACCACC CACATGGTGA CTCGGCATGT TATGAAGCCA
Group C  SB36 ....CTTGGT AAATACCACC CACATGGTGA CTTGGCATGT TATGAAGCCA
          SB1 ....CTTGGT AAATACCACC CACATGGTGA CTTGGCATGT TATGAAGCCA
         SB21 ....CTTGGT AAATACCACC CACATGGTGA CTTGGCATGT TATGAAGCCA

        19606 TGGTACTCAT GGCTCAGCCA TTTAGTTATC GTTATCCGCT AATTGAAGGA
         SB10 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT
         SB13 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT
         SB24 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT
         SB36 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT
          SB1 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT
         SB21 TGGTACTCAT GGCTCAGCCA TTTAGTTACC GCTATCCTTT AATCGAAGGT

        19606 CAGGGGAACT GGGGTTCACC AGATGATCCT AAATCTTTTG CTGCGATGCG
         SB10 CAGGGGAACT GGGGTTCACC AGATGATCCT AAATCTTTTG CTG.......
         SB13 CAGGGGAACT GGGGCTCACC TGATGATCCT AAGTCTTTTG CTGCG.....
         SB24 CAGGGGAACT GGGGCTCACC TGATGATCCT AAGTCTTTTG CTG.......
         SB36 CAGGGGAACT GGGGTTCACC AGATGATCCT AAATCTTTTG CT........
          SB1 CAGGGGAACT GGGGTTCACC TGATGATCCT AAATCTTTTG CT........
         SB21 CAGGGGAACT GGGGTTCACC AGATGATCCT AAATCTTTTG CT........
```

6.3 Outer Membrane Profiles

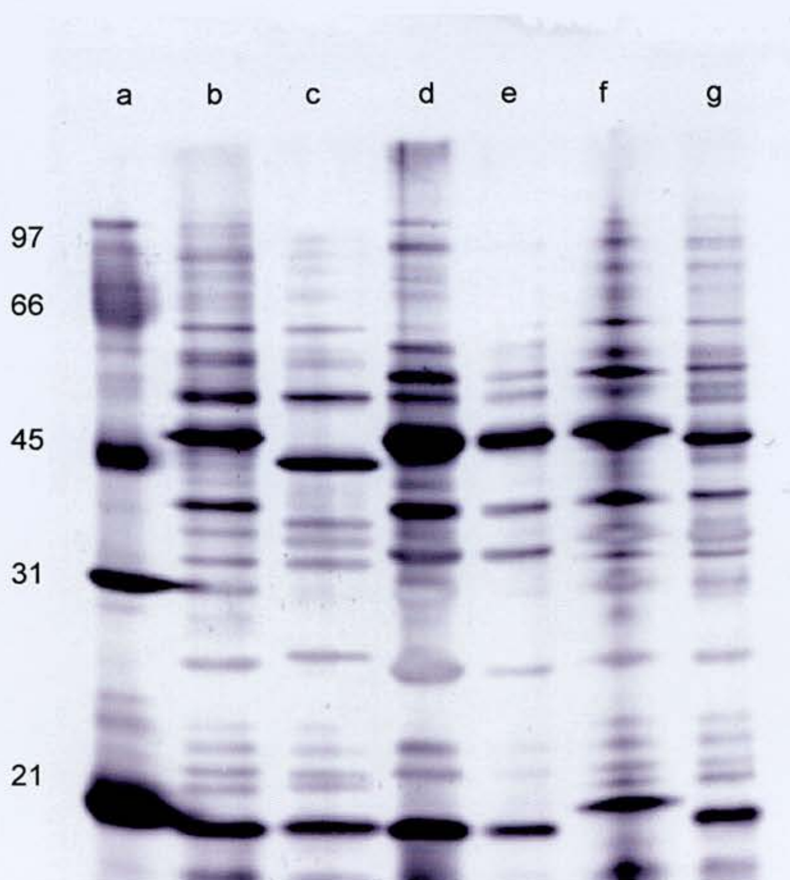
A coomassie-stained SDS-PAGE outer membrane profile of seven strains are shown in Figure 6.3. A major band of approximately 45kDa is found in all strains. Use of BioRad Quantity One software shows that this composes about 50% of the total outer membrane protein. Strains SB13, SB22 and SB24 (lanes d, e & f, Figure 6.3) have similar profiles, indicating relatedness. These strains also belong to group B and have identical *gyr*A and *par*C genes (Tables 6.2 & 6.3). There are OMP differences seen amongst the other strains but it cannot be concluded what role they play in resistance.

Figure 6.3. Coomassie-stained SDS-PAGE of clinical isolates. Lane a: MW marker, b: SB5, c:SB10, d: SB13, e:SB22, f: SB24, g: SB36, h: SB47



A silver stained SDS-PAGE OMP profile is shown in Figure 6.4. This staining technique is more sensitive than a coomassie blue stain and therefore more protein bands are visible. In this gel, the similarity between SB13 and SB22 is less pronounced than in Figure 6.3 (lanes d & e, Figure 6.4), with a band just below the major OMP not visible in SB22 but seen with SB13. This may be due to less protein loaded in the gel as lane d clearly has more protein. In this gel, SB36, in lane f, has a similar banding pattern to SB13 and SB22, however its profile in Figure 6.3 is different.

Figure 6.4. Silver stain of OMPs. Lane a: marker, b: SB1, lane c: SB5, d: SB13, e: SB22, f: SB36, g: SB47



6.4 Pulsed-Field Gel Electrophoresis

Figures 6.5, 6.6 & 6.7 show the PFGE profiles of the strains. Three of the strains, SB22, SB24 (lanes h & I, Figure 6.5) and SB13 (lane d, Figure 6.7), appear to have the same PFGE pattern. No data were given as to where the isolates came from and it is possible that they are samples from the same patient, but this is speculative. The other isolates are heterogeneous in their PFGE patterns.

Figure 6.5. PFGE of clinical isolates. Lane A, F & J; 50kb marker, B: ATCC 19606, C: SB1, D: SB4, E: SB5, G: SB21, H: SB22, I: SB24

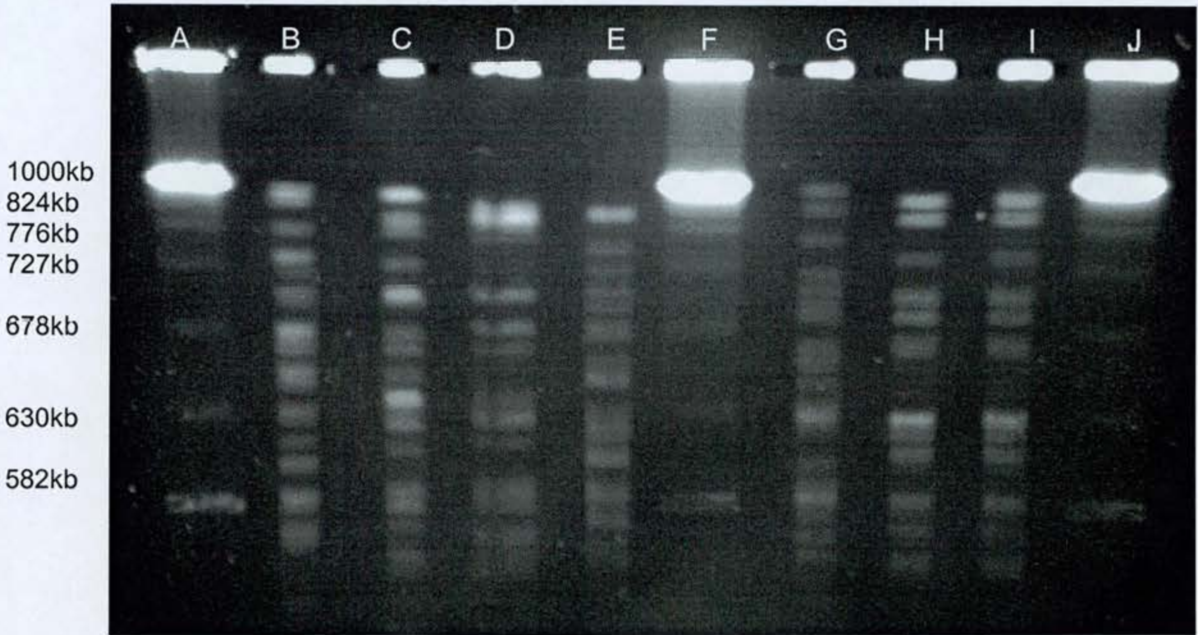


Figure 6.6. Lane A & F: 50kb marker, B: 19606, C: SB32, D: SB36, E: SB47

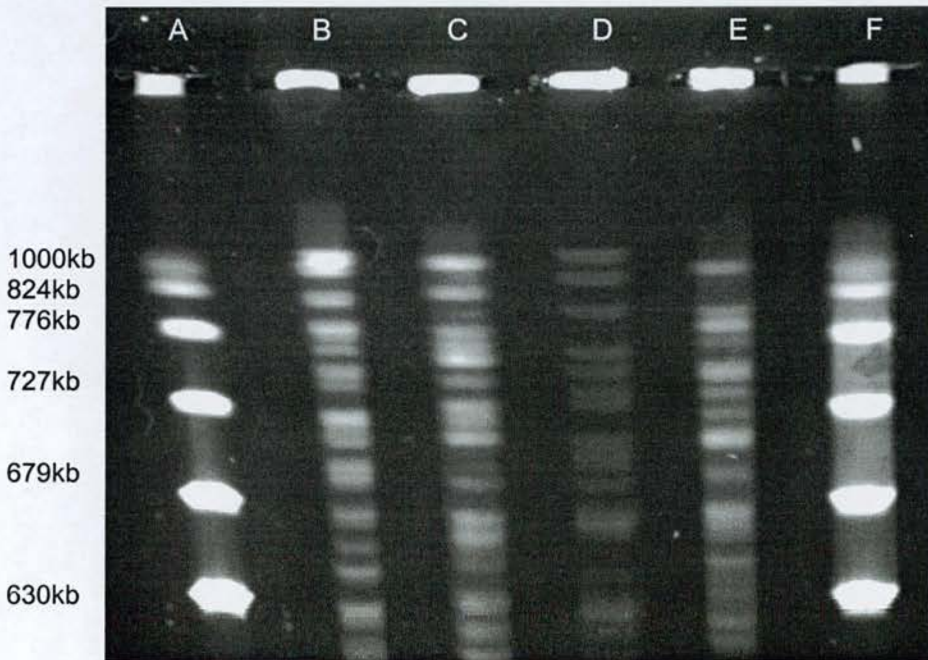
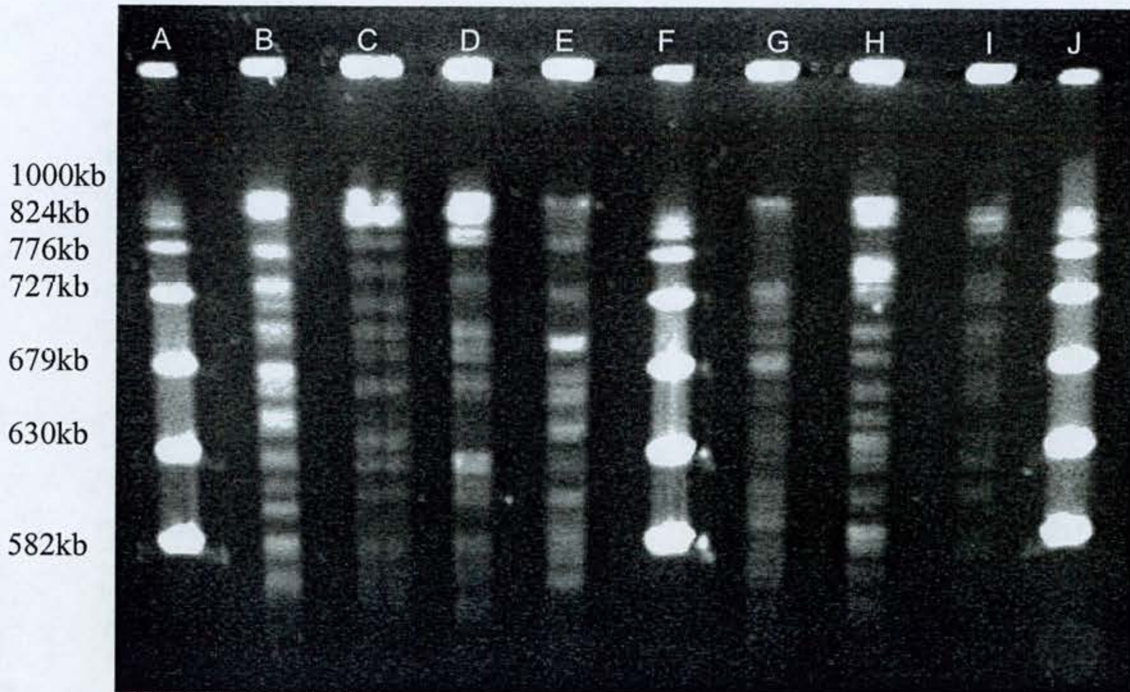


Figure 6.7. Lane A, F & J: 50kb marker, B: 19606, C: SB10, D: SB13, E: SB14, G: SB59, H: SB67, I: SB 87



6.5 Discussion

These data show that there is an associated rise in MIC when there is a serine to leucine substitution at positions 83 and 80 within the QRDR of *A. baumannii* GyrA and ParC respectively, confirming work previously done by Seward and Vila (Seward and Towner, 1998; Vila, Ruiz, Goni and Deanta, 1997; Vila, Ruiz, Goni, Marcos and de Anta, 1995). Fluoroquinolones bind to DNA gyrase and Topoisomerase IV in an area close to the mutated serine residue and this substitution reduces binding of fluoroquinolones to DNA Gyrase (Willmott and Maxwell, 1993). With the two successive mutations in *gyr*A and *par*C there is an increase in recorded MIC for all the fluoroquinolones. This rise in MIC has previously been reported in *A. baumannii* using ciprofloxacin and nalidixic acid but here it has been extended to

acid substitutions within GyrA and ParC but none were seen nor were the point mutations in the areas corresponding to their codons (Vila *et al*, 1997). However the mutations identified do not, on their own, account for the range of MICs within each group. A second mutation in *gyr*A was expected as this has been shown to be necessary for high-level resistance in *E. coli* (Heisig, 1996). The sequencing of the *gyr*A PCR product does not cover those mutations found in *E. coli* at Ala 67 and Ala 51, which, if present, could account for the MIC range (Friedman, Lu and Drlica, 2001; Yoshida *et al*, 1990).

Other gene mutations may play a part in the MIC ranges, for example changes in the QRDR of *gyr*B. These contribute to reduced fluoroquinolone sensitivity in *E. coli* (Yoshida *et al*, 1991). However Vila noted that *gyr*B mutations are not important in *A. baumannii* (Vila, 1998) but as a mechanism of resistance they should not be discounted offhand. It seems unlikely that ParE mutations are involved as they have not been found to contribute to resistance in Gram negative organisms.

Another explanation for both high fluoroquinolone and MIC ranges without extra target-site mutations could relate to the innate resistance of *A. baumannii* to the fluoroquinolones. *A. baumannii* DNA gyrase is less sensitive to fluoroquinolones than that of *E. coli* (Moreau *et al*, 1996) and it also exhibits low outer membrane permeability, which has been calculated to be 1-3% that of *E. coli* (Hancock, 1998; Sato and Nakae, 1991). A combination of these two properties could contribute to MIC differential and lack of tertiary mutations.

Evidence for permeability factors comes from SB21. It is hyper-resistant to both ciprofloxacin and gemifloxacin, both being hydrophilic drugs, but was more sensitive to the other, more hydrophobic fluoroquinolones. Moreau has shown that resistant strains of *A. baumannii* generally accumulate less drug than sensitive strains. This affects hydrophilic drugs more than hydrophobic drugs, although they found no correlation in MIC (Moreau *et al*, 1996). This relationship has also been demonstrated in *P. aeruginosa* and *E. coli* (Piddock, Jin and Griggs, 2001).

The outer membrane profiles of the strains were investigated but no conclusions could be drawn from this. This is unsurprising as outer membrane profiles have proved to be a useful tool in epidemiology (Horrevorts *et al*, 1997) and any differences seen could be due to strain-specific expression. Results of PFGE showed that 3 of the strains were related, having similar OMP and PFGE patterns, but their MICs were not equal. However changes in the outer membrane profile have been shown to contribute to fluoroquinolone resistance. For example, in *P. aeruginosa*, decreased expression of porin D2 is associated with both fluoroquinolone and carbapenem resistance (Masuda, Sakagawa and Ohya, 1995; Micheahamzehpour, Furet and Pechere, 1991) and that there is an association with fluoroquinolone resistance and overexpression of the outer membrane protein OprM (Li, Barre and Poole, 2000).

In *A. baumannii* carbapenem resistance was shown to be associated with a reduction in a 33kDa OMP (Clark, 1996). Clark used isogenic strains, thus eradicating strain difference. Other *A. baumannii* membrane proteins that have been investigated include a 43kDa and 45.5kDa porin (Nitzan *et al*, 1999; Sato and Nakae, 1991). A 38Kda OMP has been found to be a virulence factor (Ofori-Darko *et al*, 2000), therefore OMP differences between strains may not relate to resistance at all.

In conclusion, fluoroquinolone resistance in *A. baumannii* is mediated through a serine 83 mutation in GyrA. The newer fluoroquinolones, although more potent than ciprofloxacin, are also affected by this mutation although trovafloxacin records MICs that are below the breakpoint. However, as a treatment option trovafloxacin is of no use as it has been withdrawn because of severe side-effects.

Chapter 7: In-vitro Fluoroquinolone Selection

7.1 Introduction

In the previous chapter we have been looking at clinical isolates and their mechanism of fluoroquinolone resistance through target site mutations. Some questions that may come to mind is how they occur, at what frequency and in what order? The use of fluoroquinolones has been associated with the selection of ciprofloxacin resistant strains in the clinical environment (Horrevorts *et al*, 1997; Muder *et al*, 1991; Vila, 1998). We have seen that a serine 83 mutation in GyrA is found in all strains with ciprofloxacin MICs above breakpoint values and high-level resistance with a concurrent serine 80 mutation in ParC. These mutations also confer resistance to the newer fluoroquinolones as seen in chapter 6. In this study the mutation rates and associated mutations of a laboratory *A. baumannii* strain were compared between ciprofloxacin and the newer fluoroquinolones gemifloxacin and moxifloxacin. A clinical isolate carrying a serine 83 mutation in GyrA was also used to compare mutation rates once a GyrA mutation was present.

Results

7.2.1 Ciprofloxacin selection of *A. baumannii* ATCC 19606

The mutation frequencies, fluoroquinolone MICs and *HinfI* restriction analysis of the ciprofloxacin-selected mutants derived from ATCC 19606 are shown in Table 7.1. First generation mutants were selected at a frequency of 2.4×10^{-5}. These mutants could be put into two groups: (1) *gyr*A wild type (2) restriction site lost. One isolate did not show a change in MIC, suggesting that it was not a true mutant or that it had mutated and subsequently reverted. The wild type mutants showed a 2-8 fold increase in MIC to ciprofloxacin, 2-4 fold against gemifloxacin and 2-4 fold against moxifloxacin. Four of these mutants were resistant to ciprofloxacin but were still susceptible to gemifloxacin and moxifloxacin, although five and six mutants had

MICs at breakpoint values respectively. Mutants of this group retained the *gyr*A and *par*C *Hinf*I restriction site.

Table 7.1. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against gemifloxacin selected mutants of ATCC 19606

Ciprofloxacin-selected mutants							
			MIC (mg/L)			*Hinf*I restriction	
	Frequency Of Mutation	Strain	GEM	CIP	MOX	gyrA	parC
Parent		19606*	0.12	1	0.25	wt	wt
1ST generation	$2.4x10^{-5}$	C1	16	128	16	lost	wt
		C2	0.5	2	1	wt	wt
		C3	0.12	1	0.25	wt	wt
		C4	0.5	4	1	wt	wt
		C5	0.25	2	1	wt	wt
		C6	0.5	2	1	wt	wt
		C7*	16	128	16	lost	wt
		C8	0.25	4	0.5	wt	wt
		C9	0.5	4	1	wt	wt
		C10	0.5	8	1	wt	wt
2nd generation	$1.3x10^{-6}$	256C1	32	256	16	lost	wt
		256C2	32	128	16	lost	wt
		256C3	>128	>512	32	lost	wt
		256C4	32	64	8	lost	wt
		256C5	16	256	16	lost	wt
		256C6	64	512	32	lost	wt
		256C7	16	128	16	lost	wt
		256C8	16	128	4	lost	wt
		256C9	32	128	16	lost	wt
		256C10	16	128	16	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin, MOX: moxifloxacin, nt: not tested, wt: wild type cut by *Hinf*I, lost: not cut by *Hinf*I

The mutants that lost the *gyr*A restriction site, C1 and C7, showed a 128-fold increase in ciprofloxacin MIC, 133-fold increase to gemifloxacin and 64-fold increase to moxifloxacin. These mutants retained the *par*C site.

Mutant C7 was further selected on ciprofloxacin. None of the mutants derived from C7 lost the *par*C restriction site. As with the first generation, some mutants did not record an increase in MIC and interestingly, mutant 256C8 was 4-fold more sensitive to moxifloxacin. Another mutant, 256C4, was 2-fold more sensitive to ciprofloxacin and moxifloxacin but recorded a gemifloxacin MIC 2-fold greater than C7.

7.2.2 Gemifloxacin selection of ATCC 19606

The mutation frequencies, fluoroquinolone MICs and *Hinf*I restriction analysis of the gemifloxacin-selected mutants derived from ATCC 19606 are shown in Table 7.2. First generation mutants were selected at a frequency lower than ciprofloxacin. This saw a 4-fold increase in gemifloxacin MIC, a 2-4-fold increase in ciprofloxacin MIC and a 2-fold increase in MIC of moxifloxacin. One of the mutants, AP1, had a ciprofloxacin MIC greater than breakpoint level. *Hinf*I restriction analysis of the *gyr*A and *par*C gene showed no mutation at serine 80 or 83 respectively. Strain AP1 was plated onto gemifloxacin containing plates at 1mg/L to yield 2nd generation mutants at a frequency of $7x10^{-8}$. These mutants exhibited a 2-fold increase in MIC against the three fluoroquinolones. There was no change in the restriction analysis from the wild type.

Third generation mutants were selected at a lower frequency than the first two steps at $1x10^{-9}$. Three of these strains showed a 16-fold increase in MIC to gemifloxacin and ciprofloxacin and an 8-fold increase to moxifloxacin. Mutant CP4 had an 8-fold increase to gemifloxacin and ciprofloxacin but only 2-fold to moxifloxacin, even though all four of the strains had lost the *Hinf*I restriction site in *gyr*A. A 4th selection step was carried out to see if a *par*C mutation would be selected. This step yielded mutants at a frequency that was 3000 times greater than the previous step. While some of the strains had a gemifloxacin increase in MIC $\geq$ 8-fold, moxifloxacin only increased 2-3 fold. None of the mutants had lost the *par*C restriction site.

Table 7.2. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against gemifloxacin-selected mutants of ATCC 19606

Gemifloxacin-selected mutants							
	Frequency		MIC (mg/L)			*HinfI* restriction	
	of Mutation	Strain	GEM	CIP	MOX	gyrA	parC
Parent		19606*	0.12	1	0.25	wt	wt
1st	1x10^{-8}	AP1*	0.5	4	0.5	wt	wt
generation		AQ1	0.5	2	0.5	wt	wt
		AR1	0.5	2	0.5	wt	wt
2nd	7x10^{-8}	BP1*	1	8	1	wt	wt
generation		BP2	1	8	1	wt	wt
		BP3	1	8	1	wt	wt
		BP4	1	8	1	wt	wt
		BP5	1	8	nt	wt	wt
		BP6	1	8	nt	wt	wt
		BP7	1	8	nt	wt	wt
		BP8	1	8	nt	wt	wt
3rd	1x10^{-9}	CP1*	16	128	8	lost	wt
generation		CP2	16	128	8	lost	wt
		CP3	16	128	8	lost	wt
		CP4	8	64	2	lost	wt
4th	3x10^{-6}	DP1	64	>128	16	lost	wt
generation		DP2	64	>128	16	lost	wt
		DP3	64	>128	16	lost	wt
		DP4	128	>128	16	lost	wt
		DP5	>128	>128	16	lost	wt
		DP6	>128	>128	32	lost	wt
		DP7	>128	>128	32	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin,
MOX: Moxifloxacin, nt: not tested, wt: wild type – cut by *HinfI*, lost: not cut by *HinfI*

7.2.3 Analysis of *gyr*A and *par*C QRDR

Sequencing of the *gyr*A QRDR was performed and this is shown in Figure 7.1. The translated sequence is shown in Figure 7.2. These data confirm the restriction analysis in Table 7.2. The only change seen in *gyr*A was a C→T transversion at position 56 in mutant CP1 and those derived from it. Mutant M2E is a moxifloxacin-selected mutant with the same transversion. The mutation substitutes serine 83 into leucine as described in chapter 6. The *par*C QRDR of the mutants were also

sequenced and were found to be indistinguishable from the wild type parent (Figure 7.3). The translated sequence of *par*C is shown in Figure 7.4.

Figure 7.1. Alignment of the *gyr*A QRDR. The C→T transversion is in red.

```
19606 .....TCGGT AAATATCACC CGCATGGTGA CTCAGCTGTT TATGAAACCA
AP1   .....TCGGT AAATATCACC CGCATGGTGA CTCAGCTGTT TATGAAACCA
BP1   .....TCGGT AAATATCACC CGCATGGTGA CTCAGCTGTT TATGAAACCA
CP1   CGTAATCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
DP1   .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
DP7   .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
M2E   .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA

19606 GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
AP1   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
BP1   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
CP1   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
DP1   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
DP7   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT
M2E   GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT CAGGGTAACT

19606 CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
AP1   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
BP1   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
CP1   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
DP1   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
DP7   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA
M2E   CGATGGCGAT AGCGCCGCGG CAATGCGTTA TACCGAAGTC CGTATGACTA

19606 TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
AP1   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
BP1   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
CP1   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
DP1   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
DP7   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT
M2E   TGAGCTTCTT GCAGATTTAG AAAAAGACAC AGTTGACTGG GAAGATAACT

19606 ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
AP1   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
BP1   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
CP1   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
DP1   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
DP7   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
M2E   ACGACGGTTC AGCTGGCACA TCGGTTCGAT TTGTTCGTAT
```

Figure 7.2. Translated *gyr*A amino acid sequence of ATCC 19606 and mutant CP1. The mutated amino acid is shown in red. *E. coli* numbering is used.

```
        73                                                 125
19606   VIGKYHPHGDSAVYETIVRMAQDFSLRYLLVDGQGNFGSIDGDSAAAMRYTEV
CP1     VIGKYHPHGDLAVYETIVRMAQDFSLRYLLVDGQGNFGSIDGDSAAAMRYTEV

        126                                                178
19606   RMTKLAHELLADLEKDTVDWEDNYDGSERIPEVLPTRVPNLLINGAAGIAVGM
CP1     RMTKLAHELLADLEKDTVDWEDNYDGSERIPEVLPTRVPNLLINGAAGIAVGM
```

Figure 7.3. Alignment of the *par*C QRDR. The Serine 80 codon is in red

```
19606   .........C TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA
AP1     GGTGATGTAC TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA
BP1     GGNGATGTAC TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA
CP1     GGTGATGTAC TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA
DP1     .....TGTAC TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA
DP7     .....TGTAC TTGGTAAATA CCACCCACAT GGTGACTCGG CATGTTATGA

19606   AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG
AP1     AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG
BP1     AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG
CP1     AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG
DP1     AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG
DP7     AGCCATGGTA CTCATGGCTC AGCCATTTAG TTATCGTTAT CCGCTAATTG

19606   AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCTGCG
AP1     AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCTGCG
BP1     AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCTGCG
CP1     AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCTGCG
DP1     AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCTGCG
DP7     AAGGACAGGG GAACTGGGGT TCACCAGATG ATCCTAAATC TTTTGCT...
```

Figure 7.4. Translated *par*C amino acid sequence of ATCC 19606 and mutant DP7. The serine 80 residue associated with fluoroquinolone resistance is shown in red.

```
19606   GDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLIEGQGNWGSPDDPKSFAAMR
DP7     GDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLIEGQGNWGSPDDPKSFAAMR
```

7.2.4 Moxifloxacin Selection of ATCC 19606

The mutation frequencies, fluoroquinolone MICs and *Hinf*I restriction analysis of the moxifloxacin-selected mutants derived from ATCC 19606 are shown in Table 7.3. Two first generation mutants were investigated and they showed a 4-fold increase in moxifloxacin and ciprofloxacin MIC and 2-8-fold to gemifloxacin. The *Hinf*I restriction sites were unaffected. Mutant M1B was further selected on moxifloxacin to produce second generation mutants. One of the five, M2E, had lost the restriction site in *gyr*A. This strain had its *gyr*A QRDR sequenced and it had the same C→T transversion in the serine 83 codon as seen in clinical isolates (chapter 6) and gemifloxacin selected mutants (Figures 7.2 & 7.3). The other four second generation mutants, although having not lost the restriction site in either *gyr*A or *par*C, recorded higher MICs than M2E. This strain was further selected on moxifloxacin and yielded mutants that showed an 8-fold increase in moxifloxacin MIC and a 16-fold increase to ciprofloxacin and gemifloxacin. One mutant, 4M2A, had a 4-fold increase in gemifloxacin MIC. A further selection step was performed and this yielded only one mutant at a low frequency. This mutant only showed a 2-fold increase to the fluoroquinolones tested. It did not lose the *par*C restriction site.

Table 7.3. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against moxifloxacin selected mutants of ATCC19606

Moxifloxacin-selected mutants							
	Frequency Of Mutation	Strain	MIC (mg/L) GEM	CIP	MOX	*HinfI* restriction gyrA	parC
Parent		19606*	0.12	1	0.25	wt	wt
1st generation	1x10-7	M1A	0.25	4	1	wt	wt
		M1B*	1	4	1	wt	wt
2nd generation	5x10-8	M2A	2	16	2	wt	wt
		M2B	2	16	4	wt	wt
		M2C	2	16	2	wt	wt
		M2D	2	16	4	wt	wt
		M2E*	1	8	2	lost	wt
3rd generation	8x10-6	4M2A	4	128	16	lost	wt
		4M2B	16	128	16	lost	wt
		4M2C	16	128	16	lost	wt
		4M2D	16	128	16	lost	wt
		4M2E*	16	128	16	lost	wt
		4M2F	16	128	16	lost	wt
		4M2G	16	128	16	lost	wt
		4M2H	16	128	16	lost	wt
		4M2I	16	128	16	lost	wt
		4M2J	16	128	16	lost	wt
4th generation	1x10-9	32MA	32	>128	32	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin, MOX: Moxifloxacin, nt: not tested, wt: wild type – cut by *HinfI*, lost: not cut by *HinfI*

7.3.1 Ciprofloxacin selection of SB13

The clinical isolate SB13, previously described in Chapter 6, was selected with ciprofloxacin to compare mutation rates against strain ATCC 19606. Table 7.4 shows the mutation rates and associated MICs of ciprofloxacin-selected mutants. An increase in MIC against ciprofloxacin was 1-32-fold, 2-8-fold against moxifloxacin and 1-32-fold against gemifloxacin. Two of the mutants, 16CSB6 and 16CSB9, lost the *parC* restriction site. These mutants recorded the highest gemifloxacin MICs, but

their ciprofloxacin and moxifloxacin MICs were matched by mutants with the wild type restriction site.

Table 7.4. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against ciprofloxacin selected mutants of SB13

Ciprofloxacin-selected mutants							
	Frequency Of Mutation	Strain	MIC (mg/L) GEM	CIP	MOX	*HinfI* restriction gyrA	parC
Parent		SB13*	1	8	2	lost	wt
1st generation	8.5x10^{-7}	16CSB1	16	128	8	lost	wt
		16CSB2	1	8	4	lost	wt
		16CSB3	8	32	4	lost	wt
		16CSB4	8	32	4	lost	wt
		16CSB5	8	64	4	lost	wt
		16CSB6	32	128	16	lost	lost
		16CSB7	8	64	16	lost	wt
		16CSB8	8	64	16	lost	wt
		16CSB9	32	256	16	lost	lost
		16CSB10	8	64	8	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin, MOX: Moxifloxacin, nt: not tested, wt: wild type – cut by *HinfI*, lost: not cut by *HinfI*

7.3.2 Gemifloxacin Selection of SB13

The mutation frequencies, fluoroquinolone MICs and *HinfI* restriction analysis of the gemifloxacin-selected mutants derived from SB13 are shown in Table 7.5.

Table 7.5. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against gemifloxacin selected mutants of SB13

Gemifloxacin-selected mutants							
	Frequency Of Mutation	Strain	MIC (mg/L) GEM	CIP	MOX	*HinfI* restriction gyrA	parC
Parent		SB13*	1	8	2	lost	wt
1st generation	5x10^{-7}	AA1	8	32	nt	lost	wt
		AB1	16	32	nt	lost	wt
		AC1	16	128	16	lost	lost
		AD1	16	64	nt	lost	wt
		AE1*	16	64	4	lost	wt
2nd generation	5x10^{-6}	BE1	>128	256	16	lost	wt
		BE2	>128	256	8	lost	wt
		BE3	>128	256	16	lost	wt
		BE4	>128	256	16	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin, MOX: Moxifloxacin, nt: not tested, wt: wild type – cut by *HinfI*, lost: not cut by *HinfI*

These data show that SB13 mutated at a similar frequency as seen against ciprofloxacin. One mutant, AC1, lost the *par*C restriction site. Its *par*C gene was sequenced and compared to its parent SB13 in Figure 7.5. This shows a C→T transversion in the gene as found in clinical isolates. The translated sequence reveals a serine → leucine substitution corresponding to amino acid residue 80 (Figure 7.6). This mutant had the highest MIC against moxifloxacin and ciprofloxacin although it shared a gemifloxacin MIC of 16mg/L with four of the other mutants with wild-type *par*C. Mutant AE1 was further selected to produce second generation mutants at a high frequency. None of these mutants lost the *par*C restriction site and sequence analysis showed no difference from that of SB13 or AE1 (Figure 7.5). The GyrA gene was also sequenced for these mutants and is shown in Figure 7.7. There was no difference between the mutants and parents. These mutants were highly resistant to gemifloxacin and ciprofloxacin but their moxifloxacin MIC was the same as the ParC mutant AC1.

Figure 7.5. The *par*C QRDR of SB13 and mutant AC1. The mutation is shown in red.

```
SB13    GTACTTGGTA AATACCACCC ACATGGTGAC TCGGCATGTT ATGAAGCCAT
AE1     GTACTTGGTA AATACCACCC ACATGGTGAC TCGGCATGTT ATGAAGCCAT
BE1     .TACTTGGTA AATACCACCC ACATGGTGAC TCGGCATGTT ATGAAGCCAT
AC1     ...CTTGGTA AATACCACCC ACATGGTGAC TTGGCATGTT ATGAAGCCAT

SB13    GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC
AE1     GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC
BE1     GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC
AC1     GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC

SB13    AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
AE1     AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
BE1     AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
AC1     AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
```

Figure 7.6. Translated *par*C QRDR of SB13 and AC1. The serine → leucine substitution is shown in red.

```
SB13  LGKYHPHGDSACYEAMVLMAQPFSYRYPLIEGQGNWGSPDDPKSFAA
 AC1  LGKYHPHGDLACYEAMVLMAQPFSYRYPLIEGQGNWGSPDDPKSFAA
```

Figure 7.7. Alignment of the *gyr*A QRDR. The mutated *Hinf*I codon is shown in red.

```
SB13    .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
AE1     .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
BE1     .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA

SB13    TTGTTCGTAT GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT
AE1     TTGTTCGTAT GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT
BE1     TTGTTCGTAT GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT

SB13    CAGGGTAACT TCGGTTCGAT CGATGGTGAT AGCGCTGCGG CAATGCGTTA
AE1     CAGGGTAACT TCGGTTCGAT CGATGGTGAT AGCGCTGCGG CAATGCGTTA
BE1     CAGGGTAACT TCGGTTCGAT CGATGGTGAT AGCGCTGCGG CAATGCGTTA

SB13    TACCGAAGTC CGTATGACTA AGCTGGCACA TGAGCTTCTT GCAGATTTAG
AE1     TACCGAAGTC CGTATGACTA AGCTGGCACA TGAGCTTCTT GCAGATTTAG
BE1     TACCGAAGTC CGTATGACTA AGCTGGCACA TGAGCTTCTT GCAGATTTAG

SB13    AAAAGACAC  AGTTGACTGG GAAGATAACT ACGACGGTTC GGAACGTATC
AE1     AAAAAGACAC AGTTGACTGG GAAGATAACT ACGACGGTTC GGAACGTATC
BE1     AAAAAGACAC AGTTGACTGG GAAGATAACT ACGACGGTTC GGAACGTATC

SB13    CCTGAAGTAC TTCCGACACG TGTTCCAAAC TTATTAA... ..........
AE1     CCTGAAGTAC TTCCGACACG TGTTCCAAAC TTATTAATTA ACGGTGCTGC
BE1     CCTGAAGTAC TTCCGACACG TGTTCCAAAC TTATTAATTA ACGGTGCTGC

SB13    .......... .......... .
AE1     TGGTATCGCC GTAGGTATGG C
BE1     TGGTATCGCC GTAGGTATNG C
```

7.3.3 Moxifloxacin Selection of SB13

The mutation rates and associated mutations of SB13 selected on moxifloxacin are shown in Table 7.6. First step mutants did not lose the *par*C *Hinf*I restriction site as seen with ciprofloxacin and gemifloxacin selection, however the mutation frequency was similar. The moxifloxacin and ciprofloxacin MICs rose 4-8 fold and gemifloxacin 8-16 fold. Mutant M4A was further selected on moxifloxacin and the second generation mutants retained the *par*C restriction site. These mutants were heterogeneous in their MICs. One mutant, M16A, showed no change in moxifloxacin MIC but was more sensitive to gemifloxacin and ciprofloxacin. The other mutants had identical ciprofloxacin MICs. Mutant M16D was highly resistant to gemifloxacin but had a moxifloxacin MIC of 32mg/L while mutant M16G had the

potency of these drugs switched. Thus the ParC mutation is not necessary for high-level fluoroquinolone resistance.

Table 7.6. MICs to gemifloxacin, ciprofloxacin and moxifloxacin against moxifloxacin selected mutants of SB13

Moxifloxacin-selected mutants							
	Frequency Of Mutation	Strain	MIC (mg/L) GEM	CIP	MOX	*HinfI* restriction gyrA	parC
Parent		SB13*	1	8	2	lost	wt
1ST generation	$6.2x10^{-7}$	M4A*	8	64	8	lost	wt
		M4B	4	32	8	lost	wt
		M4C	4	32	8	lost	wt
		M4D	4	32	8	lost	wt
		M4E	4	32	8	lost	wt
		M4F	16	64	16	lost	wt
		M4G	16	64	16	lost	wt
2nd generation	$4x10^{-7}$	M16A	4	32	8	lost	wt
		M16B	32	256	32	lost	wt
		M16C	32	256	64	lost	wt
		M16D	256	256	64	lost	wt
		M16E	32	256	32	lost	wt
		M16G	32	256	128	lost	wt

*: strain used for further selection, GEM: gemifloxacin, CIP: ciprofloxacin, MOX: Moxifloxacin, nt: not tested, wt: wild type – cut by *HinfI*, lost: not cut by *HinfI*

7.4 Sensitivity to Non-Fluoroquinolones

The ATCC 19606 mutants were tested for their sensitivity to drugs outwith the fluoroquinolone class to investigate if non-target mutations have an effect on these drugs. These data are summarised in Table 7.7. No common pattern was observed.

First step gemifloxacin mutant AP1 recorded non-fluoroquinolone MICs that were 1 dilution higher than the parent ATCC 19606 although the imipenem MIC was unchanged. No other changes were seen after this selection step. Mutant M1B had an imipenem MIC 1-dilution higher than the parent but this change was negated at subsequent selection steps. The biggest differences were seen with the ciprofloxacin-selected mutants. Mutant C7 recorded an imipenem MIC 1 dilution

higher than the parent but a tetracycline MIC that was lower. The second step ciprofloxacin mutants could be grouped into those with an imipenem and tetracycline MIC that was 0.25mg/L and 4mg/L respectively, and the more sensitive ≤ 0.12mg/L and 1mg/L respectively. Those in the more sensitive group were the least sensitive to the fluoroquinolones (Table 7.1). Although these data show minor changes in non-fluoroquinolone sensitivity, they are not indicative of enhanced efflux. What seems more likely are changes in permeability to the selecting agent that are having minor affects to the structurally dissimilar non-fluoroquinolones.

Table 7.7. Sensitivity of ATCC19606 mutants to imipenem, sulbactam, chloramphenicol and tetracycline.

		MIC (mg/L)						MIC (mg/L)			
	Strain	**IMI**	**SUL**	**CHL**	**TET**		**Strain**	**IMI**	**SUL**	**CHL**	**TET**
P	19606	0.12	0.5	256	4	1st	C7	0.25	1	nt	2
1st	AP1	0.12	1	512	8	2nd	256C1	0.25	nt	nt	4
2nd	BP1	0.12	1	512	8		256C2	0.25	nt	nt	nt
3rd	CP1	0.12	1	512	8		256C3	0.25	nt	nt	4
4th	DP1	0.12	1	512	8		256C4	0.12	nt	nt	1
	DP7	0.12	1	512	8		256C5	0.25	nt	nt	4
							256C6	0.25	nt	nt	4
1st	M1B	0.25	0.5	256	8		256C7	0.25	nt	nt	4
2nd	M2E	0.12	0.5	256	8		256C8	0.06	nt	nt	1
3rd	4M2E	0.12	0.5	256	8		256C9	0.12	nt	nt	1
4th	32MA	0.12	nt	nt	nt		256C10	0.25	nt	nt	4

P: parent strain, IMI: imipenem, SUL: sulbactam, CHL: chloramphenicol, TET, tetracycline, nt: not tested

The non-fluoroquinolone MICs of SB13 selected mutants are shown in Table 7.8. Gemifloxacin mutants were 1 dilution more sensitive to imipenem than the parent strain. AE1 was also 1 dilution more sensitive to sulbactam, but there were no changes in chloramphenicol and tetracycline sensitivity. Mutant M4A was 1 dilution less sensitive to imipenem, chloramphenicol and tetracycline but 1 dilution more sensitive to sulbactam. Second generation moxifloxacin mutants were heterogeneous in their MICs as were ciprofloxacin selected mutants. The acquisition of fluoroquinolone resistance appears to give no change or only minor changes in non-fluoroquinolone MICs, as was also found with the ATCC 19606 derived mutants.

Table 7.8. Sensitivity of SB13 mutants to imipenem, sulbactam, chloramphenicol and tetracycline.

		MIC (mg/L)			
	Strain	**IMI**	**SUL**	**CHL**	**TET**
Parent	SB13	0.5	32	256	128
1st	AC1	0.25	32	256	128
	AE1	0.5	16	256	128
2nd	BE1	0.25	16	512	128
	BE2	0.5	16	256	128
	BE3	0.25	16	256	128
	BE4	0.5	16	256	128
1st	M4A	1	16	512	256
2nd	M16A	1	16	512	256
	M16B	0.5	16	512	256
	M16C	1	16	>512	256
	M16D	1	16	512	>256
	M16E	0.5	16	>512	256
	M16G	0.5	32	512	256
1st	16CSB1	0.5	32	512	128
	16CSB6	0.5	32	512	128
	16CSB7	1	32	>512	256
	16CSB9	0.5	16	512	128
	16CSB10	0.25	16	<200	128

P: parent strain, IMI: imipenem, SUL: sulbactam, CHL: chloramphenicol, TET, tetracycline, nt: not tested

The non-fluoroquinolone MICs show that resistance to these drugs is not selected with the fluoroquinolones tested. No strain that was sensitive to imipenem, sulbactam, chloramphenicol or tetracycline prior to selection became resistant. Conversely, if a strain was resistant prior to selection, it retained its resistance.

7.5 Discussion

It has been shown that the most important mechanism in the acquisition of fluoroquinolone resistance is the development of mutations in the target site enzymes i.e. DNA Gyrase and Topoisomerase IV. Results from clinical isolates have shown that the primary target of Gram negative organisms is GyrA and for Gram positives ParC (Gonzalez *et al*, 1998; Kanematsu *et al*, 1998; Mouneimne *et al*, 1999; Nakano *et al*, 1997; Yoshida *et al*, 1990). Since clinical isolates will most probably have been selected with ciprofloxacin or levofloxacin *in vivo*, how do the target site mutations compare against the newer fluoroquinolones moxifloxacin and gemifloxacin? Because moxifloxacin has only recently been released into the clinical armoury and gemifloxacin is still undergoing clinical trials, data on isolates that have encountered these drugs *in vivo* is lacking. Therefore *in vitro* mutation studies provide the best model.

Mutation studies have shown that in *E. coli* selected on norfloxacin or lomefloxacin, GyrA mutations are found primarily and ParC mutations are both secondary and rare (Burucoa , Lhomme and Fauchere, 1999; Tavio *et al*, 1999). Ciprofloxacin selected *S. pneumoniae* acquire a ParC mutation before a GyrA mutation (Pan *et al*, 1996). However, *S. pneumoniae* selected with clinafloxacin selects a GyrA mutation first (Pan and Fisher, 1998) and C-7 analogues of ciprofloxacin also select for GyrA mutations primarily (Alovero *et al*, 2000). With moxifloxacin selected *S. aureus*, GyrA is selected first (E. Durham, PhD thesis) but with ciprofloxacin-selected strains, a GrlA (ParC) mutation is selected primarily (Ferrero, Cameron and Crouzet, 1995). Thus it appears that target site mutations are dependent upon the selecting agent, at least in the case of Gram positive organisms.

Results obtained in this study show that the newer fluoroquinolones select for a GyrA mutation primarily, irrespective of the selecting agent and at a frequency much lower than ciprofloxacin. ParC mutations appear to be limited to selection on ciprofloxacin and gemifloxacin, although this may be strain dependant as the

laboratory strain ATCC 19606 did not mutate in its ParC QRDR when selected with any of the drugs. In spite of this, high-level resistance was recorded against the fluoroquinolones tested, therefore an alternative mechanism of resistance that is not dependant upon ParC mutations was selected. These other mutations could come from mutations in GyrB, ParE or outer membrane changes.

Indeed, both GyrB and ParE mutations have been found in both clinical and laboratory studies although their contribution is subject to speculation (Dessus-Babus *et al*, 1998; Gonzalez *et al*, 1998; Jones *et al*, 2000; Kaatz and Seo, 1997; Mouneimne *et al*, 1999; Nagai *et al*, 2000).

Active efflux has been found in laboratory mutational experiments with *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pneumoniae* (Jones *et al*, 2000; Kern *et al*, 2000; Li, Livermore and Nikaido, 1994; Mazzariol *et al*, 2001; Piddock and Jin, 1999). However, the selection of a multi-drug efflux system (MDR) was not seen in these data. Kern *et al* (2000) have shown in *Escherichia coli* that non-target mutations i.e. efflux, is associated with an 8-fold increase in non-fluoroquinolone MIC and a 100-fold increase in fluoroquinolone MIC. In this study, we have found that while the fluoroquinolone MICs do rise at least 100-fold over several selection steps without changes in the ParC QRDR, the non-fluoroquinolone MICs are subject to only minor changes, if any at all, therefore an MDR efflux system was not selected. The rise in fluoroquinolone MICs without GyrA and ParC mutations, combined with minor changes in non-fluoroquinolone MICs suggests that permeability mutants are being selected.

Evidence for this comes from the fact that moxifloxacin potency is the least affected when there is no ParC mutation. When a GyrA mutation is selected, or if there is a double GyrA/ParC mutation, gemifloxacin and moxifloxacin are effectively equipotent (see mutant AC1). However, if there is only the single GyrA mutation and no ParC mutation in subsequent selection steps, the hydrophilic gemifloxacin is surpassed in its activity by the hydrophobic moxifloxacin (mutants 256C3, DP4-DP7, BE1-BE4). Ciprofloxacin, although less potent than gemifloxacin before a

GyrA mutation, becomes equipotent without a ParC mutation. This strongly suggests that permeability mutations are being selected. Bazile *et al* (1992) has shown that there is a correlation between fluoroquinolone hydrophobicity and accumulation in Gram negative organisms. The more hydrophobic a drug is, the less it accumulates. Resistant strains also accumulate less drug, however hydrophobic drugs are less affected. If the selecting agent is hydrophilic (gemifloxacin and ciprofloxacin), it is likely that a permeability mutation against hydrophilic agents will be selected thus explaining the MIC differentials.

Permeability mutations have been found in Gram negative organisms such as *P. aeruginosa* and *K. pneumoniae* (Chevalier *et al*, 2000; Germ *et al*, 1999; Hancock, 1998). Not all permeability mutations lead to decreased fluoroquinolone sensitivity. *K. pneumoniae* porin loss in a study by Hernandez *et al* (2000) was associated with resistance to the β-lactams but not the fluoroquinolones. The slower diffusion of the β-lactam allows a β-lactamase to destroy the drug before it reaches the target. However as no drug-destroying enzyme have been found to have activity to the fluoroquinolones, they will still reach their target, albeit at a reduced rate, unless retarded diffusion is coupled to active efflux.

Recently, a new method to investigate the selection of fluoroquinolone resistance *in vitro* has been proposed. The mutant prevention concentration (MPC) represents a threshold above which the selection of resistant mutants is expected to occur only rarely (Blondeau *et al*, 2001). It is based on the number of bacteria in a human infection reaching 10^{10} cells. This number of cells are plated onto antibiotic containing plates in a similar way to determine MIC by agar dilution. The MPC is the concentration of drug where there are no recoverable cells after 48 hours incubation. Effectively, it is the MIC when 10^{10} organisms are plated onto MIC plates. However, one flaw of this method is the figure of 10^{10} organisms which does not stand up to scrutiny. This number comes from cited references that lead a paper trail to three articles where numbers of infecting organisms are given. One looked at neonatal septicaemia caused by *E. coli* (Dietzman, Fischer and Schoenknecht, 1974). A quantitative count gave a figure of 50-1000 cfu/ml. Taking the higher figure of

1000 cfu/ml, one would need 10^4L of blood to achieve 10^{10} organisms, however the human body only contains approximately 5L of blood. In another reference, the quantitative count of organisms in aspiration pneumonia was given as $\geq 10^6$ cfu/ml (Lorber and Swenson, 1974). To achieve 10^{10} organisms, the lung would have to contain 10L of fluid. Finally, bacteria recovered from endocarditis yielded up to 300 cfu/ml (Werner *et al*, 1967). Clearly not even a blue whale would have enough blood for the recovery of 10^{10} organisms. For the MPC to be of use, the number of organisms needs to be re-evaluated.

For this study, a value of 2xMIC was used to select resistant organisms. A truer picture of resistance development could come from using breakpoint values instead of multiples of MIC. However, if resistant organisms are recovered and they have an MIC above breakpoint, then further selection using breakpoints would be difficult as all organisms that are plated will grow and the recovery of secondary mutations would involve the screening of thousands of colonies. One other criticism of MIC multiples is that they are a fixed concentration of drug. *In vivo* drug concentrations will change over time as the drug is cleared from the body, something that one cannot mimic on a petri dish. Animal models exist as do *in vitro* chamber models where the concentration of drug fluctuates over time. To date there has been no comparative study that looks at selection on plates, chamber and animal models.

In vivo selection of fluoroquinolone resistance during urinary tract infections has been shown to correlate with fluoroquinolone use in *E. coli* and *K. pneumoniae* (Cizman *et al*, 2001; Deguchi *et al*, 1997; Muder *et al*; 1991). Cizman in fact recommends that fluoroquinolones should only be used as a second choice drug to prevent resistance emerging. However other studies have found no correlation between increased usage and resistance (Svetlansky *et al*, 2001; Pieroni *et al*, 1997). Thus it may be that prudent use of these drugs is called for and an overeliance will only cause problems for the future.

The high mutational rates of *A. baumannii* against ciprofloxacin presented here demonstrate the propensity for the organism to become resistant. First step mutants had lost the *gyr*A restriction site at a frequency of 2.4×10^{-5} compared to gemifloxacin

and moxifloxacin which needed three and two steps respectively in the order of 10^{-8}-10^{-9} at each step. This high rate may explain why there is a correlation between ciprofloxacin use and resistant *A. baumannii* previously discussed. Mutation rates of $1.6x10^{-7}$, $5.4x10^{-8}$ and $1.9x10^{-7}$ have been reported for *E. coli*, *P. aeruginosa* and *K. pneumoniae* respectively selected on ciprofloxacin (Gilbert *et al*, 2001; Schulte and Heisig, 2000). Perhaps it is not the organism but the selecting drug, as mutation rates of *E. coli* are considerably lower when other drugs are used for selection: $1.2x10^{-10}$ for gemifloxacin (Schulte and Heisig, 2000), $5x10^{-9}$-$1x10^{-11}$ with nalidixic acid and ofloxacin (Kern *et al*, 2000), 10^{-9}-10^{-10} with norfloxacin (Hirai *et al*, 1986b) and 10^{-9}-10^{-4} with norfloxacin and lomefloxacin (Tavio *et al*, 1999). These cited papers do not yield much information on the individual selection steps therefore it is unknown at which point a GyrA mutation was selected. The study by Kern *et al* (2000) states that they used an inoculum of 10^{10}-10^{12} organisms, however in the experience of these studies, achieving an inoculum of $>10^{9}$ organisms is difficult.

The gemifloxacin and ciprofloxacin-selected strains follow the pattern for the development of resistance seen in clinical isolates, namely that GyrA is the primary target. However mutant M2E had the *gyr*A mutation but did not record the highest MICs of it's generation. ParC mutations were not found with mutants derived from ATCC 19606, but they were found with the clinical isolate SB13, confirming its role as the secondary target. However, as a *par*C mutation was not found in all the highly resistant mutants, these data suggest that an alternative route to fluoroquinolone resistance exists with *in vitro* selected mutants.

Conclusions

If we take the acquisition of a GyrA mutation as the benchmark for the development of fluoroquinolone resistance, the use of ciprofloxacin has been shown to select for resistant organisms at high frequency. However, the newer fluoroquinolones gemifloxacin and moxifloxacin, which require 2-3 independent steps to acquire the GyrA mutation, are less likely to select for fluoroquinolone-resistant *A. baumannii*.

Chapter 8: Stability of Gemifloxacin-Selected Mutants and Identification of a Porin

8.1 Introduction

There is a thought that if antibiotic pressure is removed from a population of bacteria then resistance will disappear. In this study, the gemifloxacin-selected mutants from Chapter 7 have the stability of their mutations investigated by passage on antibiotic-free agar. Narrow-range MICs are used against some of the mutants to gain a better understanding of their sensitivity profiles. One mutant after passage is re-selected with gemifloxacin. In chapters 6 & 7, it has been shown that all isolates exhibiting high-level resistance to the fluoroquinolones have a serine 83 mutation in GyrA. However, a serine 80 mutation in ParC, although contributing to high-level resistance, is not necessary and unrelated strains with seemingly identical QRDRs can have radically diverse MICs to the same fluoroquinolone. This suggests other factors are involved besides the GyrA and ParC QRDRs. In this study, the SB13-BE1 lineage of gemifloxacin selected mutants from Chapter 7 will be examined and will show that high-level fluoroquinolone resistance is associated with an altered outer membrane profile in conjunction with the serine 83 GyrA mutation. This chapter also begins the process to characterise a porin of 45.5kDa.

8.2 Passage of ATCC 19606 Mutants

The results of ATCC 19606 mutants after 10x passage are shown in Table 8.1. The parent strain showed no difference in fluoroquinolone MIC after passage. The majority of the mutants as well as the parent strain ATCC 19606 showed no difference in their gemifloxacin, ciprofloxacin or moxifloxacin MICs.

Of the three first generation mutants, only mutant AP1 exhibits a lowering of MIC against moxifloxacin. Two of the second generation mutants show a two-fold

reduction in their fluoroquinolone MICs and one mutant a reduction in gemifloxacin and ciprofloxacin MIC. The other five mutants show no change. The third generation mutants all have a GyrA mutation which was retained after passage, however, two of the mutants have a lowering of MIC to the three fluoroquinolones. This lowering of MIC does not bring the MICs below breakpoint level. Of the fourth generation mutants, only one has a lowering of MIC to the three fluoroquinolones and mutant DP4 has a four-fold decrease in MIC to gemifloxacin.

Figure 8.1. MICs of ATCC 19606 gemifloxacin-selected mutants after passage. The pre-passage MICs are in parenthesis.

		MIC (mg/L)		
	Strain	**GEM**	**CIP**	**MOX**
Parent	19606	0.12 (0.12)	1 (1)	0.25 (0.25)
1st Gen	AP1	0.5 (0.5)	4 (4)	0.5 (1)
	AQ1	0.5 (0.5)	2 (2)	0.5 (0.5)
	AR1	0.5 (0.5)	2 (2)	0.5 (0.5)
2nd Gen	BP1	1 (1)	8 (8)	1 (1)
	BP2	0.5 (1)	4 (8)	0.5 (1)
	BP3	1 (1)	8 (8)	1 (1)
	BP4	1 (1)	8 (8)	1 (1)
3rd Gen	CP1	8 (16)	64 (128)	4 (8)
	CP2	16 (16)	128 (128)	8 (8)
	CP3	16 (16)	128 (128)	8 (8)
	CP4	4 (8)	16 (32)	2 (2)
4th Gen	DP1	16 (32)	128 (>128)	16 (16)
	DP2	64 (64)	>128 (>128)	16 (16)
	DP3	64 (64)	>128 (>128)	16 (16)
	DP4	32 (128)	>128 (>128)	16 (16)
	DP5	>128 (>128)	>128 (>128)	16 (16)
	DP6	>128 (>128)	>128 (>128)	32 (32)
	DP7	>128 (>128)	>128 (>128)	32 (32)

8.3.1 Passage of SB13 Mutants

The MICs of SB13 gemifloxacin-selected mutants after 10x passage are shown in Table 8.2. Neither the parent or the two first-generation mutants after passage showed a change in sensitivity to the fluoroquinolones, imipenem or sulbactam. SB13, AE1 and AC1 retained their GyrA mutation and AC1 retained its ParC mutation. Post-passage, the second generation mutant BE1 showed a reduction in MIC to the four fluoroquinolones and an increase in its imipenem MIC. Effectively it had reverted to the antibiotic profile of AE1 with the exception of its gemifloxacin MIC which was one dilution higher than AE1. Henceforth, this mutant will be denoted as BE1rev.

Table 8.2. MICs of SB13 derived mutants after 10x passage on antibiotic-free media. Pre-passage MICs are in parenthesis.

	MIC (mg/L)					
Strain	**GEM**	**CIP**	**MOX**	**SPA**	**IMI**	**SUL**
SB13	1 (1)	8 (8)	2 (2)	4 (4)	0.5 (0.5)	32 (32)
AC1	16 (16)	128 (128)	16 (16)	nt	nt	nt
AE1	16 (16)	64 (64)	4 (4)	8 (8)	0.5 (0.5)	16 (16)
BE1	32 (>128)	64 (256)	4 (8)	8 (16)	0. 5 (0.25)	16 (16)

8.3.2 Narrow-range MICs

Given that there were minor differences in sensitivity to moxifloxacin, sulbactam and imipenem through the selection steps, narrow-range MICs were performed on them and BE1rev to better understand their sensitivity profiles. Table 8.3 summarises the data.

Table 8.3. Narrow Range MICs (mg/L)

Strain	MOX	SUL	IMI
SB13	1.5	23	0.3
AE1	3.75	14	0.3
BE1	11.0	14	0.17
BE1rev	4.25	13	0.28

Range used:
MOX: 0.25 mg/L increments
SUL: 1mg/L increments
IMI: 0.1mg/L increment

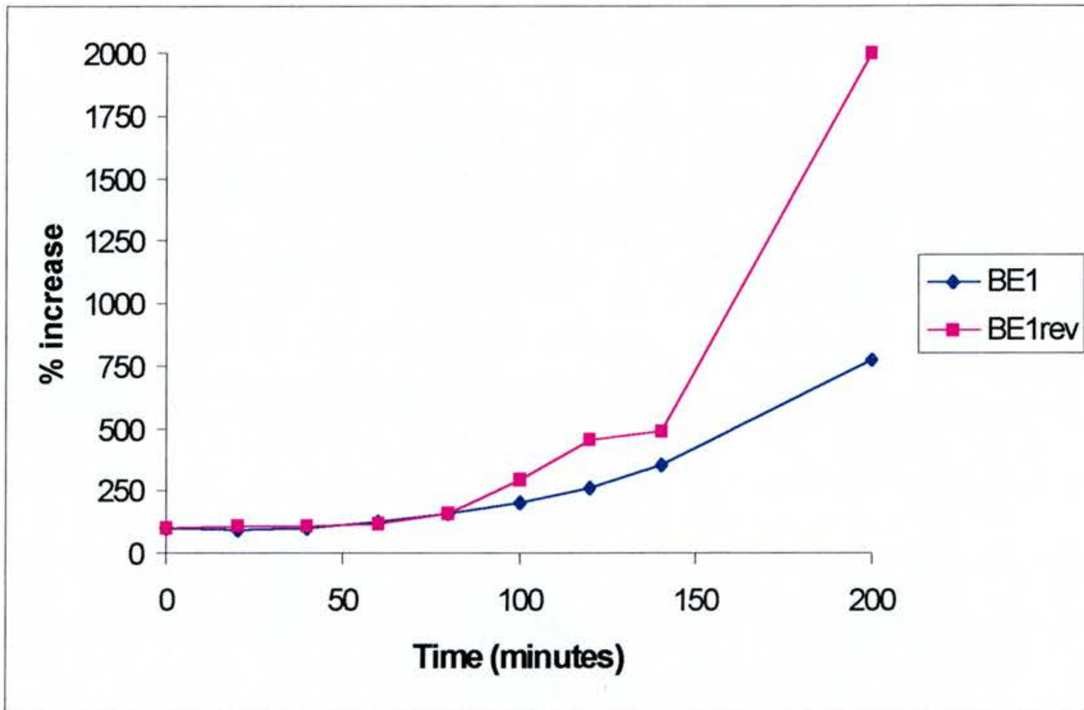
The narrow range MICs recorded are within the range one would expect when compared to doubling dilution MIC (Table 8.2). By using small increments in the amount of drug in each plate, a more exact measurement of their sensitivity to the drugs was determined. Narrow-range plates are however difficult to read as the cut-off point between growth and no growth is not as distinct as using doubling dilution. The increase in antibiotic concentration between each plate was extensively tested until a degree of confidence and reproducibility was reached. If the MIC of the organism is known, it can aid in the decision as to what range should be used.

These data show that after two selection steps, the moxifloxacin MIC has risen from 1.5mg/L to 11mg/L. This rise has been negated after passage of BE1. The first selection step led to a reduction in sulbactam resistance. This reduction was stable in both AE1 and BE1. The imipenem MIC did not decrease until the second selection step and this reduction was reversed after the passage of BE1. These data suggest that the first mutational event was stable but the second one was not.

8.4 Growth rates of gemifloxacin-selected SB13 mutants

One phenotypical difference within the SB13-BE1rev lineage was their growth rates in liquid culture and colony size on solid media. With each selection step, the growth rates and colony sizes were reduced but with the revertant strain, it increased. This can be seen in the growth curves of BE1 and BE1rev (Figure 8.1).

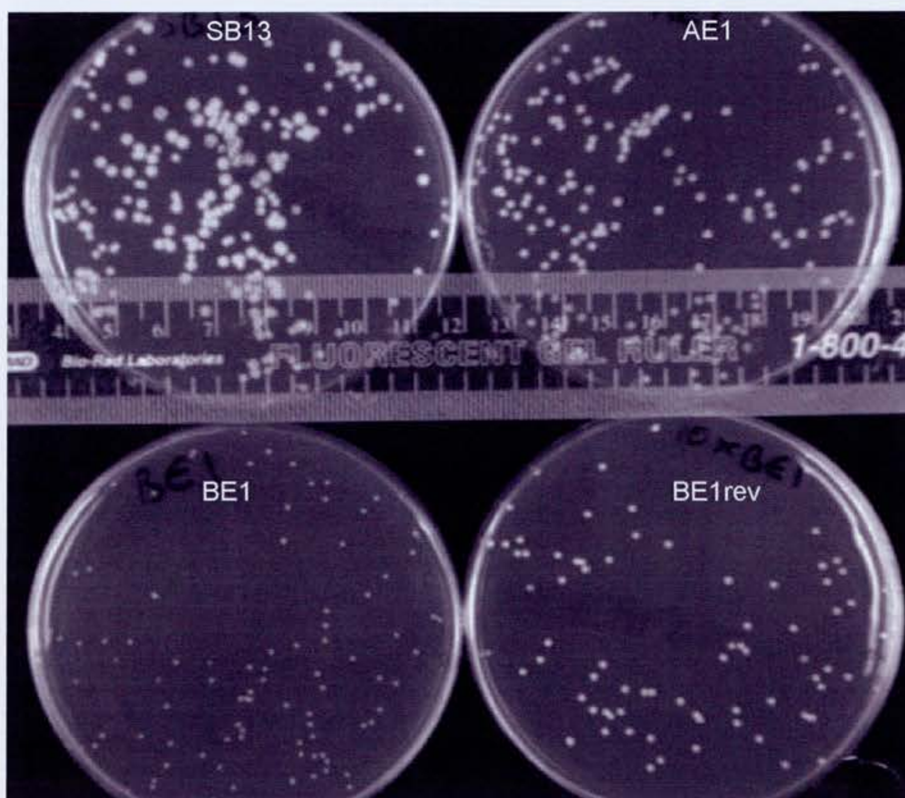
Figure 8.1. Growth rate of BE1 and BE1rev in nutrient broth



The stationary phase lasts for about 80 minutes for both organisms. However after this point, the growth rate of BE1rev increases beyond that of BE1.

This is further illustrated in Figure 8.2 where a loopful of each mutant has been streaked onto separate MacConkey agar plates. The parent SB13 has larger colonies than BE1, however the revertent colony size has increased to nearer that of AE1.

Figure 8.2. Colony size of SB13-BE1rev lineage



8.5 Analysis of BE1rev *gyr*A and *par*C QRDR

The GyrA and ParC QRDR of BE1rev was determined and is shown in Figures 8.3 and 8.4. As seen with AE1 and BE1 in Chapter 7, no differences in the QRDRs were found.

Figure 8.3. Alignment of the SB13 and BE1rev *gyr*A QRDR

```
SB13     .....TCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA
BE1rev   CGTATTCGGT AAATATCACC CGCATGGTGA CTTAGCTGTT TATGAAACCA

SB13     TTGTTCGTAT GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT
BE1rev   TTGTTCGTAT GGCTCAAGAC TTTAGCTTAC GTTATTTATT GGTTGATGGT

SB13     CAGGGTAACT TCGGTTCGAT CGATGGTGAT AGCGCTGCGG CAATGCGTTA
BE1rev   CAGGGTAACT TCGGTTCGAT CGATGGTGAT AGCGCTGCGG CAATGCGTTA

SB13     TACCGAAGTC CGTATGACTA AGCTGGCACA TGAGCTTCTT GCAGATTTAG
BE1rev   TACCGAAGTC CGTATGACTA AGCTGGCACA TGAGCTTCTT GCAGATTTAG

SB13     AAAAGACAC AGTTGACTGG GAAGATAACT ACGACGGTTC GGAACGTATC
BE1rev   AAAAAGACAC AGTTGACTGG GAAGATAACT ACGACGGTTC GGAACGTATC

SB13     CCTGAAGTAC TTCCGACACG TGTTCCAAAC TTATTAA... ..........
BE1rev   CCTGAAGTAC TTCCGACACG TGTTCCAAAC TTATTAATTA ACGGTGCTGC
```

Figure 8.4. Alignment of the SB13 and BE1rev *par*C QRDR

```
SB13     GTACTTGGTA AATACCACCC ACATGGTGAC TCGGCATGTT ATGAAGCCAT
BE1rev   ...CTTGGTA AATACCACCC ACATGGTGAC TCGGCATGTT ATGAAGCCAT

SB13     GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC
BE1rev   GGTACTCATG GCTCAGCCAT TTAGTTACCG CTATCCTTTA ATCGAAGGTC

SB13     AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
BE1rev   AGGGGAACTG GGGCTCACCT GATGATCCTA AGTCTTTTGC TGCG
```

8.6.1 SDS-PAGE of outer membrane proteins

The outer membrane profile of SB13, AE1, BE1 and BE1rev are shown in Figure 8.3. A major band of 40kDa seen in the 4 isolates has been shown to be a trimeric porin by Jyothisri *et al* (1999). Lane 4 shows increased expression of a 45.5 kDa protein (arrow) in BE1. This protein has been identified by Sato and Nakae as a porin (1991). The increased expression is not seen in AE1 (Lane 3) the parent strain of BE1 and is lost after 10x passage with the reverent BE1rev (Lane 5). Densitometric analysis of the gel shows that this 45.5kDa protein makes up 2% of the total OMPs for SB13, AE1 and BE1rev, but has increased to 4.7% of the total

increased to 4.7% of the total OMPs for BE1. The other proteins are subject to minor differences in expression levels but none of them have a >2-fold increase in expression.

Figure 8.3. SDS-PAGE outer membrane profile from isogenic mutants and parent strain. Lane 1, molecular weight marker: Lane 2, SB13: Lane 3, AE1: Lane 4, BE1: Lane 5, BE1rev: Lane 6, molecular weight marker. The 45.5kDa porin is indicated with an arrow.

These data suggest that there is an association between high-level fluoroquinolone resistance and increased expression of the 45.5kDa porin. The remainder of this chapter deals with determining the amino acid and nucleotide sequence of the porin.

8.6.2 Purification of the 45.5kDa Porin

The purification of the 45.5kDa porin was attempted using ion exchange chromatography. Two different columns were used; cation exchange and anion exchange using a salt gradient to elute the proteins. This did not purify the porin therefore it was isolated by electroblotting an SDS-PAGE gel onto a PVDF membrane, coomassie staining the membrane and excising the band of interest with a scalpel.

8.7.1 N-Terminal Sequencing of the Porin

The excised PVDF membrane-bound porin was N-terminally sequenced at GlaxoSmithKline laboratories. A 40 amino acid sequence was determined and is shown in Figure 8.5.

Figure 8.5. 40 amino acid fragment of the 45.5kDa OMP from BE1

SEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKDTSSVAQ

The sequence was analysed in BLAST and was found to have 45% identity to porin OprE3 and 42.5% identity to OprD2, both from *P. aeruginosa* (Appendix VII).

8.7.2 Determination of the nucleotide sequence of 28 amino acids of the Porin

The 40 amino acid sequence was used to design degenerate primers to amplify and sequence the gene. Appendix I shows the amino acid sequence and the possible codons. Because serine has six possible codons, the primers were derived from an area of less degeneracy corresponding to 28 of the amino acids. PCR of the gene gave a product of 87bp. This was sequenced using the degenerate primers (Figure

8.6). However, the plus and minus strands did not overlap therefore a consensus sequence was not possible. The nucleotide sequence of three amino acids in the middle portion of the fragment were not identified (Figure 8.6). The reason for this is that when sequencing a PCR product, the first 30-40bp, including the primer region, does not yield a readable sequence. This means that primer 1 gave the sequence of the 3' end of the fragment and primer 2 the 5' end of the fragment. As there was no overlap, this resulted in the missing segment. The primer 2 sequence was reverse complemented before translation. The translated sequence did not fully match the original amino acid sequence with one amino acid difference (Figure 8.6). Nevertheless, inverse PCR primers were designed in order to sequence upstream and downstream of the known area.

Figure 8.6. Amino acid and nucleotide sequence of the OMP using degenerate primers. In bold is the amino acid that differs from that using N-terminal sequencing (in parenthesis).

Amino	E	A	K	G	F	V	E	D	A	N
Nucleotide	GAG	GCG	AAA	GGG	TTT	GTG	GAG	GAT	ACG	AAC

Amino	G	S	I	L	F	R	T	G	Y	L
Nucleotide	GGC	TCC	ATT	No sequence			ACA	GGT	TAT	CTA

Amino	T	**G**(R)	D	K	K	Q	G	A
Nucleotide	ACC	GGC	GAC	AAA	AAA	CAA	GGC	GCA

8.7.3 Inverse PCR

Inverse PCR was attempted using primers derived from the sequence in Figure 8.6. Double digests of whole genomic DNA from BE1 was used as template. After restriction of genomic DNA, re-ligation and PCR, there was no PCR product visible on the gel. Using software that identifies restriction sites within DNA, a novel restriction site (*Nla*IV) was found in the 87bp sequence. Attempts to cut the 87bp

PCR product failed, therefore suspicion on the integrity of the sequence meant that the inverse PCR was shelved. Another method was then used to determine the sequence.

8.7.4 Cloning of the PCR fragment

The PCR product was cloned into a pCR 4-TOPO plasmid by Richard Gibbs. The vector contains M13 priming sites 88bp upstream and 75bp downstream of the insert. After the plasmid was extracted, it was subjected to PCR using M13 primers to give a PCR product of 252bp. This PCR product was sequenced using the M13 primers. The sequence, when translated, matched the original amino acid sequence (Figure 8.7a). The nucleotide sequence from the clone is compared against the sequence from using the degenerate primers (Figure 8.7b).

Figure 8.7. (a) Nucleotide and translated amino acid sequences from the cloned PCR fragment. **(b)** comparison of the nucleotide sequence from 1: using degenerate primers and 2: the cloned PCR fragment. Mismatched nucleotides are in red.

```
(a)  gaggcgaaggggtttgtggaagatgcgaacggttctattctcttccgtaca
      E  A  K  G  F  V  E  D  A  N  G  S  I  L  F  R  T

     ggttatctaacccgcgacaaaaaacagggcgc
      G  Y  L  T  R  D  K  K  Q  G

(b)  1.   GAGGCGAAAGGGTTTGTGGAGGATGCGAACGGCTCCATT.........ACAG
          |||||||| |||||||||||| |||||||||||| || |||         ||||
     2.   GAGGCGAAGGGGTTTGTGGAAGATGCGAACGGTTCTATTCTCTTCCGTACAG

     1.   GTTATCTAACCGGCGACAAAAAACAAGGCGC
          |||||||||||| ||||||||||||| |||||
     2.   GTTATCTAACCCGCGACAAAAAACAGGGCGC
```

The difference in the nucleotide sequence between using the degenerate primers to sequence the PCR product and that from the clone are clearly seen in Figure 8.7b. There are 5 nucleotide differences between them. The missing 9bp fragment has also

been sequenced using the clone. The nucleotide sequence was put into BLAST but there was no sequence similarity in the database.

8.8 Re-Selection of BE1rev on Gemifloxacin

The revertant BE1rev was re-selected on gemifloxacin containing agar plates to see if a ParC mutation would be selected. The MICs of the mutants from this selection are shown in Table 8.8. The loss of the *gyr*A restriction site was carried over but none of the mutants had lost the *par*C restriction site.

Table 8.8. Selection of mutants from BE1rev (nt: not tested)

	MIC (mg/L)								*Hinf*I Restriction	
Strain	GEM	CIP	MOX	SPA	IMI	SUL	CHL	TET	*gyr*A	*par*C
BE1rev	32	64	4	8	0.25	16	256	128	not cut	cut
G32A	>128	>256	16	16	nt	16	512	128	not cut	cut
G32B	64	128	8	16	nt	8	256	128	not cut	cut
G32C	>128	256	8	16	nt	16	128	128	not cut	cut
G32D	64	128	8	8	nt	16	256	128	not cut	cut
G32E	>128	>256	32	32	1	8	512	256	not cut	cut
G32F	>128	>256	16	16	nt	16	256	128	not cut	cut
G32G	>128	>256	32	32	nt	8	512	256	not cut	cut
G32H	64	256	8	8	0.25	8	256	64	not cut	cut

A mutational frequency of 1×10^{-5} was recorded for this selection which was higher than that to produce the mutants BE1-BE4 from AE1. A rise in MIC against the fluoroquinolones was seen in all mutants except for two which showed no rise in their sparfloxacin MIC. However, these mutants recorded a 2-fold increase to gemifloxacin, ciprofloxacin and moxifloxacin. Two mutants, G32E and G32G, recorded sparfloxacin and moxifloxacin MICs of 32mg/L and were the only ones to show a rise in resistance to tetracycline, from 128mg/L to 256mg/L. These two strains saw a lowering of their resistance to sulbactam and G32E also saw its imipenem MIC rise 4-fold.

8.9 Discussion

In many respects, the stability of a mutation is equally as important, if not more, than the mutation frequency. If an organism has the ability to mutate to an antibiotic it is of great and obvious concern to the clinician. However if this mutation compromises the organism to such a degree that it cannot compete with other organisms, then it will not persist in the environment.

Jones *et al (2000)* have investigated the stability of mutations by growing *S. aureus* for 500 generations in an antibiotic-free environment. The strains used carried multiple mutations conferring resistance to the fluoroquinolones. These included target-site mutations and those in the efflux pump NorA and its promoter. All the mutations were stably inherited after passage. Strains that were grown for 500 generations with a subinhibitory concentration of ciprofloxacin were found to accumulate more mutations and their ciprofloxacin MICs rose accordingly. This has also been demonstrated with clonal lineages of *S. aureus* in the clinical environment over a period of years (Jones *et al*, 1999).

In the present study, the stability of gemifloxacin-selected *A. baumannii* mutants was investigated. The mutants from laboratory strain ATCC 19606 did not lose their GyrA mutation although a few strains did become slightly more sensitive to the fluoroquinolones. However these mutants, when resistant, did not record MICs below published breakpoint values (Andrews, Ashby, Jevons and Wise, 1999; British Society for Antimicrobial Chemotherapy, 1991; Wise and Andrews, 1999), except for BP2 which became sensitive to moxifloxacin.

A similar picture emerged with mutants derived from the clinical isolate SB13. The parent strain and the first generation mutants did not show any difference in their sensitivity to fluoroquinolones and non-fluoroquinolones after passage. The GyrA and ParC mutations were carried through passage. Only the second generation mutant BE1 reverted but it did not become clinically sensitive to the

fluoroquinolones. The *gyr*A and *par*C gene showed no difference between the revertent and strain SB13.

The reselection of mutants from the revertant was at a high frequency and high-level resistance to the fluoroquinolones resulted. One mutant recorded a four-fold increase in its imipenem MIC. This suggests that even if a mutation is unstable, the organism can re-mutate and at a higher frequency.

The selection of SB13 mutants had an effect on their growth rates. Strain BE1 grew slower than its parents. This was visible in their colony sizes after an overnight incubation on solid media and in their growth curves. The effects of fluoroquinolone mutations on growth rate has been reported in *E. coli* (Bagel *et al*, 1999). It was found that a GyrA serine 83 or aspartic acid 87 mutation had no effect on doubling time. However, a combination of GyrA and a ParC mutation in high-level resistance mutants did have an effect on doubling time. The mutant BE1 only had a serine 83 mutation in GyrA, therefore the slower rate of growth is not caused by target-site mutations. As a reduction in permeability is hypothesised to be the resistance mechanism combined with innate efflux, these must be having a knock-on effect on growth.

The narrow-range MICs show that one-dilution differences seen with doubling dilution MIC can be significant. However they are sometimes difficult to interpret as there is no clear cut-off point when growth is inhibited, rather the growth on the antibiotic plates fades gradually. They should not be used for routine MIC investigations but are useful if permeability mutations are suspected. The data in this study show that there is a small but significant lowering of sulbactam MIC in the first mutational step of SB13. The second mutational step gave a small but reproducible increase in imipenem sensitivity. This lowering of MIC was negated after passage of BE1, as was the MIC to the fluoroquinolones. Given that the mutation in BE1 compromised the growth of the organism, any back-mutation would outgrow the parent genotype which is what was found.

Resistance to antibacterials mediated through low outer membrane permeability and porin loss has been well documented in *E. coli* and *P. aeruginosa* (Gotoh and Nishino, 1990; Heisig and Tschorny, 1994; Tavio *et al*, 1999; Trias and Nikaido, 1990). In *A. baumannii*, intrinsic antibiotic resistance has been attributed to a small number of porins that results in the outer membrane being 1-3% as permeable as *E. coli* (Sato and Nakae, 1991). Resistance to imipenem in *A. baumannii* is associated with the loss of a 33-36kDa porin (Clark, 1996) and a 23kDa porin (Bou *et al*, 2000). However, Moreau *et al* (1996) and Vila *et al*(1995) found fluoroquinolone resistance involved gyrase mutations but not OMP differences.

In Chapters 7 and 8, high-level fluoroquinolone resistance was recorded in mutant BE1 with a serine 83 mutation in GyrA, no change in ParC and increased expression of a 45.5kDa porin. The porin was sequenced and was found to have 45% identity to OprE3 and 42.5% identity to porin D2 of *P. aeruginosa*. OprE3 does not appear to play a role in antibiotic resistance. Okamoto *et al* (1999) found that there was no difference in antibiotic sensitivity between *P. aeruginosa* strains with or without OprE3. Porin D2 has been shown to form an imipenem permeable pore (Yoneyama *et al*, 1992) and to catalyse the diffusion of carbapenems and sparfloxacin (Micheahamzehpour, Furet and Pechere, 1991). When introduced into lipid bilayers, D2 allows the passage of imipenem and fluoroquinolones but not tetracycline (Ishii and Nakae, 1996). In *P. aeruginosa*, when D2 expression is lowered, the imipenem MIC is raised (Gotoh and Nishino, 1990; Micheahamzehpour *et al*, 1991). Conversely, the more D2, the lower the imipenem MIC. The mutant BE1 overexpressed the porin (4.7 % vs 2 % of total OMPs) and was more sensitive to imipenem than SB13, AE1 and BE1rev. Micheahamzehpour *et al* (1991) speculate that because both imipenem and sparfloxacin have a carboxyl and protonated nitrogen approximately 1nm apart, they act as substrates for the porin. However, moxifloxacin does not have the nitrogen moiety and in the present study was equipotent with sparfloxacin against BE1. Given that there was more of the porin, one would expect the sparfloxacin MIC to be lower in BE1 than AE1. The opposite effect was seen. This of course is speculative, as only the N-terminal 40 amino acids are known, which is approximately 10% of the whole protein. The functional part of

the porin that makes up the pore is unknown and it is possible that this portion may not share homology to D2.

One other factor that contributes to fluoroquinolone accumulation is the molecular weight of the drug, with heavier drugs accumulating less (Piddock, Jin and Griggs, 2001). Moxifloxacin, with a molecular weight of 437.9g is the heaviest of the drugs tested and recorded the lowest MICs. Ciprofloxacin has a molecular weight of 331.4g and records the highest MICs, while gemifloxacin has a molecular weight of 374.3g. Sato and Nakae (1991) found that the 45.5kDa porin preferentially allows the diffusion of low molecular weight drugs over high molecular weight. This does not explain the greater potency of moxifloxacin over gemifloxacin unless the lighter drugs are preferentially pumped out of the cell. Given that the greatest difference in drug potency between the "heavy" and "light" drugs was with the mutant overexpressing the porin is suggestive of a permeability mutation. This will need to be tested using accumulation assays.

Given that reduced permeability alone will not contribute significantly to fluoroquinolone resistance is suggestive that the porin may be working in tandem with an efflux system that preferentially pumps out hydrophilic fluoroquinolones (Li, Livermore and Nikaido, 1994). Indeed, the hydrophilic ciprofloxacin and gemifloxacin recorded higher MICs than the hydrophobic moxifloxacin and sparfloxacin. The 40 amino acid fragment does not show any sequence similarity to known efflux components, but from these data the involvement of efflux cannot be discounted.

The fluoroquinolone MICs were elevated in BE1, especially gemifloxacin and ciprofloxacin, and reverted to parent sensitivity with the reduction in expression of the porin. There were no differences in the GyrA or ParC QRDRs between the mutants. These data show that there is an association between fluoroquinolone resistance and the porin, and that it was selected in preference to a ParC mutation.

Why would a permeability mutation be selected before or instead of a target site mutation? Gram negative bacteria have only one *gyr*A and one *par*C gene but have several different porins. For example, *P. aeruginosa* has to name a few, D2, OprE3, OprM, OprJ, OprN, of which the latter three are overexpressed in resistant organisms (Masuda, Sakagawa and Ohya, 1995). The only mutation in GyrA and ParC found in this thesis is a C→T transversion in the codons serine 83 (GyrA) and serine 80 (ParC). Two point mutations out of a whole genome! However, a mutation in a porin could come from at least four different avenues: (1) downregulation mediated through a mutation in a repressor gene leading to slower diffusion; (2) it can also be downregulated by a mutation in the promoter that results in inhibition of expression; (3) structural modifications to the porin that change its affinity to the drug; (4) if the porin is linked to an efflux system then a mutation in the efflux pump may make it work more efficiently or be overexpressed and hence keep intracellular levels of drug below inhibitory concentrations. As there is more than one type of porin per cell, more mutations are theoretically possible, and the randomness of mutations makes it more likely that they will be selected. However, the mutation may compromise a cells fitness and once the drug is removed, a back mutation may occur as is seen with BE1. These ideas have backing from the literature.

Overexpression of the efflux system AcrAB, its regulatory gene *mar*R and deletion of OmpF have been shown to contribute to fluoroquinolone resistance in laboratory mutants and clinical isolates of *E. coli* (Mazzariol *et al*, 2001; Oethinger *et al*, 2000; Tavio *et al*, 1999). Deletion of *acr*AB also lowers the MIC of tetracycline 4-8 fold and 16-64 fold for chloramphenicol as well as that of the fluoroquinolones (Oethinger *et al*, 2000). In the present study, the chloramphenicol MIC was two-fold less in the revertant but the tetracycline MIC was unchanged, suggesting that multidrug efflux, if present, was not to the same degree as described above.

Hirai *et al* (1986a, 1986b) has shown that fluoroquinolones of low hydrophobicity enter through OmpF and those with high hydrophobicity enter through both OmpF and LPS. This has led Heisig and Tschorny (1994) to suggest that the hydrophilic ciprofloxacin will select for a permeability mutation and the hydrophobic nalidixic

acid a gyrase mutation. However the results from chapter 7 dispute this as ciprofloxacin selected a gyrase mutation in the first selection step and the more hydrophobic moxifloxacin a gyrase mutation at the second step. However there may be a degree of truth in their hypothesis, but not in gyrase as the target, rather ParC, as the hydrophobic moxifloxacin did not select a mutation in this enzyme but the hydrophilic gemifloxacin and ciprofloxacin did.

In conclusion, *A. baumannii* selected on gemifloxacin retain both GyrA and ParC mutations. Non-target mutations have an effect on the growth rate of the mutants. This is reversible. However those strains that lost their mutations exhibited a higher mutation frequency upon subsequent challenge but did not show a ParC mutation. The isogenic mutant BE1 overexpressed a 45.5kDa porin more than two-fold over its parent AE1. This overexpression was lost after serial passage on antibiotic-free media and was associated with antibiotic MICs of AE1.

Chapter 9: Conclusions

These data presented in this thesis show that the newer fluoroquinolones, compared to ciprofloxacin and levofloxacin, exhibit increased potency against *A. baumannii*. The mechanism of resistance to these drugs is the same irrespective of the drug: GyrA is the primary target and possession of the serine 83 mutation confers fluoroquinolone resistance, however, GyrA mutants are still susceptible to trovafloxacin, recording MICs just below breakpoint levels.

The *A. baumannii* clinical isolates showed a large variation in their susceptibilities to the fluoroquinolones even when their target enzymes were seemingly identical; this suggests other factors contribute to decreased sensitivity. These factors can include GyrB and ParE mutations, drug efflux and reduced permeability. However, what has to be remembered is that any of these factors, if present, do not on their own cause resistance. No isolate was found to be resistant that did not have the GyrA mutation.

The presence of a ParC mutation raises the MIC even higher. This mutation is found only in isolates in possession of a GyrA mutation. Selection of fluoroquinolone resistant mutants *in vitro* demonstrate that a GyrA mutation is selected before a ParC mutation, however not all mutants mutated in their ParC gene, confirming that other factors are involved in reduced sensitivity.

The selection of mutants is far higher with ciprofloxacin compared to gemifloxacin or moxifloxacin. This suggests that the development of resistant *A. baumannii in vivo* will be less frequent when the new-generation of fluoroquinolones are introduced for clinical use. However this will need to be tested using animal models as there are fundamental differences between *in vitro* and *in vivo* selection. *In vitro*, the concentration of drug is fixed at multiples of the MIC. *In vivo*, drug concentrations will rise to peak serum levels after drug dosing and then fall over time as the drug is cleared from the system until the cycle is repeated with further dosing.

The mutation study raises some interesting questions:

- why was a ParC mutation not selected with ATCC 19606?
- why did moxifloxacin not select for a ParC mutation when both gemifloxacin and ciprofloxacin did against SB13?

Only two strains were used, a laboratory strain and a clinical isolate. One can speculate that the differences seen with the selection of mutants may be strain-related and not species-specific, therefore further work using a larger number of clinical isolates need to be conducted.

Further studies need to be done before firm conclusions can be drawn about the 45.5 kDa porin and its role in fluoroquinolone resistance. Its overexpression in BE1, combined with the raising of hydrophilic fluoroquinolone MIC but lesser effect on hydrophobic fluoroquinolones, suggests a permeability mechanism of resistance. However, reduced permeability on its own will not be enough to confer resistance unless it is coupled with efflux. For this reason its overexpression is an association and not the cause of resistance. The whole gene sequence needs to be identified as only 40 amino acids of the N-terminal was determined. It should also be compared to that found in its parent strains (SB13 & AE1) to determine if there is a mutation within the porin that alters its fluoroquinolone affinity. Northern blotting can be used to further quantify its level of expression. Finally, the role of fluoroquinolone efflux from intact cells needs to be investigated.

Degenerate primer ABOMP1

AMINO ACID	S	E	Q	S	E	A	K	G	F	V	E	D	A	N	G	S	I	L	F	R
	SER	GLU	GLN	SER	GLU	ALA	LYS	GLY	PHE	VAL	GLU	ASP	ALA	ASN	GLY	SER	ILE	LEU	PHE	ARG
CODON	TCT	GAA	CAA	TCT	GAA	GCT	AAA	GGT	TTT	GTT	GAA	GAT	GCT	AAT	GGT	TCT	ATT	TTA	TTT	CGT
	TCC	GAG	CAG	TCC	GAG	GCC	AAG	GGC	TTC	GTC	GAG	GAC	GCC	AAC	GGC	TCC	ATC	TTG	TTC	CGC
	TCA			TCA		GCA		GGA		GTA			GCA		GGA	TCA	ATA	CTT		CGA
	TCT			TCT		GCG		GGG		GTG			GCG		GGG	TCT		CTC		CGG
	AGT			AGT												AGT		CTA		AGA
	AGC			AGC												AGC		CTG		AGG
PRIMER 5'-3'					GAR	GCI	AAR	GGI	TTY	GTI	GAR	GAY	GCI	AAY	GGI					

PRIMER 1: 5' GARGCIAARGGITTYGTIGARGAYGCIAAYGGI 3'

Degenerate primer ABOMP2

AMINO ACID	T	G	Y	L	T	R	D	K	K	Q	G	A	K	D	T	S	S	V	A	Q
	THR	GLY	TYR	LEU	THR	ARG	ASP	LYS	LYS	GLN	GLY	ALA	LYS	ASP	THR	SER	SER	VAL	ALA	GLN
CODON	ACT	GGT	TAT	TTA	ACT	CGT	GAT	AAA	AAA	CAA	GGT	GCT	AAA	GAT	ACT	TCT	TCT	GTT	GCT	CAA
	ACC	GGC	TAC	TTG	ACC	CGC	GAC	AAG	AAG	CAG	GGC	GCC	AAG	GAC	ACC	TCC	TCC	GTC	GCC	CAG
	ACA	GGA		CTT	ACA	CGA					GGA	GCA			ACA	TCA	TCA	GTA	GCA	
	ACG	GGG		CTC	ACG	CGG					GGG	GCG			ACG	TCT	TCT	GTG	GCG	
				CTA		AGA										AGT	AGT			
				CTG		AGG										AGC	AGC			
PRIMER 5'-3'		GGI	TAY	YTI	ACI	MGI	GAY	AAR	AAR	CAR	GGI	GCI	AAR	GAY	ACI					
PRIMER 3'-5' (reverse)		CCI	ATR	RAI	TGI	KCI	CTR	TTY	TTY	GTY	CCI	CGI	TTY	CTR	TGI					

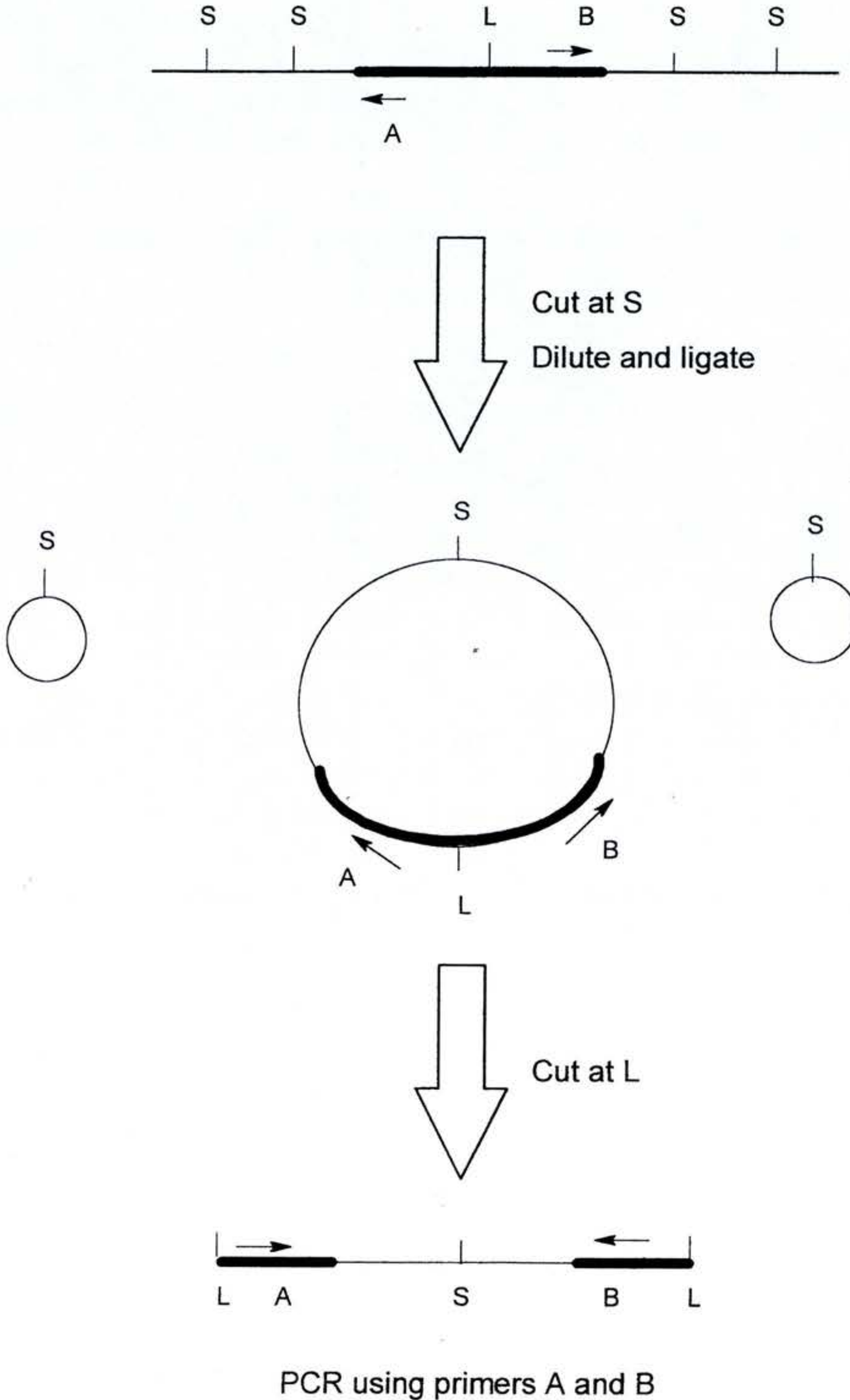
PRIMER 2: 5' IGTRTCYTTIGCICCTTGYTTYTTRTCICKIGTIARRTAICC 3'

Key to bases

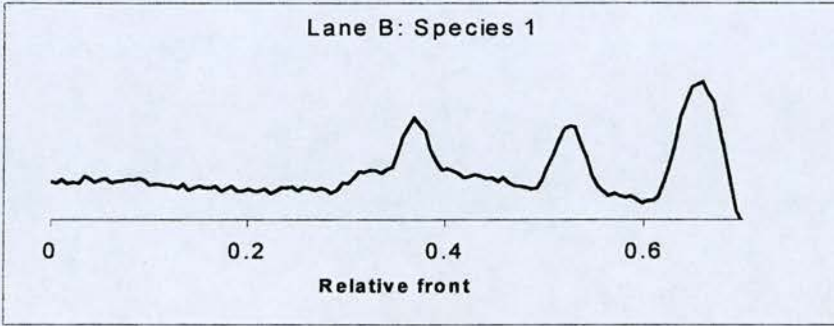
Base	T, C, A, G	T or C	A or G	A or C	A or T	C or G	T or G
Replace with	I	Y	R	M	W	S	K

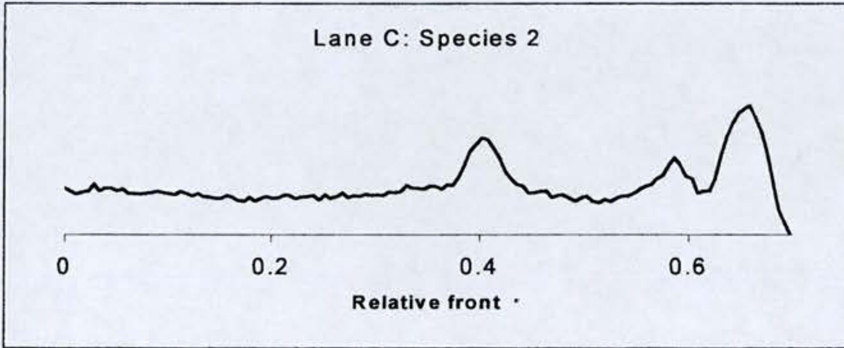
Appendix II. Schematic Diagram of Inverse PCR

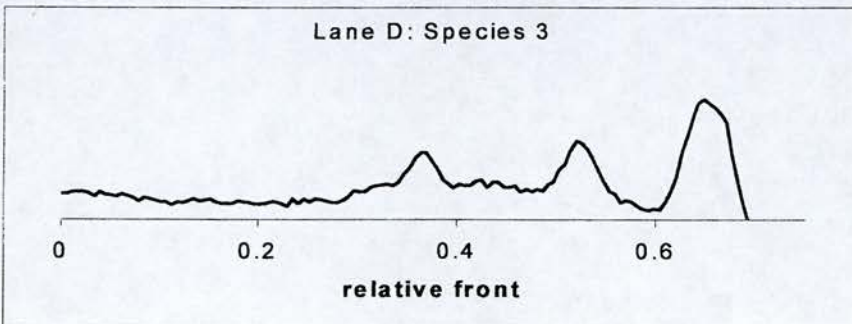
Piece of known DNA sequence (bold) and primers A and B with restriction site (L) and unknown DNA containing random restriction sites (S)

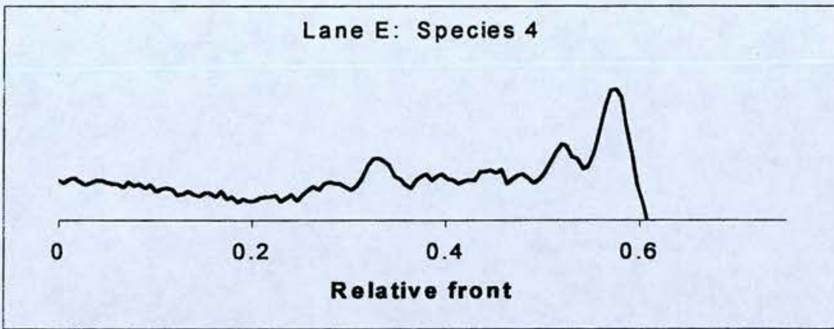


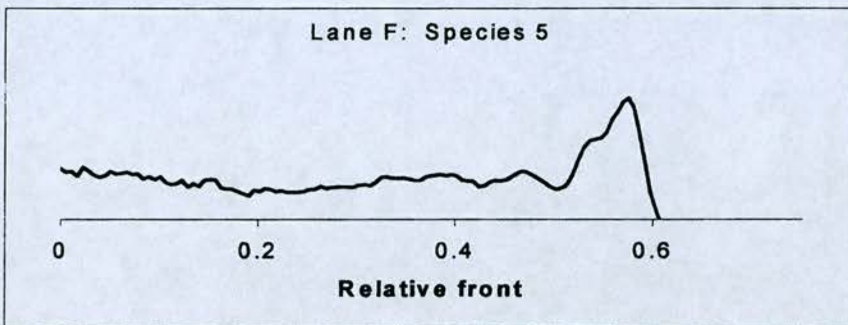
Appendix III: tRNA Fingerprinting profiles from Figure 3.1

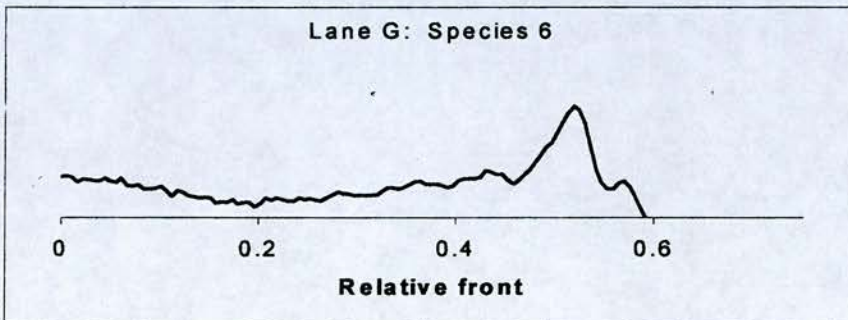


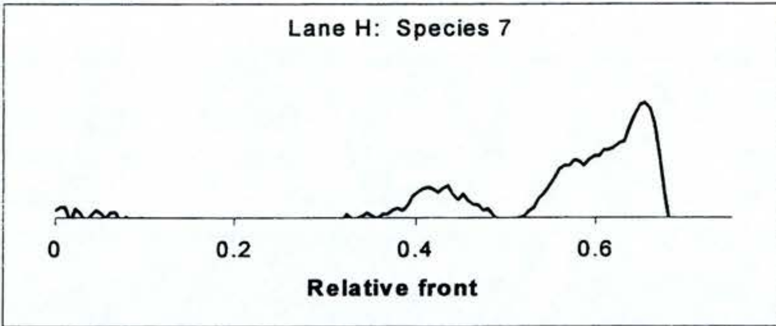


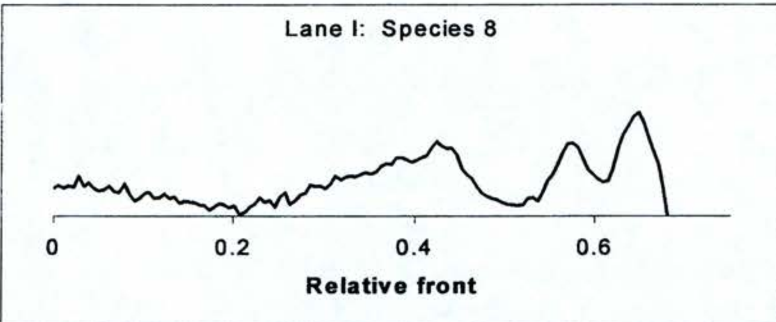


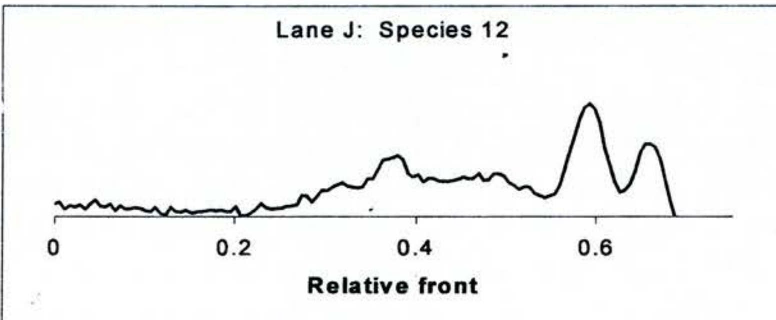

Lane E: Species 4
0
0.2
0.4
0.6
Relative front


Lane F: Species 5
0
0.2
0.4
0.6
Relative front


Lane G: Species 6
0
0.2
0.4
0.6
Relative front


Lane H: Species 7
0
0.2
0.4
0.6
Relative front


Lane I: Species 8
0
0.2
0.4
0.6
Relative front


Lane J: Species 12
0
0.2
0.4
0.6
Relative front

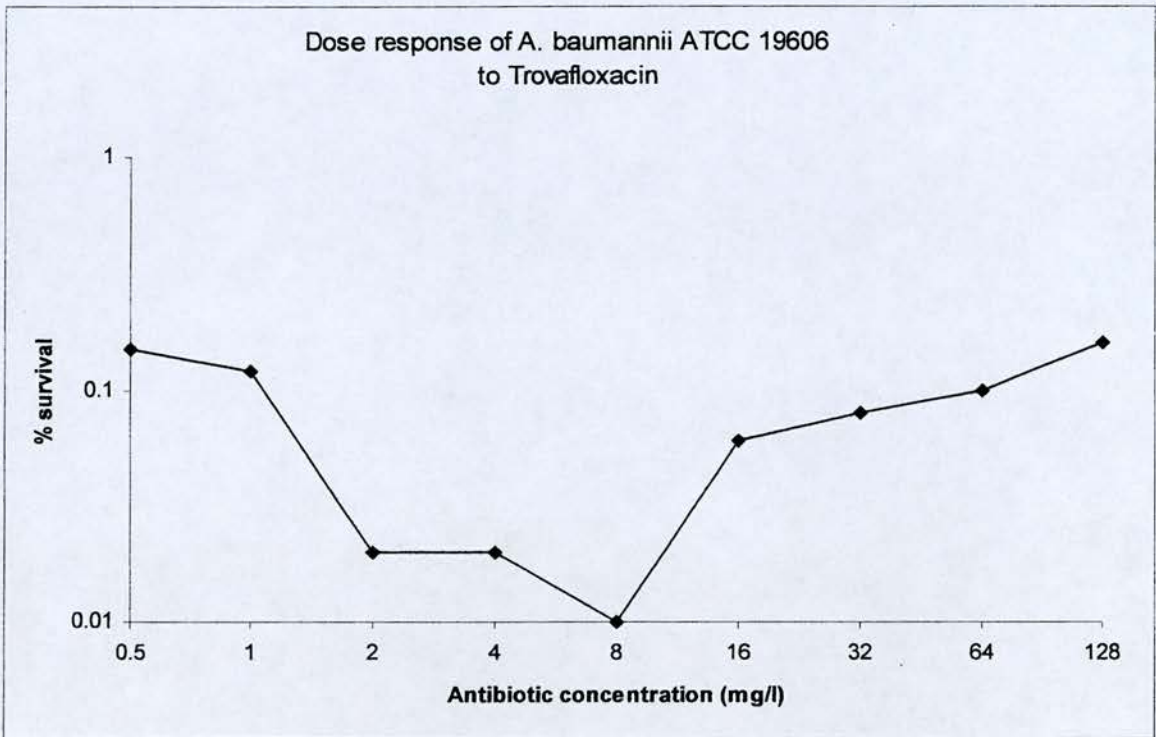
Appendix IV: *Acinetobacter* spp. identification by tRNA

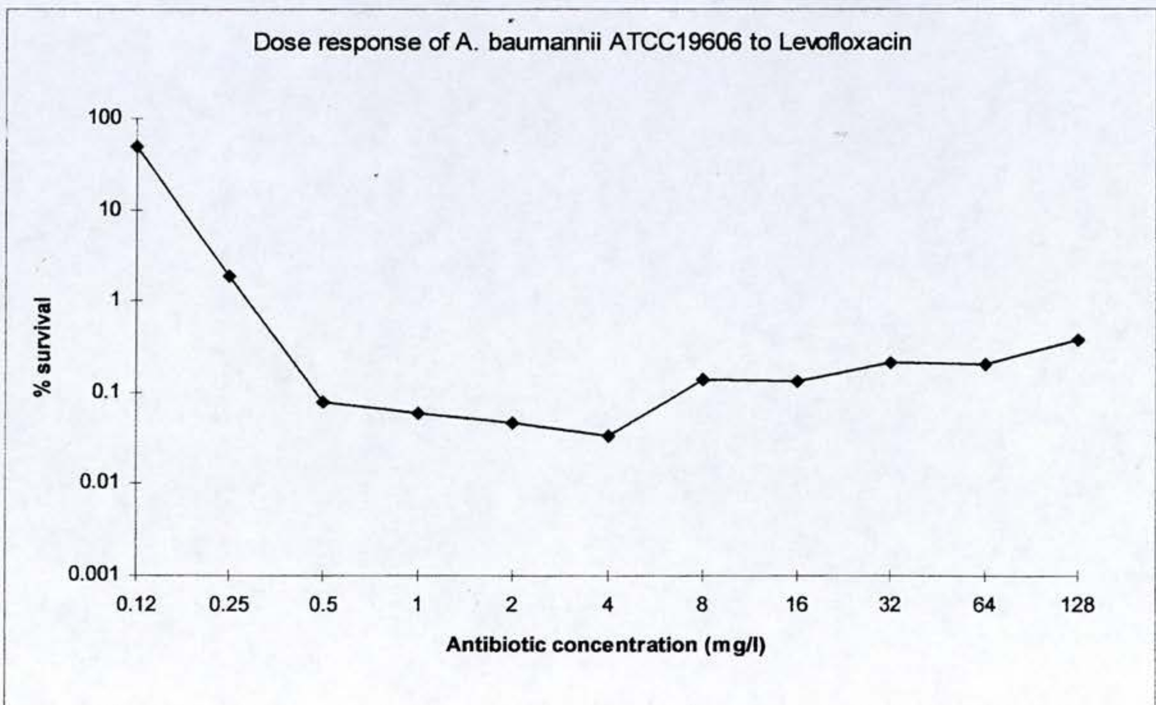
Strain	Speciation by tRNA	Speciation by Vitek
SB3	1/3	1
SB16	1/3	2
SB40	1/3	1
SB42	1/3	*A. anitratus*
SB44	1/3	2
SB57	1/3	2
SB65	1/3	2
SB72	1/3	1
SB73	1/3	1
SB26	4	8
SB33	4	8
SB50	4	1
SB56	4	2
SB58	4	8
SB92	4	2
SB100	4	*A. anitratus*
SB18	5	8
SB54	5	2
SB31	7	8
SB48	7	8
SB39	8	2
SB2	12	8
SB70	12	8
SB1	2	2
SB4	2	*A. anitratus*
SB5	2	2
SB6	2	2
SB7	2	spp
SB8	2	8
SB9	2	2
SB10	2	2

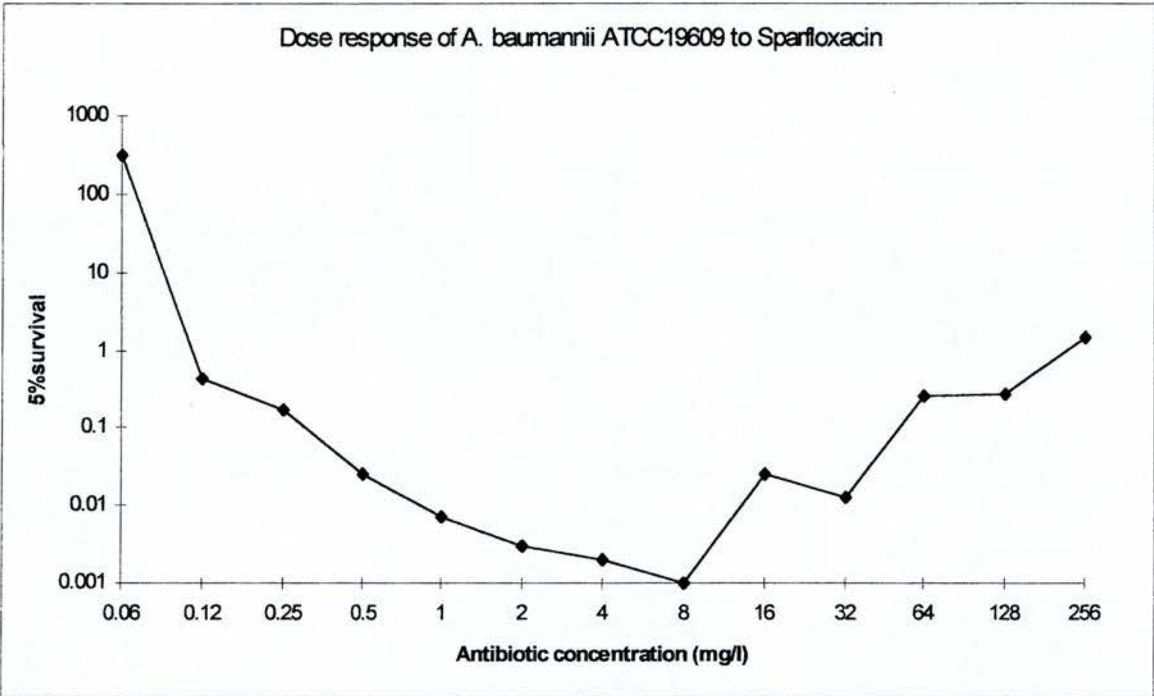
Strain	Speciation by tRNA	Speciation by Vitek
SB11	2	2
SB12	2	spp
SB13	2	8
SB14	2	*A. anitratus*
SB15	2	2
SB17	2	2
SB20	2	2
SB21	2	2
SB22	2	8
SB23	2	2
SB24	2	2
SB25	2	2
SB27	2	*A. anitratus*
SB28	2	*A. anitratus*
SB29	2	2
SB30	2	2
SB32	2	*A. anitratus*
SB34	2	*A. anitratus*
SB35	2	2
SB36	2	2
SB38	2	1
SB41	2	2
SB43	2	2
SB45	2	2
SB46	2	2
SB47	2	2
SB49	2	1
SB51	2	2
SB52	2	8
SB53	2	*A. anitratus*
SB59	2	spp
SB60	2	2
SB61	2	*A. anitratus*
SB62	2	2
SB63	2	1
SB64	2	2
SB66	2	2
SB67	2	*A. anitratus*
SB68	2	*A. anitratus*
SB69	2	2
SB71	2	2
SB74	2	8

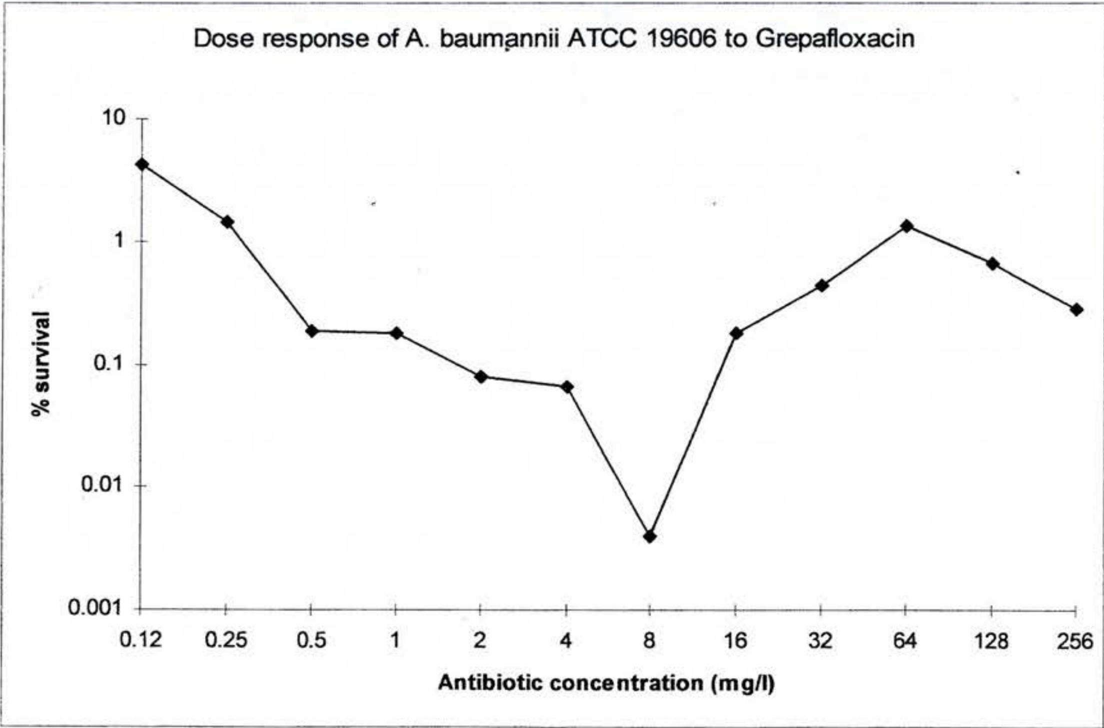
Strain	Speciation by tRNA	Speciation by Vitek
SB75	2	*A. anitratus*
SB76	2	*A. anitratus*
SB77	2	*A. anitratus*
SB78	2	1
SB79	2	2
SB80	2	2
SB81	2	2
SB82	2	2
SB83	2	*A. anitratus*
SB84	2	*A. anitratus*
SB86	2	2
SB87	2	1
SB88	2	1
SB89	2	1
SB90	2	2
SB91	2	2
SB93	2	8
SB97	2	2
SB98	2	2
SB99	2	2
SB101	2	2

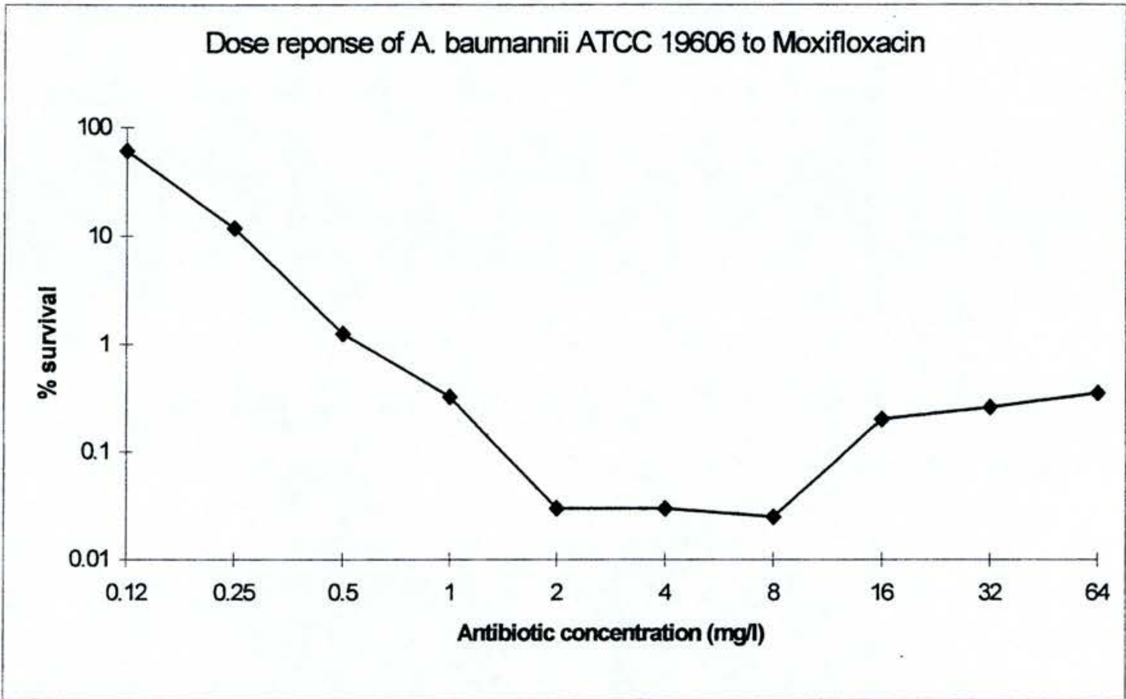
Appendix V: Dose response curves to *A. baumannii ATCC 19606*

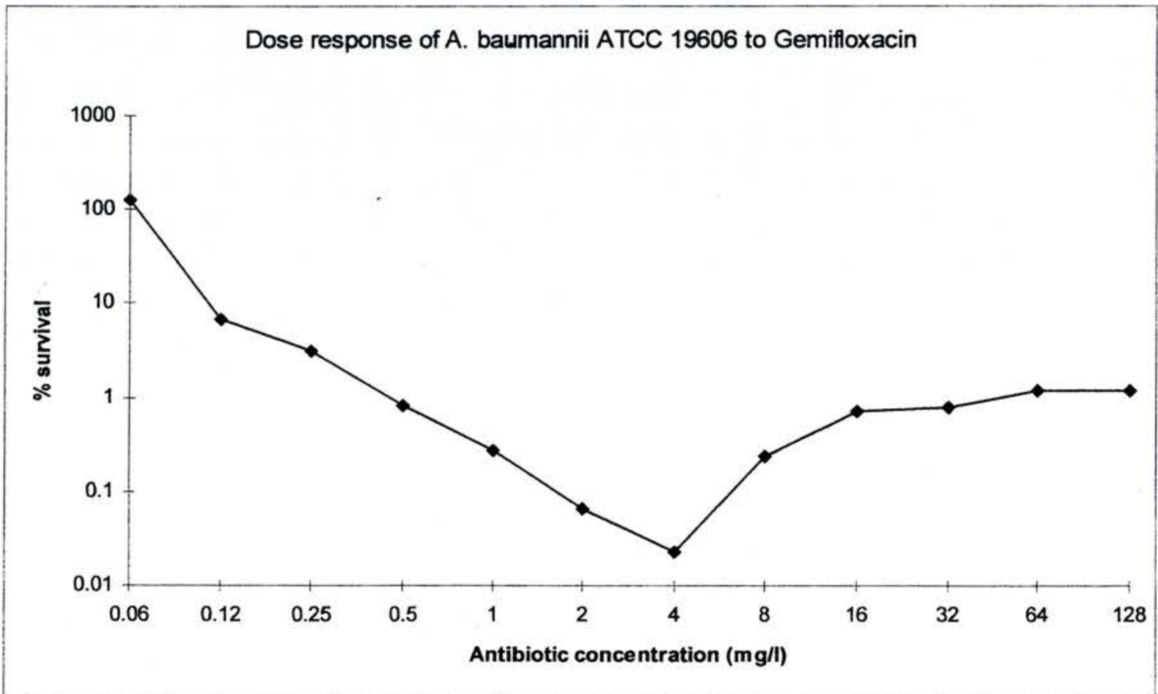


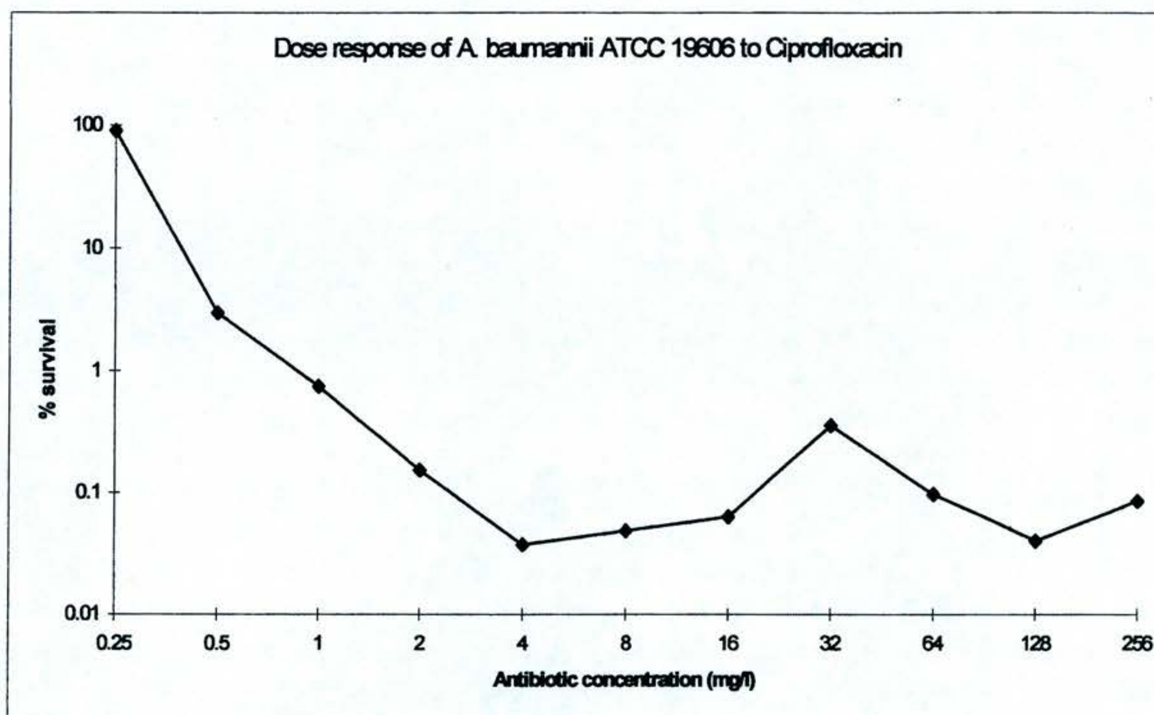



Dose response of A. baumannii ATCC19609 to Sparfloxacin
1000
100
10
1
0.1
0.01
0.001
5%survival
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
Antibiotic concentration (mg/l)


Dose response of A. baumannii ATCC 19606 to Grepafloxacin
10
1
0.1
0.01
0.001
% survival
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
Antibiotic concentration (mg/l)


Dose reponse of A. baumannii ATCC 19606 to Moxifloxacin
100
10
1
0.1
0.01
% survival
0.12
0.25
0.5
1
2
4
8
16
32
64
Antibiotic concentration (mg/l)


Dose response of A. baumannii ATCC 19606 to Gemifloxacin
1000
100
10
1
0.1
0.01
% survival
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
Antibiotic concentration (mg/l)


Dose response of A. baumannii ATCC 19606 to Ciprofloxacin
100
10
1
0.1
0.01
% survival
0.25
0.5
1
2
4
8
16
32
64
128
256
Antibiotic concentration (mg/l)

Appendix VI: MICs of Species 1/3

Gem; gemifloxacin, Cip; ciprofloxacin, Mox; moxifloxacin, Tro; trovafloxacin, Imi; imipenem, Cef; cefuroxime, Gat; gatifloxacin, Azi, azithromucin, Lev; levofloxacin, Ofl; ofloxacin, Spa; sparfloxacin, Gre; grepafloxacin

Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
SB3	0.06	0.12	0.12	0.06	0.12	128	0.06	1	0.12	0.5	0.06	0.06
SB16	0.03	0.25	0.12	0.03	0.12	64	0.06	2	0.12	0.5	0.03	0.06
SB40	0.06	0.5	0.12	0.03	0.12	128	0.06	4	0.5	1	0.06	0.12
SB42	0.06	0.5	0.03	0.03	0.12	32	0.03	2	0.25	0.5	0.03	0.06
SB44	16	128	8	8	0.12	128	8	4	16	32	8	32
SB57	0.12	0.5	0.12	0.03	0.06	128	0.25	2	0.25	0.5	0.06	0.12
SB65	0.03	0.25	0.12	0.06	0.12	64	0.12	4	0.12	0.25	0.03	0.06
SB72	0.12	0.5	0.12	0.06	0.12	64	0.25	2	0.25	0.5	0.06	0.12
SB73	0.06	0.25	0.12	0.03	0.25	64	0.25	2	0.12	0.5	0.03	0.06

MICs of Species 4

Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
SB26	0.06	0.12	0.12	0.06	0.12	128	0.12	0.5	0.5	1	0.06	0.12
SB33	0.03	0.12	0.12	0.03	0.12	64	0.06	1	0.25	0.5	0.015	0.03
SB50	0.06	0.25	0.12	0.03	0.12	32	0.12	1	0.25	0.5	0.03	0.06
SB56	0.03	0.12	0.12	0.03	0.12	16	0.25	2	0.12	0.25	0.03	0.06
SB58	0.12	0.06	0.06	0.015	0.06	2	0.12	2	0.12	0.25	0.06	0.06
SB92	0.06	0.25	0.12	0.03	0.12	16	0.25	2	0.25	0.5	0.06	0.06
SB100	0.06	0.25	0.12	0.015	0.25	32	0.12	1	0.25	0.5	0.015	0.03

MICs of Species 5, 7, 8 & 12

Species	Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
5	SB18	0.06	0.12	0.06	0.03	0.12	8	0.12	0.5	0.12	0.25	0.03	0.06
5	SB54	0.12	0.5	0.12	0.03	0.12	32	0.25	2	0.25	0.5	0.06	0.06
7	SB31	1	16	0.5	0.5	0.25	64	4		4	8	0.5	0.5
7	SB48	0.06	0.12	0.06	0.015	0.008	64	0.06	0.25	0.25	0.5	0.015	0.03
8	SB39	0.015	0.12	0.06	0.008	0.25	32	0.03	0.5	0.12	0.25	0.008	0.015
12	SB2	0.06	0.06	0.25	0.12	0.5	>128	0.06	16	0.12	0.25	0.12	0.12
12	SB70	0.06	0.25	0.06	0.03	0.12	32	0.12	0.5	0.25	0.5	0.03	0.06

MICs of Species 2

Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
SB1	32	>128	16	8	0.25	>128	4	32	16	32	16	64
SB4	0.25	2	1	0.12	0.06	32	0.5	32	0.5	1	0.25	0.25
SB5	0.06	0.25	0.12	0.06	0.12	128	0.06	4	0.25	1	0.03	0.06
SB6	32	>128	64	16	0.25	128	64	32	64	128	32	32
SB7	32	>128	16	8	2	>128	8	32	16	32	8	32
SB8	0.03	0.03	0.03	0.015	0.03	8	0.03	0.25	0.12	0.12	0.008	0.015
SB9	32	128	32	16	0.25	>128	8	32	16	32	16	32
SB10	0.12	1	0.25	0.06	0.12	128	0.25	4	0.5	1	0.06	0.12
SB11	32	128	16	16	1	>128	8	32	16	32	8	16
SB12	0.12	0.5	0.06	0.03	0.12	64	0.03	1	0.25	0.5	0.008	0.03
SB13	1	8	2	0.5	0.5	64	2	32	1	4	4	2
SB14	0.12	0.5	0.12	0.03	0.008	32	0.12	4	0.25	0.5	0.03	0.06
SB15	0.03	0.25	0.03	0.015	0.12	64	0.06	1	0.12	0.25	0.015	0.03
SB17	1	4	1	0.25	0.12	32	1	>128	1	4	0.5	0.5
SB20	0.06	0.5	0.06	0.03	0.008	64	0.06	4	0.25	0.5	0.015	0.03
SB21	>128	>128	64	32	0.25	16	64	128	32	64	32	64
SB22	1	8	2	0.5	0.25	128	4	32	4	8	1	1
SB23	0.06	0.5	0.12	0.03	0.12	64	0.06	1	0.25	0.5	0.03	0.06
SB24	1	16	4	0.5	0.25	64	2	32	4	8	1	1
SB25	0.03	0.25	0.06	0.015	0.12	128	0.25	1	0.12	0.25	0.03	0.03
SB27	0.12	0.5	0.12	0.03	0.12	128	0.03	1	0.12	0.25	0.06	0.12
SB28	0.06	0.5	0.12	0.03	0.12	128	0.06	2	0.25	0.5	0.03	0.06
SB29	0.06	0.25	0.06	0.03	0.12	128	0.12	4	0.25	0.5	0.03	0.12
SB30	0.06	0.25	0.06	0.03	0.12	64	0.12	16	0.25	0.5	0.06	0.06
SB32	0.06	1	0.12	0.06	0.06	64	0.06	2	0.5	1	0.03	0.06
SB34	0.06	0.5	0.12	0.03	0.06	64	0.06	4	0.25	1	0.03	0.06
SB35	0.06	0.5	0.06	0.015	0.12	64	0.06	2	0.25	0.5	0.015	0.06

MICs of Species 2 cont.

Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
SB36	32	64	16	16	0.5	>128	16	32	16	32	16	32
SB38	32	>128	16	16	0.25	>128	16	64	32	32	16	32
SB41	8	128	16	8	0.12	64	16	16	8	16	8	16
SB43	0.06	0.25	0.06	0.03	0.12	32	0.06	4	0.5	0.5	0.03	0.06
SB45	16	128	16	8	0.5	>128	8	32	16	32	8	16
SB46	0.03	0.25	0.06	0.03	0.12	64	0.06	4	0.25	0.5	0.015	0.03
SB47	16	128	16	16	1	>128	8	16	16	32	16	64
SB49	0.12	1	0.12	0.03	0.008	64	0.06	1	0.25	0.5	0.03	0.06
SB51	0.03	0.25	0.03	0.015	0.06	16	0.03	1	0.25	0.5	0.03	0.06
SB52	0.03	0.25	0.06	0.03	0.12	128	0.25	2	0.25	0.25	0.06	0.06
SB53	0.06	0.5	0.06	0.06	0.12	128	0.25	1	0.25	0.5	0.03	0.06
SB59	0.008	0.03	0.008	0.008	0.008	2	0.008	0.12	0.008	0.03	0.008	0.015
SB60	0.06	0.5	0.12	0.03	0.12	64	0.03	2	0.25	0.25	0.03	0.06
SB61	0.03	0.25	0.06	0.015	0.12	64	0.06	2	0.12	0.25	0.015	0.03
SB62	0.06	0.25	0.06	0.03	0.12	32	0.12	2	0.25	0.5	0.03	0.06
SB63	0.06	0.5	0.12	0.06	0.06	64	0.25	2	0.25	0.5	0.03	0.03
SB64	16	>128	16	16	0.25	256	8	32	8	16	8	32
SB66	0.015	0.03	0.03	0.015	0.03	8	0.03	0.12	0.12	0.25	0.008	0.015
SB67	16	128	16	8	2	>256	8	32	16	16	8	16
SB68	0.03	0.25	0.06	0.015	0.25	32	0.12	2	0.12	0.25	0.015	0.015
SB69	0.03	0.25	0.06	0.015	0.12	64	0.12	2	0.12	0.25	0.015	0.03
SB71	0.015	0.12	0.015	0.015	0.12	64	0.03	1	0.06	0.12	0.008	0.015
SB74	0.015	0.015	0.015	0.008	0.015	4	0.12	0.12	0.06	0.12	0.008	0.015
SB75	32	64	16	8	0.5	256	32	64	8	16	16	64
SB76	32	128	32	16	0.25	256	16	64	8	16	16	32
SB77	64	128	32	16	0.25	256	16	64	8	16	16	32
SB78	0.015	0.25	0.06	0.03	0.12	64	0.25	2	0.12	0.25	0.015	0.03

MICs of Species 2 cont.

Strain	Gem	Cip	Mox	Tro	Imi	Cef	Gat	Azi	Lev	Ofl	Spa	Gre
SB79	0.06	0.5	0.12	0.03	0.25	256	0.12	2	0.25	0.5	0.03	0.06
SB80	0.06	0.25	0.12	0.03	0.06	16	0.25	2	0.25	0.25	0.03	0.06
SB81	0.06	0.25	0.06	0.03	0.25	256	0.25	2	0.12	0.25	0.03	0.06
SB82	0.06	0.25	0.06	0.03	0.06	64	0.25	2	0.12	0.5	0.03	0.06
SB83	0.03	0.25	0.06	0.03	0.25	256	0.25	2	0.12	0.25	0.03	0.06
SB84	0.06	0.5	0.12	0.03	0.06	32	0.25	2	0.12	0.25	0.03	0.06
SB86	0.12	1	0.25	0.12	0.06	128	0.5	4	0.25	0.5	0.12	0.12
SB87	64	>128	64	32	1	256	128	32	64	>238	32	32
SB88	0.015	0.12	0.03	0.03	0.06	64	0.03	1	0.12	0.12	0.03	0.06
SB89	0.06	0.5	0.06	0.015	0.12	128	0.12	1	0.06	0.5	0.015	0.03
SB90	0.03	0.5	0.06	0.015	0.12	64	0.06	2	0.25	0.5	0.015	0.03
SB91	16	>128	16	16	0.25	>256	16	64	16	32	8	16
SB93	0.06	0.25	0.06	0.015	0.12	32	0.12	2	0.12	0.25	0.03	0.06
SB97	0.06	0.25	0.12	0.06	0.12	128	0.12	2	0.12	0.25	0.03	0.06
SB98	16	>128	16	8	0.25	256	16	64	16	64	8	16
SB99	0.06	0.25	0.12	0.12	0.12	128	0.25	1	0.25	0.25.	0.03	0.06
SB101	0.03	0.25	0.06	0.06	0.06	64	0.25	2	0.12	0.5	0.015	0.06

Appendix VII. Comparison of OMP sequence in BLAST

```
Sequences producing significant alignments:

gi|15597956|ref|NP_251450.1|  (NC 002516) probable outer mem... 40
gi|2425172|dbj|BAA22267.1|  (AB006797) OprE3 [Pseudomonas ae... 40
gi|15596155|ref|NP_249649.1|  (NC 002516) outer membrane por... 38
gi|5712720|gb|AAD47624.1|  (AF153710) porin-like protein [Ps... 35
gi|15598784|ref|NP_252278.1|  (NC 002516) probable porin [Ps... 30

Alignments
 gi|15597956|ref|NP 251450.1| (NC 002516) probable outer membrane
protein [Pseudomonas aeruginosa]
 gi|11351566|pir||G83299 probable outer membrane protein PA2760 -
Pseudomonas aeruginosa (strain PAO1)
 gi|9948840|gb|AAG06148.1|AE004704 2 (AE004704) probable outer
membrane protein [Pseudomonas aeruginosa]
          Length = 425

 Score = 40.4 bits (93), Expect = 0.004
 Identities = 18/40 (45%), Positives = 27/40 (67%)

Query: 1  SEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKDTSSVAQ 40
          ++Q  AKGFVED++  + FR GY++RD K G +D +   Q
Sbjct: 22 NDQEAAKGFVEDSHLDLFFRNGYISRDYKHGRQDKAEWGQ 61


>gi|2425172|dbj|BAA22267.1| (AB006797) OprE3 [Pseudomonas
aeruginosa]
          Length = 425

 Score = 40.4 bits (93), Expect = 0.004
 Identities = 18/40 (45%), Positives = 27/40 (67%)

Query: 1  SEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKDTSSVAQ 40
          ++Q  AKGFVED++  + FR GY++RD K G +D +   Q
Sbjct: 22 NDQEAAKGFVEDSHLDLFFRNGYISRDYKHGRQDKAEWGQ 61


>gi|15596155|ref|NP 249649.1| (NC 002516) outer membrane porin
protein OprD precursor [Pseudomonas aeruginosa]
 gi|417518|sp|P32722|PORD PSEAE PORIN D PRECURSOR (OUTER MEMBRANE
PROTEIN D2) (IMIPENEM/BASIC AMINO ACID-SPECIFIC OUTER MEMBRANE PORE)
 gi|94811|pir||S23771 porin oprD precursor - Pseudomonas aeruginosa
 gi|11350707|pir||E83527 outer membrane porin protein OprD precursor
PA0958 [imported] - Pseudomonas aeruginosa (strain PAO1)
 gi|45371|emb|CAA44855.1| (X63152) protein D2 [Pseudomonas
aeruginosa]
 gi|45373|emb|CAA78448.1| (Z14065) OprD porin [Pseudomonas
aeruginosa]
 gi|9946864|gb|AAG04347.1|AE004529 13 (AE004529) outer membrane
porin protein OprD precursor [Pseudomonas aeruginosa]
 gi|444429|prf||1907161D porin [Pseudomonas aeruginosa] Length = 443
```

```
 Score = 37.7 bits (86), Expect = 0.028
 Identities = 17/34 (50%), Positives = 24/34 (70%)

Query: 1  SEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKD 34
           S+Q+EAKGF+ED++  +L R  Y  RD K G+ D
Sbjct: 28 SDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGD 61
 gi|5712720|gb|AAD47624.1| (AF153710) porin-like protein
[Pseudomonas sp. BG33R] Length = 249

 Score = 34.7 bits (78), Expect = 0.20
 Identities = 16/40 (40%), Positives = 27/40 (67%)

Query: 1  SEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKDTSSVAQ 40
           ++Q ++KGFVED++ +I  R  Y++RD K G +D +   Q
Sbjct: 21 NDQEQSKGFVEDSHLNIAARNAYISRDYKNGKQDKAEWGQ 60


 gi|15598784|ref|NP 252278.1| (NC 002516) probable porin
[Pseudomonas aeruginosa]
 gi|11351752|pir||F83197 probable porin PA3588 - Pseudomonas
aeruginosa (strain PAO1)
 gi|9949743|gb|AAG06976.1|AE004779 8 (AE004779) probable porin
[Pseudomonas aeruginosa] Length = 416

 Score = 29.6 bits (65), Expect = 7.3
 Identities = 12/27 (44%), Positives = 18/27 (66%)

Query: 6  AKGFVEDANGSILFRTGYLTRDKKQGA 32
           A+GF+ED+  S+  R  Y+ RD + GA
Sbjct: 24 AEGFLEDSRASLALRNFYMNRDFRDGA 50
```

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