Evolution of fluoroquinolone resistance in *Burkholderia cepacia*

By

Cassie Francesca Pope

A thesis submitted to University College London in fulfilment of the requirement for the degree of Doctor of Philosophy

Centre for Medical Microbiology,
Royal Free and University College Medical School,
Hampstead Campus,
Rowland Hill Street,
London
NW3 2PF

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

'I, Cassie Pope, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis'.

Signed:

Abstract

This study investigates the evolution of fluoroquinolone resistance in *Burkholderia cepacia* and assesses fitness of clinical isolates of the *B. cepacia* complex. *B. cepacia* was chosen as a clinically relevant model of antibiotic resistance because these bacteria cause chronic infections in cystic fibrosis patients, are highly resistant to killing by many antimicrobials and consequently require long term antibiotic treatment.

Fluoroquinolones are a widely used class of antimicrobials, increasingly used in medical and veterinary practice. A method was optimised and used to determine the rate of mutation occurring in topoisomerase genes that confer resistance to fluoroquinolones. The fitness cost associated with fluoroquinolone resistance mutations was assessed as a measure of the stability of resistance in the bacterial population. Clinical isolates were assessed for hypermutability using mutation to fluoroquinolone resistance as a selective tool.

In Gram-negative bacteria resistance to fluoroquinolones occurs via three major mechanisms; drug efflux, reduced permeability and target alteration. The spectrum of fluoroquinolone resistance mutations occurring *in vitro*, the rate at which they arise, and the fitness costs of characterised topoisomerase mutations was investigated, using models relevant to transmission of the *Burkholderia cepacia* complex. Previous studies have shown that single point mutations in DNA gyrase, conferring resistance, have no or low cost. Only double mutations in *gyrA* and *parC* conferred a fitness cost. Second step mutations occur at a faster rate than first step mutations. Mutation in *gyrA*, therefore, may predispose the genome to mutation in topoisomerase genes.

Acknowledgments

I am indebted to many people who have either helped me personally or have contributed to the work in this thesis.

I gratefully acknowledge the support of my supervisors Dr. Timothy McHugh and Prof. Stephen Gillespie and thank them for having faith in me. I am indebted to Dr. Tim McHugh for his kindness, generosity and positivity and to Prof. Stephen Gillespie for his encouragement.

Dr Bambos Charalambous assisted me with statistical analysis and I am grateful for his advice. Additionally I am grateful to Dr. Jonathan Pratten for help with biofilm work and his patience and good humour. Also to Dr. John Moore for providing clinical isolates.

I thank Fitzroy Hall and Betty Thaine for providing an autoclave service. Also thanks to Anne Dickens and Marina Bogovac for ordering of reagents. Among other things Clare Ling and Claire Jenkins have provided sequencing training and I am grateful to them both. My colleagues have provided daily support and have provided a fun working environment. In this regard I am grateful to the clinical scientists and research staff in the department. In particular I have relied on Dr Denise O'Sullivan for advice and friendship. I thank Marcus for his patience and support. I am grateful to all for making this experience a happy one.

LIST OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

Abbreviations

AFLP:	amplified fragment length polymorphism
AHLs:	acyl homoserine lactones
API:	analytical profiling index
ANOVA:	analysis of variance between groups
Bcc:	*Burkholderia cepacia* complex
C:	number of parallel cultures
CCCP:	carbonyl cyanide m-chloro phenylhydrazone
CDFF:	constant Depth Film Fermenter
CF:	cystic fibrosis
cfu/mL:	colony forming units/mL
CLSI:	Clinical and Laboratory Standards Institute
cm:	centimetres
CO_2:	carbon dioxide
DNA:	deoxyribonucleic acid
EM:	electron microscopy
FQ:	fluoroquinolone
kbp:	kilobase pair
LB:	Luria Bertani
LPS:	lipopolysaccharide
m:	number of mutations
MATE:	multidrug and toxic compound extrusion family
MDR:	multi drug resistant
MFS:	major facilitation family
MIC:	minimum inhibitory concentration
min:	minutes
MRSA:	methicillin resistant *Staphylococcus aureus*
MPC:	mutant prevention concentration
μ:	mutation rate
NCTC:	National Collection of Type Cultures
N_0:	initial inoculum
N_t:	final cell number
O_2:	oxygen
OD:	optical density
ORF:	open reading frame
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PFTE:	polytetrafluoroethylene
PMF:	proton motive force
PVC:	polyvinyl chloride
rpm:	revolutions per minute
QRDR:	quinolone resistance determining region
RND:	resistance nodulation cell division family
SCLM:	scanning confocal laser microscopy
SEM:	standard error of the mean
SDS PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDW:	sterile distilled water
SHV:	sulfhydryl variable
SMR:	small multidrug resistance family
SSCP:	single strand conformation polymorphism
SPS:	sodium polyanetholesulfonate
TBE:	Tris Borate EDTA
TIFF:	tagged image format file
VRE:	vancomycin resistant Enterococci

1.0 General Introduction

1.1 Antibiotic Resistance

1.1.1 Significance of antibiotic resistance

The number of infections caused by antibiotic resistant bacteria has been increasing worldwide, resulting in decreased efficacy of antimicrobial therapy. This problem has been exacerbated by the limited number of new antibiotics developed. The increase in the frequency of antibiotic resistance can be attributed to a number of factors, including the increase in immunocompromised patients and invasive procedures, the overuse and misuse of antibiotics in healthcare and animal husbandry (Witte, Klare, & Werner 1999) and breaches in infection control. This results in raised healthcare costs and increased patient mortality. For example, in the intensive care setting the widespread use of antibiotics for treatment of immunocompromised patients has allowed the selection of drug resistant bacteria e.g. *Acinetobacter baumannii* (Wroblewska *et al.* 2006).

The primary aim of *in vitro* susceptibility testing of clinical isolates is to assess the susceptibility to an antibiotic in order to guide therapy. A pathogen is classed as resistant if the Minimal Inhibitory Concentration (MIC) is greater than the defined breakpoint; the discriminatory antibiotic concentration used to define isolates as susceptible, intermediate and resistant. In most infections the *in vitro* susceptibility values correlate with the effectiveness of therapy. However in some situations such as infection of the cystic fibrosis (CF) lung by *Pseudomonas aeruginosa* the correlation is poor. The results of susceptibility testing, therefore, should be treated with caution.

The community and nosocomial spread of antibiotic resistance in numerous bacterial pathogens is causing concern. These include methicillin resistant *Staphylococcus aureus*

(Fluit *et al.* 2001), vancomycin-resistant Enterococci (VRE) (Courvalin 2006; Kolar *et al.* 2006), extended spectrum β-lactamase producing *Enterobacteriacae* (ESBLs) (Bedenic & Zagar 1998; Gangoue-Pieboji *et al.* 2005) and non-fermentative Gram-negative bacilli, including *P. aeruginosa*, *A. baumannii* and *Stenotrophomonas maltophilia* (McGowan, Jr. 2006). A number of European and worldwide studies and surveillance programmes monitor the frequency of infections caused by antibiotic resistant pathogens. Examples of these are the SENTRY antimicrobial resistance surveillance programme (Fluit *et al.* 2001) and the European Network for Antimicrobial Resistance and Epidemiology (ENARE) (Voss *et al.* 1994).

1.1.2 Mechanisms of Antibiotic Resistance

Clinically meaningful levels of resistance arise via a number of mechanisms that enable bacteria to survive the effects of antimicrobial agents. These include the alteration of antibiotic targets (e.g. penicillin binding proteins or DNA gyrase), production of enzymes that inactivate the antibiotic (e.g. β-lactamases and aminoglycoside modifying agents), reduction of permeability and active efflux to reduce the antibiotic concentration in the bacterial cell and bypass of the metabolic pathway (e.g. resistance to trimethoprim and sulphonamides; Neu 1989; Dever & Dermody 1991).

1.1.2.1 Alteration of Target

Resistance to a number of antibiotic classes including β-lactams, glycopeptides, fluoroquinolones, aminoglycosides, rifampicin and fusidic acid occurs due to alteration of target molecules. These alterations occur via nucleotide mutations which result in a modification of the protein structure of the target so that the antibiotic can no longer bind and exert an effect, resulting in decreased susceptibility. For example, resistance to

rifampicin occurs due to mutation within the *rpoB* gene that alters the β-subunit of RNA polymerase. Rifampicin, therefore, cannot bind to the ribosome and large increases in resistance occur. Fluoroquinolone resistance is another example of target alteration conferring resistance. The primary mechanisms of resistance to fluoroquinolones are target alteration mediated by point mutations in DNA gyrase (encoded by the genes *gyrA* and *gyrB*) and topoisomerase IV (encoded by the genes *parC* and *parE*). These enzymes are essential for DNA replication as they alleviate topological stresses associated with progression of the replication fork (Drlica & Zhou 1997). These mutations occur in the conserved quinolone resistance determining regions (QRDR) of these genes.

1.1.2.2 Modification of Antibiotic

Antibiotics can be inactivated so that they can no longer have an effect on their target by hydrolysis of the antibiotic or addition of chemical groups (e.g. an acetyl group is added to chloramphenicol by acetyltransferase). These mechanisms are generally specific for a single drug or drug class.

β-lactamases achieve hydrolytic cleavage of the β-lactam ring of cephalosporins and penicillins by binding via the serine present at the active site of the enzyme. This was the first reported example of an antibiotic resistance mechanism (Abraham & Chain 1940).

β-lactamases that render bacteria resistant to first, second and third generation cephalosporins, aztreonam and penicillins by hydrolysis are known as extended spectrum β-lactamases and were first described in 1983 (Knothe *et al.* 1983). These enzymes are produced by single or double mutations within the sulfhydryl variable (SHV) encoding gene.

A further example of antibiotic modification is the inactivation of macrolides, which exert an antimicrobial effect by interfering with protein synthesis. Macrolide esterases that cleave the ester bond of the lactone ring and inactivate the antibiotic were first described in *Escherichia coli* in 1984 (Barthelemy *et al.* 1984). Although not a common resistance mechanism, this results in very large increases in MIC. Another example is fosfomycin, an epoxide antibiotic that alters a gene essential for the production of N-acetylmuramic acid, can also be deactivated by epoxidases.

Modification by group transfer is a common way by which bacteria inactivate antibiotics. These enzymes modify antibiotics by addition of a chemical group e.g. phosphate and acetyl groups. This alters the antibiotic structure, impairing binding, thereby inactivating the antibiotic. For example, aminoglycoside modifying enzymes include acetyltransferases, phoshotransferases and nucleotidyl transferases (Mingeot-Leclercq, Glupczynski, & Tulkens 1999). These enzymes alter the structure of the antibiotic so that it can no longer bind to the ribosome. One or more enzymes may be expressed and the resistance conferred and spectrum of activity can vary.

A further enzymatic mechanism, other than hydrolysis and group transfer, is oxidation of antibiotics, although this is a rare mechanism among pathogens. An example of this is the oxidation of tetracycline by TetX. This gene was initially found in a plasmid in *Bacteroides* and can confer tetracycline resistance to *E. coli* (Speer & Salyers 1988; Speer & Salyers 1989).

1.1.2.3 Reduction in Permeability

The cell wall of Gram-positive bacteria is more permeable to antibiotics than the Gram-negative cell wall. This is because the Gram-positive cell wall is a structure made of peptidoglycan attached to teichoic acid and lipoteichoic acid and this alone does not restrict the entry of antibiotics by diffusion. However the cell wall of Gram-negative bacteria is comprised of a thinner layer of peptidoglycan but additionally contains an outer membrane and the periplasmic space. The outer membrane lipopolysaccharides and phospholipids prevent penetration of antibiotics. The degree of impermeability varies between species.

Most antibiotics need to pass through outer membrane porins as they are hydrophilic in nature. Spontaneous mutations that prevent expression of porins can occur during therapy and some resistance in Gram-negative bacteria is attributable to altered expression of these porins. Imipenem resistant *P. aeruginosa*, for example, are deficient in the OprD porin, which facilitates uptake of imipenem and amino acids, preventing penetration of the cell (Wang & Mi 2006). Furthermore reduction in the permeability of the outer membrane of *Burkholderia cepacia* can confer high level resistance to chloramphenicol (Burns *et al.* 1989).

The cytoplasmic membrane does not restrict the entry of lipophilic agents such as fluoroquinolones, chloramphenicol, trimethoprim and rifampicin but does restrict hydrophilic antibiotics such as erythromycin, clindamycin, sulfonamides and aminoglycosides. The ingress of hydrophobic antibiotics is dependent on the active uptake via transport proteins and resistance can be caused by alterations in their action. For

example aminoglycoside resistance can be conferred by inactivation of the pumps that require proton motive force (PMF) (Mingeot-Leclercq, Glupczynski, & Tulkens 1999).

1.1.2.4 Efflux

Efflux pumps are proteins that are involved in the extrusion of toxic substances from within the cell and are found in Gram-positive and Gram-negative bacteria (Van Bambeke, Balzi, & Tulkens 2000). Efflux is dependent on the proton motive force (PMF) or the hydrolysis of ATP. Efflux systems that have a role in antibiotic resistance have been described in many pathogenic bacteria including *P. aeruginosa*, *Campylobacter jejuni*, *Streptococcus pneumoniae*, *Salmonella typhi*, *S. aureus* and *E. coli* (Lin, Michel, & Zhang 2002; Poole 2000).

Genes encoding efflux pumps, that extrude toxic compounds from the cell, have been found in all sequenced bacterial genomes and confer antibiotic resistance in many pathogenic bacteria. Efflux pumps can be categorised into five superfamilies. These are the major facilitation family (MFS), small multidrug resistance family (SMR; Paulsen *et al.* 1996b, resistance nodulation cell division family (RND; Saier *et al* 1994) and multidrug and toxic compound extrusion family (MATE; Li & Nikaido 2004). The MFS and SMR are more common in Gram-positive organisms while most efflux pumps in Gram-negative bacteria are of the RND type. Antibiotic efflux pumps use the proton motive force to generate ATP to export antibiotics out of the cell and are members of the resistance nodulation cell division, major facilitation and multidrug and toxic compound extrusion superfamilies (Pao, Paulsen, & Saier, Jr. 1998; Paulsen, Brown, & Skurray 1996; Poole 2000).

P. aeruginosa and *E. coli* contain two of the most well studied efflux pump systems; the MexAB-OprM system of *P. aeruginosa* (Li, Nikaido, & Poole 1995) and the AcrB system of *E. coli* (Ma *et al.* 1995). Increased expression of these pumps can occur by mutation in structural genes, regulatory genes or horizontal acquisition of plasmids or transposons.

1.1.2.5 Metabolic Bypass

Some antibiotics target enzymes in metabolic pathways. Resistance to antibiotics can be mediated by use of alternative metabolic pathways that the antibiotic can not inhibit. Resistance to sulphonamide and trimethoprim antibiotics occurs via metabolic bypass by production of altered dihydropteroate synthetase and dihydrofolate reductase, respectively (Then 1982).

1.1.3 Genetic Basis of Antibiotic Resistance

The genetic mechanisms whereby bacteria become resistant to antibacterial agents develop by three ways: acquisition of resistance genes via plasmids and other transposable elements (Guiney, Jr. 1984; Lacey 1984; Maiden 1998; Ochman, Lawrence, & Groisman 2000; Shapiro 1997), recombination of foreign DNA into the genome (Campbell 1962) and spontaneous mutational events in chromosomal genes (Davies 1994).

In the absence of a selective pressure mutations in the chromosome are stochastic in that the rates of beneficial mutations do not occur at higher frequencies than those that are neutral or disadvantageous. However there has been some controversial evidence to suggest that mutations can be directed to enhance growth (Cairns, Overbaugh, & Miller 1988). This is discussed later (section 1.2.3 and 1.2.8). For bacterial cells there is a finite

probability that a mutation will occur conferring the resistant phenotype, and unless a revertant mutation occurs, all the progeny of such a cell will also be resistant. Spontaneously occuring mutations occur in the range of 10^{-6} to 10^{-8} mutations/ cell division (Drake 1991). Resistance to streptomycin, rifampicin, fusidic acid, fluoroquinolones and oxazolidinones occurs via mutation.

1.1.3.1 Transformation

Transformation is the uptake of naked DNA and is dependent on bacteria being competent, a state in which they are able take up extraneous DNA. This ability was first demonstrated in *S. pneumoniae* by Fred Griffiths in 1928 (Griffiths 1928). In 1944 Avery, McCarty and Macleod showed that DNA was the vehicle for transformation (Avery, MacLeod, & McCarty 1944). Some bacteria are naturally competent e.g. *Streptococcus pneumoniae*, *Bacillus subtilis*, *Neisseria gonorrhoeae* and *Haemophilus influenzae* and others can be induced to take up DNA by calcium chloride or heat shock treatment (Johnsborg, Eldholm & Havarstein 2007).

1.1.3.2 Conjugation

Conjugation is a process by which DNA is transferred from the donor cell to the recipient cell via the conjugation apparatus when cell surfaces are in contact. Conjugative transfer of plasmids or transposons is responsible for the majority of bacterial gene transfer in the environment and therefore the horizontal transfer of genes conferring antibiotic resistance. However this process is not restricted to resistance genes. These processes have a wide host range and allow the transfer of resistance genes across bacterial genera to remote taxa. The same resistance gene has been found in varied bacterial species sampled from

the gastrointestinal tract and from the environment. For example, alleles of TetM have been found in a variety of Gram-positive and Gram-negative bacterial species (Roberts, Chung, & Roe 1996; Salyers *et al.* 1995).

Conjugative transposons are DNA segments of 18 to 150 k.b.p. that are integrated in the chromosome. These elements can excise to form non-replicating circular intermediates, which can integrate into the recipient genome.

The importance of these mechanisms is emphasised by the fact that between 10% and 16% of the *E. coli* chromosomal genome is foreign DNA arising from horizontal transfer (Lawrence & Ochman 1997).

1.1.3.3 Transduction

During transduction DNA is transferred from a donor bacterium to a recipient through the lifecycle of bacteriophages and this occurs in many pathogenic bacteria (Davison 1999). This genetic transfer can occur in two ways. In the first, known as generalised transduction, any portion of donor DNA can replace bacteriophage genetic material. However the second, known as specialised transduction, involves replacement of bacteriophage genes by a specific region of the host chromosome adjacent to the phage attachment site. The transducing bacteriophages in both generalised and specialised transduction are likely to be defective and cannot cause infection as essential genes have been replaced. Not all bacteria are transducible and not all bacteriophages can transduce. Bacteriophages may encode virulence factors, including shiga toxin in *E. coli* (Waldor & Mekalanos 1996). Transduction of imipenem, cefotaxime and kanamycin resistance

determinants has been described in nosocomial isolates of *P. aeruginosa* (Blahova, Kralikova & Krcery 1992).

1.1.3.4 Stability of Acquired Elements

Many strains found in the environment carry resistance genes even if these strains are not exposed to antibiotics (Andersen & Sandaa 1994; Gotz *et al.* 1996). A plasmid will be maintained in a bacterial population if it contains genes that confer an added advantage in addition to the resistance genes, e.g. genes that promote colonisation. Antibiotic genes can also be propagated by integrons and transposons. Integrons are promoterless genes that can be carried on conjugative plasmids and transposons, containing an integrase gene and a cassette integration site into which gene cassettes can insert (Stokes *et al.* 1997). A gene cassette usually contains a promoterless gene with a recombination site known as the 59 base element. Presence of integrons has been shown to be associated with increased probability of the multidrug resistant phenotype in the Enterobacteriaceae (Leverstein-van Hall *et al.* 2003). Transposons are mobile genetic elements found on plasmids or integrated into the chromosome. These genes are composed of an attachment site, an integrase and a promoter (Maiden 1998). Transposons frequently contain heavy metal resistance genes in addition to antibiotic resistance genes and therefore increase the likelihood of the antibiotic resistance genes being maintained in the population. Acquisition of multiple antibiotic resistance determinants, including aminoglycoside modifying enzymes, in strains of *Acinetobacter calcoaceticus* during a nosocomial outbreak has been attributed to transposon transfer (Devaud, Kayser & Bächi 1982).

1.1.3.5 Persister Cells

Within bacterial populations a small number of cells can survive exposure to antibiotic concentrations that kill the majority of the cells. Survivors are known as persister cells and were first described in *Staphylococcus* spp. exposed to penicillin by Bigger in 1944 (Bigger 1944). At this time Bigger proposed that these cells were dormant and it has since been confirmed that these cells, when exposed to antibiotics, have limited growth with downregulation of biosynthetic pathways (Balaban *et al.* 2004). Although the majority of cells are not persisters their presence may allow the survival of a population following antibiotic exposure and are likely to have a role in recalcitrant bacterial infections. Surprisingly, unlike resistant mutants, this resistance is nonheritable and cultures grown from persister cells have the same antibiotic susceptibility as the non-persister parent cells. Persistence genes, such as the high persistence mutant gene (*hip*), have been identified that result in an increased proportion of persisters (Moyed & Bertrand 1983; Moyed & Broderick 1986). Biofilms favour formation of persister cells where the proportion of persister cells has been reported to be 10^{-2} (Spoering & Lewis 2001), as opposed to 10^{-5}-10^{-6} in planktonic cultures (Moyed & Bertrand 1983). As well as *S. aureus* persister cells have been identified in *P. aeruginosa*, *E. coli* and *Candida albicans* biofilms (Brooun, Liu, & Lewis 2000; Spoering & Lewis 2001; Lafleur, Kumamoto & Lewis 2006).

1.1.4 Use of Antibiotics and Antimicrobial Resistance

Few studies have investigated the effect of reduction in antibiotic use in humans, and subsequent levels of resistance in bacterial populations. Reduction in antibiotic use results in an observed decrease in the frequency of penicillin resistance in pneumococci (Nowak

1994). However resistance in the population does not disappear and some resistant organisms will persist. If the selective pressure of the antibiotic is restored then these resistant strains can take over the previously susceptible population. This has been demonstrated by Gerding and colleagues during their study of the return to use of gentamicin in a hospital and observed the rapid increase gentamicin resistant isolates (Gerding *et al.* 1991).

Reduction in macrolide use within an outpatient population in Finland during the 1990s resulted in a decline in erythromycin resistance in Group A streptococci isolated from throat swabs and pus samples (Seppala *et al.* 1997). However a series of studies that evaluated the effect of antimicrobial use on penicillin resistant *S. pneumoniae* carriage in children demonstrated that carriage of penicillin resistant isolates increased despite reduction in antibiotic use for respiratory tract infection (Arason *et al.* 2002; Arason *et al.* 2006). A similar pattern of increased resistance despite reduced antibiotic use has been observed in Sweden (Hogberg *et al.* 2006).

An increase of 16% in fluoroquinolone use in Spain between 1997 and 2001, due to the introduction of levofloxacin and moxifloxacin, did not result in the expected corresponding increase in ciprofloxacin resistance in *S. pneumoniae* (Garcia-Rey, Martin-Herrero, & Baquero 2006). No significant association between fluoroquinolone use in hospitals and fluoroquinolone resistance in *P. aeruginosa* and *S. aureus* has been demonstrated (MacDougall *et al.* 2005).

Austin *et al* attempted to quantify the relationship between antibiotic use and frequency of resistance using epidemiological models. Their findings suggested that significant

reduction in antibiotic use is required to cause a significant decline in resistance. This decline in resistance is likely to occur at a lower rate than the initial emergence of the resistance (Austin, Kristinsson, & Anderson 1999).

1.2 Mutation Rates

1.2.1 Mutation and Mutagens

A mutant organism is defined as a bacterium that varies in a characteristic from its parental strain and that can pass on this new characteristic to its progeny. For this variation to be inherited, a change, or a mutation, must occur in the genome of the organism. Variation in bacteria can also be by the acquisition or loss of genetic elements, such as plasmids and transposons but bacterial genotypes are generally stable. Selection may lead to predominance of the new variant characteristic. Of all the genes present in a genome only a subset will be expressed in any given set of conditions. These expressed genes will represent the phenotype of the organism as this confers the detectable characteristics of the organism. Therefore the phenotype of an organism may be very variable.

Mutations occur without any transitional state and arise continuously at low frequencies even in favourable growth conditions. Induction of mutation may be due to cellular function, interaction with the environment or exposure to a mutagenic agent (mutagen). Many spontaneous mutations occur following errors during replication and repair. Cellular repair systems remove most of the erroneous bases but a small proportion will remain without being corrected. Many mutagens are encountered by bacteria and mutagenic activity often occurs by production of reactive oxygen species (OH radicals, superoxide anions and hydrogen peroxide) that result in damage to DNA (table 1.1).

Mutagenic Agent	Method
Ionizing Radiation	Causes breakage of the phosphate-deoxyribose of DNA. Single or double stranded breaks produced.
Ultraviolet Light	Formation of thymine dimers. Cause DNA distortion. Mostly base substitutions.
Deaminating agents e.g. nitrous acid	Modify bases. DNA polymerase will not recognise base causing erroneously pairing and mutation.
Base analogues e.g. 5-bromouracil and 2-aminopurine	Integrated into DNA by polymerase. Different pairing capacities with bases depending on the tautomeric form.
Alkylating Agents e.g. ethylmethane sulphonate	Covalently bind alkyl residues to bases, causing random pairing.
Intercalating agents e.g. ethidium bromide, acridine orange	Flat molecules intercalate between bases. Causes frameshift mutations.
Cross linking agents e.g. mitomycin C	Covalent binding of bases on opposite strands of DNA helix. Stop unwinding of helix and block DNA synthesis

Table 1.1 Modes of action of mutagens (Griffiths *et al.* 2000)

The consequences of single point mutations depend on the nature and location of the base substitution within the codon. These mutations can cause alterations in the amino acid residue encoded (missense mutation) or may leave the amino acid unchanged (silent mutation). There are two types of base pair substitution; transitions (purine to purine or pyrimidine to pyrimidine) or transversions (purine to pyrimidine or pyrimidine to purine). Deletions and inversions may also occur and can have a greater effect. These mutations can change the phenotype of the cell by causing changes in the reading frame, and these are termed frameshift mutations.

An antibiotic resistant mutant may occur due to mutation in a resistance conferring gene of a susceptible strain. The initial isolate is referred to the 'wild type' or susceptible parent. The term 'wild type' is arbitrary and should be used to describe the first observed state of the gene in question, in this case susceptibility to an antibiotic. Subculture of this

'wild type' may allow mutations to arise causing deviation from the initial state. Therefore efforts should be made to maintain the genotype of the characterised reference strains.

1.2.2 Mutation Rate versus Mutation Frequency

A *mutation rate* is an estimation of the probability of a point mutation occurring at each cell division and corresponds to the probability of a mutation occurring in the lifetime of a bacterial cell. A *mutation frequency* is simply the proportion of mutant bacteria in a culture. These two terms are often wrongly used interchangeably, causing confusion. Mutation frequency gives no indication of when the mutation occurred in the lifetime of the cell; if a mutation occurred early in the lifetime of the cell then a large number of mutant clones will be present, resulting in a high mutation frequency. This is known as a 'Jackpot Culture' and is a rare event. This phenomenon was first described in 1943 by Luria and Delbrück during their classic set of experiments on bacterial mutation (Luria & Delbrück 1943).

1.2.3 The Fluctuation Test of Luria and Delbrück

Luria and Delbrück demonstrated that bacteriophage resistant mutant colonies of *E. coli* arise from a sensitive culture if T1 bacteriophage was present in excess (Luria & Delbrück 1943). The *E. coli* culture was grown up in either nutrient broth (containing 0.5% NaCl) or asparagin-glucose synthetic medium. Their initial inocula contained between 50 and 500 bacteria. Resistance was detected by lack of clearing of the culture. Resistant colonies appeared from sensitive cultures, in which there was clearing within a couple of hours. These bacteria were resistant to bacteriophage T1 but sensitive to other viruses active on

that strain of *E. coli*. They showed that reversion to sensitivity must be a rare event as when the bacteriophage was mixed with resistant bacteria no increase in the titre of the bacteriophage occurred. In a growing culture the proportion of resistant bacteria will then increase with time. If the presence of the phage was needed to trigger the change to resistance then the distribution of mutant colonies should demonstrate a Poisson distribution. However the high variance in the mutant numbers led Luria and Delbrück to hypothesize that resistant mutants were present in the culture before bacteriophage exposure and that the mutation arose independently (Luria & Delbrück 1943). At the time this settled the issue of whether such resistant bacteria arise via random mutation or directed mutation from a selective pressure as it proved that mutations occur randomly during non selective growth. If mutations are random, the mutational events that are occurring are random in the sense that they do not occur at a higher rate if they are advantageous and that natural selection occurrs. If mutations are directed this would mean that the mutation rate conferring an advantage to a cell would be higher. For both of the hypotheses the resistance is inherited. If mutations are random, i.e. are not directed, then mutations will be clustered within a small subset of the population. If mutations are directed, the mutants will be more evenly spread throughout the whole population. However there has since been much debate over whether mutations can be directed towards being advantageous to the cell (see section 1.2.8).

Luria and Delbrück assumed that, for a bacterium, there was a small fixed chance that a resistance conferring mutation could occur per unit of time, if the bacteria are 'in an identical state'. The numbers of mutated cells in a culture depend on how early the mutation occurred during growth of the bacterial population. If mutation occurs early in the culture, the number of mutated cells will be higher than if it occurs later. This makes

mutation frequencies inaccurate as it does not reflect the stochastic nature of mutant accumulation. Mutation rates, on the other hand, are more accurate and reproducible.

1.2.4 Determination of Mutation Rate

Broadly, there are two methods for determination of mutation rate. These are the mutation accumulation method and fluctuation analysis (Rosche & Foster 2000). The mutant accumulation method can be very accurate but is involved and time consuming. This is because the culture needs to be sampled at a series of time points. In this method cultures are grown exponentially until probability dictates that a mutant will be present. If we assume that the growth rate of wild type bacteria and mutant bacteria is the same then the proportion of mutants will increase linearly with time. Furthermore if the number of mutants and the total number of bacterial cells is known, at various time points, then the mutation rate (μ) is the gradient of the line of number of mutants against generation number. Terms are defined in Table 1.2.

Term	Definition
m	Number of mutations per culture
μ	Mutation rate
r	Observed number of mutants
$\bar{r}$	Median number of mutants
C	Number of cultures
p_0	Proportion of cultures without mutants
N_0	Initial Number of Cells
f	Mutant frequency
n	Number of generations

Table 1.2 Definition of Terms

For this method, a very large total cell number is needed with a long time period between N_1 (number of cells at first time point) and N_2 (number of cells at second time point). Serial dilutions would make this easier to perform but this introduces sampling errors. If available, continuous culture would be an alternative but this would allow selection of waves of bacteria, each better suited than the generation before, to take over the culture. Moreover Billington *et al* have shown that single point mutations can impose a significant fitness deficit which undermines one of the basic premises of the mutant accumulation method (Billington, McHugh, & Gillespie 1999). For these reasons a fluctuation analysis method is more commonly used. The introduction of this technique was a pivotal event in mutational research.

1.2.5 Fluctuation Analysis

A fluctuation analysis involves estimating the mutation rate from the distribution of mutants in a number of parallel cultures. This method was pioneered by Luria and Delbrück (Luria & Delbrück 1943). Briefly an initial inoculum of cells (of known culture volume) from a growing culture is added to a broth, which is then incubated at appropriate conditions in the absence of selective pressure. The cultures are then centrifuged to concentrate the cells. Screening for antibiotic resistant cells is performed by plating the cell deposit onto solid media containing a concentration of antibiotic, usually at 2 - 4 times that of the MIC. This will kill all sensitive cells and only the resistant mutants will remain. A plate count is performed on a proportion of the culture, as a measure of the number of cells in the cell deposit. A series of log dilutions of the cell deposit are performed and inoculated on agar not containing the test antibiotic. The number of colony forming units per mL of the deposit can then be determined. Luria and Delbrück then suggested two

methods for estimating the overall mutation rate for the population: the p_0 method which is based on the proportion of cultures in which no mutants are observed and the method of the mean which relies on the determination of the mean number of mutants. Both methods assume a Poisson distribution with a mean and variance equal to the product of the probability of a mutation and the number of bacteria i.e. the ratio of variance of the mean is one. All of the methods described (Table 1.3) utilise the fluctuation analysis and use an estimate of the probable number of mutations that are the responsible for the distribution of mutations (m). This m is the number of mutations, not mutants, and will be controlled by the amount of growth and the mutation rate (μ) itself. The estimated value of m can be divided by the total number of cells to give the mutation rate. There are a number of different methods available for the estimation of mutation rate. Different methods are appropriate depending on the predicted number of mutations per culture (m).

Method	Value of m (number of mutations per culture)	References
p_0 Method	$0.3 \leq m \leq 2.3$	Luria & Delbrück 1943
Method of the Mean	None	Luria & Delbrück 1943
Lea and Coulson Method of the Median	$1.5 \leq m \leq 15$	Lea & Coulson 1949
Drake Formula	$m \geq 30$	Drake 1991
MSS Maximum Likelihood Method	All	Sarkar, Ma, & Sandri 1992
Koch's Quartiles Method	$2 \leq m \leq 14$ (See Koch *et al* for values outside range)	Koch 1982
Jones Mediator Method	Unknown Works when $1.5 \leq m \geq 10$	Jones *et al.* 1994

Table 1.3 Mutation rate estimation methods which are appropriate for different numbers of mutations per culture

1.2.5.1 The p_0 method

The p_0 can be used for selection of mutants on either liquid or solid media. This is the simplest method to calculate and was originally described by Luria and Delbrück (Luria and Delbrück, 1943). It is most suitable when the number of mutational events in a culture is low. This was initially apparent as few cultures (if any) exhibited resistant colonies. The proportion of cultures without mutants (p_0) is the zero term of the Poisson distribution given by equation 1.

$$p_0 = e^{-m} \text{ Eq [1]}$$

This method should only be used if the proportion of cultures without mutants is between 0.1 and 0.7 i.e. the number of mutations per culture (m) is between 0.3 and 2.3. The formula can be rearranged to give the number of mutations (Equation 2).

$$m = -\ln p_0 \text{ Eq [2]}$$

As cultures are scored as positive or negative there is no need to enumerate the number of colonies (Foster, 2004).

The precision of m (number of mutations per culture) varies depending on the value of p_0. Compared with other methods the p_0 method requires more cultures for the same level of precision. The method is not affected if the mutant growth rate is slower than that of the wildtype. However this is only true if each clone of mutants gives rise to a colony, post selection. If the clone does not give rise to a colony then that mutant will contribute to the proportion of cultures without mutants. Conditions need to be chosen so that the

proportion of resistant colonies is not too large or too small. When the proportion of mutants detected is known then the actual value of *m* can be calculated. When this method is chosen the investigator should design the experiments so that 10-70 % of the cultures have no mutants and the number of mutations is between 0.3 and 2.3. This can be achieved by limiting the volume of the culture or by plating only a proportion of the culture.

1.2.5.2 Lea and Coulsons Method of the Median

Median methods should not be used if more than half the plates are devoid of mutants. Such methods are of most use when all or most of the cultures give rise to visible mutant colonies. Numbers of mutations per culture (*m*) is determined by equation 3.

$$\bar{r}/m - \ln(m) - 1.24 = 0 \quad [3]$$

1.2.6 Parameters

For each mutation rate experiment there are three main parameters which need to be considered (Rosche & Foster 2000). The first of these is that the expected number of mutations (not mutants!) per culture (*m*) should not be less than 0.3. The number of mutations depends on the mutation rate itself and the amount of growth. The value of *m* can be varied by plating different volumes of culture but this can introduce errors. The method chosen will depend on the expected value of *m*.

The second parameter is the number of parallel cultures chosen to represent the bacterial population. For all methods the precision of m is a function of $1/\sqrt{C}$ and increases as C increases. Therefore if more cultures are tested then precision is increased. For the p_0 method a precision level of 20 % is considered necessary to estimate the number of mutations per culture (Rosche & Foster 2000).

The final parameter is the size of the initial inoculum (N_0). As this inoculum should not contain any pre-existing mutants or generate too many mutants, the initial inoculum should be small. This inoculum, however, needs to be large enough to represent the bacterial population. For example Luria and Delbrück used an initial inoculum of between 50 and 500 bacteria. The smaller the initial inoculum, the longer the incubation period that is required. This is especially important when working with slow growing cultures e.g. *Mycobacterium tuberculosis*. There are other complications involved in growing small numbers of organisms. For example many organisms monitor the density of cells via 'quorum sensing' and only switch on virulence genes after a certain cell density of bacteria are present (Parsek & Greenberg 2000). They do this by the production and detection of moieties such as N-acyl-L-homoserine lactones (AHLs). In *M. tuberculosis* resuscitation promoting factors, originally identified in *Micrococcus luteus* (Mukamolova *et al.* 1998; Mukamolova *et al.* 2002), have been identified that promote recovery from a non replicating phase and increase cell growth. Therefore it may be necessary to stimulate the growth of small numbers of cells by stimulatory proteins. In each parallel culture the final cell number (N_t) should be the same. N_0 should always be negligible compared to N_t (a ratio of at least <1/1000 is desirable). However a culture started from a small number of cells will take longer to grow. In addition a small inoculum may produce a reduction in viability resulting in greater variation in the final number of cells. Variations in N_t can be

eliminated be using a large initial inoculum. Rosche and Foster have found that in their experiments a good rule of thumb is to use an initial inoculum of total cells of $mN_t/10^4$ (Rosche & Foster 2000). This represents a compromise between the above factors.

In order to observe a mutation, it is necessary to have a large enough volume of cells, therefore the volume of the broth used will depend on the mutation frequency. If the frequency is high then a small broth culture can be used. However if the rate is low then large cultures should be used. To avoid inclusion of pre-existing mutants in the initial inoculum counterselectable markers or fluorescence-activated cell sorting methods can be used (Rossman, Goncharova, & Nadas 1995). The initial inoculum of cells should contain cells that are in the same phase of the growth cycle.

All of the above parameters should be kept constant between experiments.

1.2.7 Assumptions of Fluctuation Analysis

There are a number of assumptions that are made in order to perform a fluctuation analysis. These assumptions are summarised (Table 1.4).

The probability of a mutation occurring is constant per cell lifetime
The probability of this mutation occurring does not vary between growth phases
There is no cell death
Revertants occur at a negligible rate
Mutation occurs only during cell division and results in only one mutant
Growth rates of mutants and non mutants are the same
Initial cell numbers are negligible compared to final cell numbers,
All mutants are detected and no mutants occur after selection is imposed.

Table 1.4 Assumptions of mutation rate estimation

It should be remembered that all of the available methods only provide a way of estimating mutation rates. Each method relies on a set of pragmatic assumptions that are made in order to make estimations possible and therefore each mutation rate is only an estimate and can not represent exactly what is happening *in vivo*.

1.2.8 Directed Mutation Controversy

Strategies that are used to estimate mutation rates assume that mutagenesis is spontaneous and random in the sense that it is not directed. Mutations in the chromosome are therefore stochastic in that the rates of beneficial mutations do not occur at higher frequencies than those that are neutral or disadvantageous.

Luria and Delbrück's experiments provided evidence that mutations do not occur in a directed manner (Luria and Delbrück 1943). However this has since been an area of controversy. Experiments have been performed that provide evidence on both sides, although the evidence suggests that directed mutation is unlikely. In 1952 Lederberg and Lederberg used replica plating from non selective media to selective media as way to indirectly select mutants (Lederberg & Lederberg 1952). They noted that the location of mutants on the replica plate corresponded to the location on the master plate. Therefore these mutants must have been present prior to the selection. This provided further evidence for random mutation. However in 1988, it was observed mutations from Lac^- to Lac^+ in *E. coli* occur more frequently if lactose is present than if there is no lactose present (Cairns, Overbaugh, & Miller 1988). Subsequently it was pointed out that the low variance to mean ratio noted by Cairns, Overbaugh and Miller may have been due to deviations from the underlying assumptions (Lenski, Slatkin, & Ayala 1989). For example resistant mutants that grow on selective media may exhibit slower growth on non selective

media. This will result in fewer progeny cells and would decrease the variance to mean ratio. This may become a problem if mutations are directed. However this can be overcome. If mutations occur after plating then the rate of mutation will reflect not only the mutations occur during growth in the aliquots but also those appearing on the solid media. This risk can be reduced by counting the number of mutants soon after plating and post plating mutations can be considered negligible.

Hendrickson *et al* have proposed a mechanism by which apparent directed mutation can be explained by natural selection (Hendrickson *et al.* 2002). During growth limitation bacterial cells are selected that contain an amplification of a mutant *lac* gene that enables the cells to utilise the available lactose. This selection increases the probability of a reversion occuring to lac $^{+}$. This appears to direct mutation to allow bacterial growth.

1.2.9 Deviations from the Assumptions

Mutation rates may be higher during the growth phases where there is a faster rate of replication i.e. in the exponential phase. Resistant mutants may have a lower growth rate than the wild types due to a physiological fitness costs (Andersson & Levin 1999; Billington, McHugh, & Gillespie 1999; Gillespie & McHugh 1997). The net effect of this is to increase the variance of distribution. It is also possible that not all mutations are detected. For example mutations that occur late in the culture may not give rise to visible colonies and these mutants will not be included in the estimation. This phenomenon is known as phenotypic lag. It is also conceivable that mutations may occur after selection has been imposed i.e. mutants may arise on the antibiotic containing solid media.

Reversions to the sensitive phenotype are assumed to be negligible. In the absence of selective pressure reversion mutations can and do occur, however. For example reversion to linezolid susceptibility in clinical isolates of *S. aureus* has been shown to occur (Meka *et al.* 2004). Reversion to wildtype may occur by mutation, loss of a gene or by a compensatory mutation in a resistance gene (Bjorkman *et al.* 2000).

There are a number of other factors that complicate calculation of mutation rates. For example mutation rates are not constant in a population of cells. They can vary depending on antibiotic concentration (Kohler, 1997), availability of the carbon source (Hughes & Anderson 1997) and whether the bacterium is under stress or not (Shapiro 1997). Under stress the mutation rate is likely to be higher (O'Sullivan, McHugh, & Gillespie 2005). Additionally the presence of cells prone to mutation (containing mutations in repair genes) (LeClerc *et al.* 1998) and so will exhibit an increased mutation rate of the whole population. Such mutator phenotypes can be beneficial to bacteria as variation may provide an advantage in changing conditions. However once the bacterium has adapted to the selective pressure then cells with high mutation rates may lose their advantage as some important function that is not needed for immediate survival may be lost (Giraud *et al* 2001). Classical evolutionary theory would consider bacterial cells with the highest rates of replication as the fittest organisms. However a slow replication rate may still increase fitness as in the presence of a selective pressure, e.g. an antibiotic, a mutation may confer enough of a benefit to the slower replicator so that it can displace the fast replicator (Burch & Chao 2000). This has been confirmed using 'digital organisms' where at high mutation rates, faster replicating organisms were outcompeted by organisms with the lower replication rate with a selective advantage (Wilke *et al.* 2001). Some antibiotics can also increase the frequency of mutations in the genome (Mamber *et al.* 1993). For

example fluoroquinolones have been shown to induce the SOS response in *E. coli* and *Salmonella* (Mamber *et al.* 1993). During the SOS response, which is controlled by products of *lexA* and *RecA*, the expression of polymerases is upregulated but these have a higher error rate as gaps, which are opposite thymidine dimers, are filled in by replication and not recombination. This increases the mutation rate (Gillespie *et al* 2005).

Sarkar *et al* attempted to improve the Luria and Delbrück model by considering the effect of nonsynchronous division and the effect of cell death (Sarkar 1991). It was proposed that mutants have a lower mutation frequency than wildtype cells. This may be due to a reduction in fitness or a reversion to the susceptible phenotype. It was suggested that this could be addressed by plating only a proportion of the final culture and calculating moments of the distribution. However Sarkar failed to consider the concept of phenotypic lag, which caused discrepancies between calculated mutation rates and *in vivo* rates. Phenotypic lag, describes the observation that mutations that occur late in the culture may not give rise to colonies. Armitage *et al* (Armitage 1952) identified the existence of phenotypic delay for the mutation described by Luria and Delbrück. Most methods do not take this factor into account, but Armitage *et al* subsequently introduced this extra parameter into the Lea and Coulson model (Armitage 1953). The results of this approach indicate that the outcome of fluctuation analysis tests may depend on the size of the original inoculum, however Armitage did not address this. A possible explanation is that a small proportion of cells in the inoculum may result in progeny that have a different mutation rate than the average cell. Therefore smaller inocula may not contain such cells. Phenotypic lag will increase the variance of the final distribution and will affect the lower end of the distribution more than the upper end. This alters the shape of the distribution as mutants that arise near the end of the culture period will not be expressed and will

therefore be underestimated in the culture. Koch has suggested estimating the duration of the lag in generations (n) and calculating the values of *m* using values of *r* in quartiles that are lowered to 2^n of the values which are observed (Koch 1982). The value of *m* is then multiplied by 2^n-1 to give the final estimated number of mutations.

It is assumed that all mutants are detected. However Ma *et al* (Ma & Sandri 1992) and Jones *et al* (Jones *et al.* 1994) have altered their fluctuation analysis method to allow for only an aliquot of the culture volume to be plated. Crane *et al* have proposed a modified fluctuation assay for estimation of mutation rates where small increases in mutation rate are expected (Crane, Thomas, & Jones 1996). In their method a proportion of large broth is examined as opposed to the original method of using a small culture. This allows the detection of small increases in mutation rate using small numbers of cultures.

1.2.10 Detection of Resistant Mutants

For all mutation rate estimation methods a number of parallel cultures are inoculated with a small number of wildtype bacteria i.e. no resistant mutants will be present in the initial inoculum. The culture is incubated and the number of cells in the culture at N_t is calculated by plating out the culture onto non selective media. To determine the number of resistant mutants the aliquots are cultured onto solid selective media containing the antibiotic. Antibiotic resistance of mutants is used as a selective marker for estimation of mutation rate but there may be other markers that could be used (see section 6.5). Using an antibiotic as a tool is distinct from the examination of the effect of antibiotics on the mutation rate.

No satisfactory solution of the Luria and Delbrück distribution has been found that effectively describes the distribution numerically. Therefore extensive attempts have been made to improve the accuracy of the estimates (Asteris & Sarkar 1996; Kepler & Oprea 2001). It is important to note that mutation rates estimated via different methods cannot be compared.

1.2.11 Hypermutability

Within populations of pathogenic bacteria, some bacterial clones have a higher spontaneous mutation rate than the majority of the population. These variants exhibit the mutator phenotype and are therefore known as 'mutators' and owe their high mutation rates to defects in proof reading and repair mechanisms. These defects are likely to be in a methyl directed mismatch repair system that is encoded by *mutH*, *mutL*, *mutS*, *dam* and *uvrD* and defects causing an increase in mutation rate are commonly due to loss of activity in *mutS* (LeClerc, Li, & Payne 1996; Matic *et al.* 1997; Miller 1996; Sniegowski, Gerrish, & Lenski 1997). Mutation rates may be between 10 – 10,000 fold higher than the non mutator wildtype but this depends on the nature of the mutator allele. Mutator bacteria exist in populations of pathogenic bacteria and have been described in *E. coli*, (LeClerc, Li, & Payne 1996; Matic *et al.* 1997) *Salmonella* spp. and *P. aeruginosa* (Oliver *et al.* 2000; Oliver *et al.* 2004).

1.2.12 High Mutation Rate leads to Adaptation

As stress is ever present for bacteria in the environment an organism that can adapt to changes is more likely to survive. Therefore it can be beneficial for the bacterial

population to be heterogenous, increasing the probability that a subset of cells will survive. This 'bet-hedging' may be a useful strategy in uncertain environments and may promote survival. However few mutations are beneficial, most are deleterious. Therefore the mutation rate that has evolved represents a compromise between allowing adaptation and avoiding excessive deleterious mutations. Bacteria that have a higher spontaneous mutation rate than the majority of the population are more likely to gain resistance via mutation in chromosomal genes.

Mutation rates may be higher for individual mutants within a bacterial population as a whole. Genotypes with lower mutation rates than the mutator may also exist in the population but these mutation rates are still higher than rates in the wild-type (Giraud *et al.* 2001). During colonisation of the mouse gastrointestinal tract by *E. coli* a higher mutation rate was observed. This could prove advantageous as initial adaptation to a new environment may occur more readily. However deleterious mutations that occur after colonisation may accumulate and become problematic (Giraud *et al.* 2001).

1.2.13 Mutator Phenotypes Select for Antibiotic Resistance

Homology in DNA sequence is required to allow recombination to occur between bacteria. This prerequisite acts as a barrier to exchange between species. However it has been shown that recombination can occur between *E. coli* and *S. typhimurium* in mutants defective in the mismatch repair genes *mutL*, *mutS* and *mutH* (Rayssiguier, Thaler, & Radman 1989). Therefore mutators that are defective in their methyl-directed mismatch repair genes display both a hypermutable phenotype and are more likely to transfer genetic elements across species barriers. This may result in increased selection and spread of

antibiotic resistance genes. These mutators are also more likely to accumulate compensatory mutations which may reduce the physiological cost of the target site modifications that decrease susceptibility (Andersson, Bjorkman, & Hughes 1998; Bjorkman *et al.* 2000). These bacteria therefore represent a challenge for reducing rates of antimicrobial resistance.

1.2.14 Fitness of Mutators

It is possible that mutators may have an increased growth rate due to reduced use of cell resources on repair of damaged DNA. However there is very limited evidence for this increased fitness (Sniegowski *et al.* 2000). Through the use of competition assays between the mutator and non-mutator phenotypes Chao and Cox demonstrated that mutators have a small but measurable decrease in growth rate (Chao & Cox 1983). Progeny will also have defects in repair mechanisms and may accumulate further costly mutations. However mutators can be at an advantage due to increased rate of mutations that confer some advantage to the cell. There may be a selective pressure for mutators that increase the frequency of the phenotype in the population as the long term fate of the mutator allele is linked to the mutations that occur as a result. If beneficial mutations occur the mutator phenotype may 'hitch-hike' on the back of the beneficial mutation that provides a selective advantage (Chao & Cox 1983). For this to occur the beneficial mutations must occur in the mutator population before they occur in the wild type population. As the numbers of mutators is lower than non-mutators then the mutation rate must be increased to the point that allows for this. Relative numbers of mutators are unlikely to exceed 10^{-3} compared to the rest of the population. Therefore the mutation rate must be at least 1000 fold than the wild-type for the mutators to be maintained in the general population (de

Visser, 2002). Certain loci in the genome may have an increased mutation rate while housekeeping genes may be expected to have a lower rate (Moxon *et al.* 1994). This allows the exploitation of adaptability but minimises the detrimental effects of accumulation of costly mutations.

1.2.15 Stability of Mutators

If a mutator allele is eventually fixed in a population then it is possible that all cells in the population will have increased mutation rates. It is unlikely that reversion to fully functional repair genes will occur. Horizontal acquisition of functional genes is more likely (Brown *et al.* 2001). It has been shown that the mutator phenotype is retained in *E. coli* following 11500 generations of growth (Sniegowski, Gerrish, & Lenski 1997). Therefore it is unlikely that mutators will be lost from the population.

1.3 Fitness

1.3.1 Importance of fitness

Fitness is a complex characteristic that encompases the ability of a genotype to reproduce within a host, be transmitted and survive in defined environments.

When bacteria are exposed to antibiotics a mutation conferring resistance to that antibiotic is likely to give the bacterium an advantage in the presence of the antibiotic. However it is accepted dogma that a resistant organism pays a physiological price for resistance, particularly resistance mediated by chromosomal mutations (Levin, Perrot, & Walker 2000). It is believed that following rational use of antibiotics, antimicrobials are removed

from the environment and in the absence of a selective pressure, resistant mutants will be out-competed by their susceptible counterparts and will be lost from the population. Although studies have shown that acquisition of antibiotic resistance can incur a biological cost (Andersson & Levin 1999; Gillespie & McHugh 1997) there is evidence that some mutations conferring resistance may result in low or no fitness deficit (Gillespie 2001; Gillespie, Voelker, & Dickens 2002; Kugelberg *et al.* 2005). Thus, quantification of fitness costs is important when determining the stability of antibiotic resistance in a population.

1.3.2 Fitness and Antibiotic Resistance

The major factors that influence the frequency of antibiotic resistance in a population of bacteria are the extent of antibiotic exposure, the cost of resistance and the extent that the bacteria can compensate for this cost. Within a population of bacteria different genotypes must compete with each other to fill a niche. Therefore, the incidence of resistance can be reduced by the rational use of antibiotics as resistant bacteria can be selected against in the absence of antibiotics due to a fitness cost. However, resistance will not disappear from the population, if mutations conferring resistance have a low fitness cost, or no cost, then these mutants may remain at high levels in the bacterial population if antibiotic use is withdrawn or may return to high frequencies if antibiotic pressure is reintroduced.

1.3.3 Measuring Fitness

In vitro models of fitness have been used in order to investigate the evolution of antibiotic resistance and to assess the physiological price associated with acquisition of resistance. The growth rate of bacteria in liquid culture medium is a commonly used model for evaluating fitness (Bennett, Dao, & Lenski 1990; Kugelberg *et al.* 2005; Lenski *et al.*

1998; Lenski, Simpson, & Nguyen 1994; Nguyen *et al.* 1989). Relative fitness of a resistant mutant compared to the susceptible parent is often determined by assay of competition between isogenic antibiotic susceptible and antibiotic resistant bacteria in culture or in animal models. Fitness costs have been measured in a number of ways. Growth rate in culture media is a good model for evaluating fitness, as are paired competition experiments (Billington, McHugh, & Gillespie 1999; Davies *et al.* 2000; Gillespie *et al.* 2002; Komp *et al.* 2005). In liquid culture relative fitness is defined by the difference in number of generations that have occurred between the susceptible parent and the mutant. These methods minimise observed variation and allow fitness costs to be calculated in terms of generations.

The models selected should aim to reflect growth and environmental survival conditions of the bacterial species of interest, and can be confounded by variations in measurements in experimental procedures. No one method in isolation is likely to be sufficient to describe *in vivo* fitness and multiple models are required. The models selected will depend on the organism and its mode of transmission. Fitness deficits will vary depending on the resistance mutation, the organism and the model used to quantify the cost. For example Sanchez *et al* assessed the fitness costs associated with overproduction of multidrug efflux pumps in *P. aeruginosa* using survival in water, maintenance on dry surfaces, biofilm formation, nematode killing, production of pyocyanin and pyoverdin and quantification of proteases (Sanchez *et al.* 2002). Mutants were shown to have fitness costs in terms of resistance to dessication, survival in water, loss of quorum sensing response and loss of virulence in the nematode killing model. However the *nalB* mutant exhibited greater biofilm formation than the wild type (Sanchez *et al.* 2002).

Most studies investigating fitness costs use *in vitro* models while few have used animal or human *in vivo* models. These *in vivo* studies commonly use competitive colonisation to measure fitness (Johnson *et al.* 2005). Fitness of fluoroquinolone resistant *C. jejuni*, was assessed via colonisation and persistence in chickens in the absence of antibiotic selective pressure (Luo *et al.* 2005). It is unrealistic to assume that *in vitro* assays, using biological rich media, will accurately reflect the fitness costs experienced by the pathogen during infection. Fitness deficits may be affected by growth conditions (Durso, Smith, & Hutkins 2004; Remold & Lenski 2001). Therefore use of minimal media may be more appropriate if *in vivo* models are not possible. Competitive colonisation of human skin by *Staphylococcus epidermidis* has been used to measure fitness costs associated with fluoroquinolone and fusidic acid resistance (Gustafsson, Cars, & Andersson 2003).

1.3.4 Cost of Fitness

1.3.4.1 Chromosomal Mutations

Antibiotic targets that are altered by chromosomal mutations to confer resistance include DNA gyrase, RNA polymerase, the cell wall or the ribosome and these alterations can cause a reduction in fitness (Andersson & Levin 1999; Gillespie & McHugh 1997; Levin, Perrot, & Walker 2000). Mutations in *rpsL* confer streptomycin resistance in *Salmonella typhimurium* due to changes in the ribosomal protein S12. These mutants have been shown to be less fit than the wild type due to a decrease in peptide elongation rate, resulting in decreased protein synthesis and growth rate (Bjorkman, Hughes, & Andersson 1998). Chromosomal mutations in RNA polymerase (*rpoB*) that confer resistance to rifampicin are associated with a fitness cost in *S. aureus* and *M. tuberculosis* (Billington, McHugh, & Gillespie 1999; Moorman & Mandell 1981; Wichelhaus *et al.* 2002). The

extent of this fitness cost depends on the nature of the resistance mutation (Billington, McHugh, & Gillespie 1999).

In 1953, Barnett and colleagues showed that resistance to isoniazid in *M. tuberculosis* ameliorated disease in a guinea pig model (Barnett, Busby, & Mitchison 1953). Molecular tools have since shown that point mutations in *katG* confer isoniazid resistance. Functional *katG*, integrated to the genome, restored virulence to wild type levels (Wilson, de Lisle, & Collins 1995). In the mouse model, resistant strains of *M. tuberculosis* vary in virulence (Ordway *et al.* 1995), however the degree of drug resistance was not correlated with a reduction in virulence.

A number of mutations, conferring antibiotic resistance, which do not incur measurable fitness costs have been described. These include mutations in topoisomerase genes conferring fluoroquinolone resistance in *S. pneumoniae* (Gillespie, Voelker, & Dickens 2002), vancomycin resistance in Enterococci (Ramadhan & Hegedus 2005) and ribosomal mutations conferring aminoglycoside and spectinomycin resistance in *Borrelia burgdorferi* (Criswell *et al.* 2006).

1.3.4.2 Plasmids

The carriage of plasmids has been shown to reduce the fitness of bacteria (Lee & Edlin 1985; Nguyen *et al.* 1989; Warnes & Stephenson 1986). Insertion of a plasmid reduced fitness of the strain compared to the plasmid free strain. However, this fitness deficit was reduced following 500 generations (Nguyen *et al.* 1989). Restoration of fitness may be due to loss of plasmid containing bacteria from the population as plasmid free bacteria outgrow them (Lenski & Bouma 1987). This would suggest that following rational

antibiotic use the frequency of resistant bacteria may decline, reducing the spread of antibiotic resistance. Subsequently, it has been demonstrated that, with time, chromosomal changes occur that increase the fitness of the plasmid carrying bacteria (Lenski, Simpson, & Nguyen 1994). Over many generations of association the effects of fitness can be decreased extensively by compensatory mutations which restore reproductive potential (Bouma & Lenski 1988; Modi *et al.* 1991).

1.3.4.3 Other Genetic Elements

In *S. aureus* methicillin resistance occurs due to acquisition of the Staphylococcal Chromosomal Cassette (SCCMec) element and this incurs a fitness cost. Fitness cost was measured via transformation of SCCMec into the susceptible parent strain. Growth rates were determined in paired competition assays. The generation time of *S. aureus* containing SCCMec was 29 +/- 0.1 min compared to 40 +/- 0.1 min to the parent strain (Ender *et al.* 2004). Lee *et al* measured fitness costs of type I and type IV SCCmec elements in glucose limited continuous culture. A fitness cost was measured for type I as measured by growth rate and cell yield but no similar cost was found for type IV (Lee *et al.* 2007).

The *bla* $_{SME-1}$ β-lactamase is only found in carbapenem resistant *Serratia marcescens* strains and has not been identified on mobile genetic elements. However its mode of horizontal transfer is unknown. Using growth competition assays a fitness cost was associated with presence of this element and this may limit dissemination of the β-lactamase among *S. marcescens* isolates (Marciano, Karkouti & Palzkill 2007).

1.3.4.4 Compensatory Mutations

A deleterious mutation may be lost from the population, revert to susceptibility or be compensated for by another mutation. In bacteria which are less fit, selection of mutations which compensate for this deficit may occur and restore reproductive potential. These mutations occur at another site and ameliorate the cost incurred by the initial resistance mutation without the loss of the resistance. These mutations can accumulate and restore fitness, stabilising the population of resistant bacteria. The compensated strain may then outcompete the wildtype strain. These mutations have been observed in a number of bacterial species. For example, compensation of fluoroquinolone resistance in *S. aureus* occurs by decreased expression of topoisomerase IV (Ince & Hooper 2003). It has also been demonstrated that the fitness cost of mutations in *rpsL*, conferring streptomycin resistance in *E. coli*, can be compensated to a restored rate of protein synthesis following adaptation (Schrag, Perrot, & Levin 1997). Similarly, adaptation experiments in *M. tuberculosis* have demonstrated that rifampicin resistant *rpoB* mutants lose the fitness deficit following serial passage (Billington, McHugh, & Gillespie 1999).

Most compensatory mutations that restore fitness are not revertants to susceptibility. This may be because the mutation rate for other mutations is higher due to multiple targets (Levin, Perrot, & Walker 2000).

Isoniazid resistant *M. tuberculosis* with mutation in *katG*, resulting in loss of a functional catalase, accumulates compensatory mutations that result in increased expression of the *ahpC* promoter. The *ahpC* gene encodes an alkyl hydroperoxidase reductase and it has been proposed that these mutations increase the expression of this enzyme which protects

M. tuberculosis from oxidative stress and compensates for the loss of catalase (Sherman *et al.* 1996).

There is evidence to suggest that antibiotic resistance may reduce the ability of an organism to be transmitted and cause disease in another host. Drug resistant strains of *M. tuberculosis* have been shown to cause fewer secondary cases of tuberculosis than susceptible strains (Burgos *et al.* 2003). Some drug resistant strains have been shown to be as likely to occur in clusters, as compared to the wildtype and others less likely (van Soolingen *et al.* 1999).

1.3.5 Fitness Landscapes

Fitness or adaptive landscapes can be used to visualise relationships between genotypes and replication rates (Wright 1932). The fitness of all mutants can be compared via construction of a topographical map with fitness on the ordinate and genomes/phenotypes on the abscissa. A single large peak indicates that a single genotype has optimum fitness and that any deviations result in a decrease in fitness. Multiple peaks indicate that a number of possible genotypes may have similar fitness values.

1.3.6 Mullers Ratchet

In a culture a deleterious mutation is more likely to occur than a favourable mutation. Therefore, over numerous generations, an irreversible decline in fitness will be observed and the fittest individuals can be lost from the population. This has been referred to as 'Muller's ratchet' (Muller 1964) and occurs in RNA virus populations; the effect is exacerbated in asexual populations where populations are small and mutation rates are

high. However Andersson and Hughes showed that Muller's ratchet also operates in *S. typhimurium* (Andersson & Hughes 1996).

1.4 Bacterial Biofilms

1.4.1 Definition

Bacteria may exist in sessile or planktonic states. It is accepted that planktonic cells are necessary for replication and colonisation of new habitats and adherent cells are important for perseverance. Classic planktonic laboratory culture experiments have elucidated many aspects of bacterial physiology and molecular biology. Although planktonic cells are convenient to study, it is now commonly recognized that bacteria grow as biofilms both within the human body and in natural environments (Costerton *et al.* 1987). Bacterial biofilms have been isolated from 3.2 billion year old deep sea hydrothermal rocks and therefore represent an ancient mode of existence (Rasmussen 2000). Purely planktonic populations of bacteria are likely to be rare. In order to guide treatment of infections caused by biofilms, bacteria should be studied as biofilms because planktonic and biofilm cells are inherently different. The definition of a microbial biofilm has evolved over the last twenty years and may now be regarded as a complex community of interdependent microbial cells enclosed within a self produced extracellular polymer matrix that are associated with a biotic or abiotic surface or interface that express a distinct phenotype.

1.4.2 Biofilms in Human Disease

Although it is accepted that biofilms are common in natural environments the significance of biofilms in infectious diseases is not yet clear. However, it is estimated that 65% of infections in the developed world are caused by biofilm forming organisms (Potera 1999). Many chronic bacterial infections, including otitis media (Fergie *et al.* 2004), endocarditis (Hyde, Darouiche, & Costerton 1998), dental caries (Marsh 1995), peridontitis (Darveau, Tanner, & Page 1997), biliary tree infection (Leung, Sung, & Costerton 1989) and lung infection in cystic fibrosis patients (Singh *et al.* 2000) owe their persistence, in part, to the formation of biofilms and the intrinsic resistance of these biofilms cells to antimicrobial killing and to the immune response of the host (Costerton, Stewart, & Greenberg 1999). Many nosocomial infections are associated with colonisation of indwelling medical devices include those relating to urinary catheters (Morris, Stickler, & McLean 1999), central venous catheters (Passerini *et al.* 1992), orthopaedic devices and prosthetic heart valves.

1.4.3 Biofilm Development

Biofilm development is a complex process and five stages have been suggested (Stoodley *et al.* 2002), see figure 1.1.

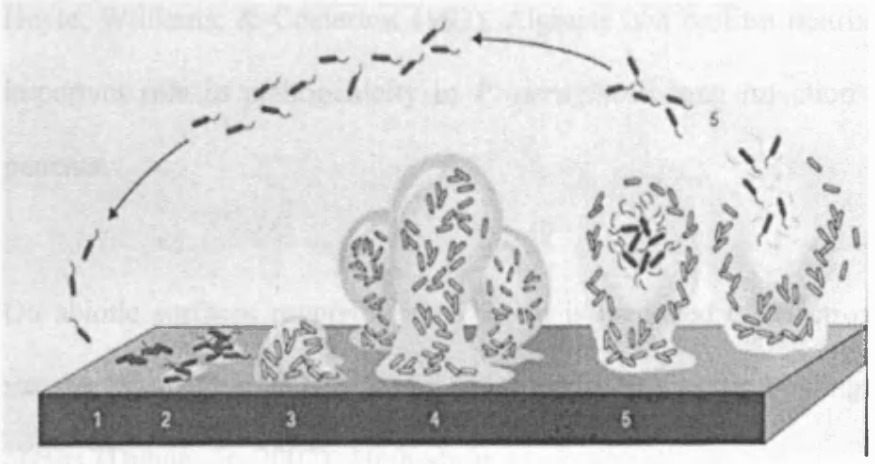


Figure 1.1 Stages of Biofilm Development (obtained from Stoodley *et al.* 2002). Stage 1 involves reversible initial attachment to a surface. Stage 2 involves irreversible binding via exopolysaccharide (EPS) production. Stages 3 and 4 represent differentiation of an initial biofilm to a mature biofilm. During stage 5, dispersion of single planktonic cells from the biofilm occurs.

Mature biofilms consist of 3D mushroom and pillar structures separated by water channels and the shape of these structures is affected by nutrient status (Stoodley *et al.* 1999). This allows the transport of oxygen and nutrients and the removal of metabolic waste products. Flow within these channels has been documented (Stoodley, Debeer, & Lewandowski 1994).

1.4.4 Adhesion

Bacteria initially adhere to the surface in a reversible, then irreversible manner to form a structured community. Adhesion triggers changes in gene expression. For example attachment of *P. aeruginosa* cells during the initial stages of biofilm formation stimulates production of alginate from the *algC* promoter (Davies, Chakrabarty, & Geesey 1993;

Hoyle, Williams, & Costerton 1993). Alginate is a biofilm matrix polymer that plays an important role in pathogenicity in *P. aeruginosa* lung infection in cystic fibrosis (CF) patients.

On abiotic surfaces reversible attachment is mediated by electrostatic, hydrophobic and van der Waals interactions but on tissue surfaces, specific binding of lectins and adhesins occurs (Dunne, Jr. 2002). High shear environments can promote biofilm formation. It is hypothesized that this represents a survival strategy as planktonic cells will be removed (Donlan & Costerton 2002).

1.4.5 Quorum Sensing

Quorum sensing is the regulation of cell density dependent expression of genes and allows coordination of virulence factor expression by the release of small molecules. Motility, production of antibiotics, exchange of DNA, and biofilm production have been shown to be controlled by quorum sensing systems. Quorum sensing was first described over 25 years ago in the light producing marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Nealson & Hastings 1979). The *luxCDABE* luciferase operon encodes the enzymes responsible for light production. Emission of light was dependent on the accumulation of signalling molecules and only occurred at high population densities (Nealson & Hastings 1979). The quorum sensing circuits identified in Gram-negative bacteria contain homologues of the *luxI* and *luxR* genes. The *luxI* gene encodes the autoinducer homoserine lactone signalling molecules (AHLs). These are small diffusible molecules that accumulate with increases in cell population until a threshold is reached. The *luxR* gene binds autoinducers and can then activate expression of target genes (Hanzelka &

Greenberg 1995). Quorum sensing has been described in a number of Gram-negative pathogens including *P. aeruginosa* (Passador *et al.* 1993), which contains two systems, *lasRI* and *rhlRI* (Latifi *et al.* 1995). The quorum sensing systems of *P. aeruginosa* activate the expression of a number of virulence factors including; elastase, encoded by *lasB*; exotoxin A, encoded by *toxA*; alkaline phosphatase, encoded by *aprA*; siderophores and is involved in biofilm formation (Parsek & Greenberg 2000; Pearson, Pesci, & Iglewski 1997; Whiteley, Lee, & Greenberg 1999).

1.4.6 *P. aeruginosa* grows as a biofilm in the CF lung

There are three separate lines of evidence to support the observation that *P. aeruginosa* grows as a biofilm in the lungs of CF patients (Lam *et al.* 1980; Singh *et al.* 2000). Cells isolated from CF sputum form microcolonies surrounded by a matrix that are visible using electron microscopy (Lam *et al.* 1980). Furthermore the ratio of quorum sensing acyl homoserine lactones expressed supports the role of the biofilm phenotype in infection of CF patients (Singh *et al.* 2000). This is because biofilm cells of *P. aeruginosa* produce more butryl (C4) acyl homoserine lactone than oxyodecanoyl (C12) acyl homoserine lactone, while planktonic cells produce more of the C12 signal (Singh *et al.* 2000) and this is also observed in the CF lung. *B. cepacia* grow as biofilms *in vitro* (Al Bakri, Gilbert, & Allison 2004; Conway, Venu, & Speert 2002; Tomlin *et al.* 2005; Tomlin, Clark, & Ceri 2004; Tomlin, Coll, & Ceri 2001) and therefore it is likely that growth within the lung is as the biofilm phenotype.

1.4.7 Biofilm Resistance to Antimicrobial Killing

Biofilms growing in association with epithelial cells in the lungs of CF patients can cause persistent infections that are difficult to eradicate, partially because biofilms are highly resistant to killing to bactericidal antibiotics. Various mechanisms have been proposed to account for the increased resistance to antimicrobials and it is likely that multiple mechanisms act synergistically. However the reasons for increased tolerance to antimicrobials remain unclear. The cells that persist exhibit increased resistance to antibiotics (Stewart & Costerton 2001) have been shown to be up to 1000 fold more resistant than planktonic cells (Ceri *et al.* 1999). Accepted resistance mechanisms such as mutation in antibiotic target genes, modification enzymes and efflux pumps (Walsh 2000) are unlikely to be responsible as cells sacrificed from biofilms regain their susceptibility (Anwar *et al.* 1989). This implies that genetic events have not occurred. However derepression of a chromosomal β-lactamase in *P. aeruginosa* has been shown to occur over time (Bagge *et al.* 2004), suggesting although conventional mechanisms alone do not explain resistance, they may contribute to persistence.

There are three resistance mechanisms that have been proposed for cells growing in a biofilm. These are reduced penetration of the antibiotic, development of an altered environment and formation of a protected, resistant phenotype (Mah & O'Toole 2001). The first mechanism is more likely to be due to the deactivation of the antibiotic in the biofilm or by binding of positively charged antibiotics, e.g. aminoglycosides, to the negatively charged matrix (Kumon *et al.* 1994; Shigeta *et al.* 1997) than reduced penetration. It has been shown that some antibiotics readily penetrate biofilms, in particular the fluoroquinolones (Anderl, Franklin, & Stewart 2000; Ishida *et al.* 1998;

Stewart 1996; Vrany, Stewart, & Suci 1997) and that reduced transport has only a minor effect on biofilm antibiotic resistance. In addition the biofilm matrix readily allows antibiotic sized molecules to pass through (Stewart 1998). The second hypothesis relies on the development of an altered environment and nutrient gradients within the biofilm. Antibiotic action could be antagonised by accumulation of acidic waste products or reduced in zones of depleted oxygen levels, inhibiting action of antibiotics such as the aminoglycosides which have less activity in anaerobic conditions. Conditions within the biofilm, such as nutrient limitation may cause the bacterial cells to enter a non-growing state and so antibiotics that target the bacterial cell wall are likely to have reduced efficacy. This hypothesis has been supported by visualisation of *P. aeruginosa* biofilms, which has shown that the majority of the biofilm consists of metabolically inactive cells with metabolically active cells being only at the biofilm/liquid interface (Xu, McFeters, & Stewart 2000). Planktonic cells in the stationary phase are more tolerant to antibiotics than biofilm cells (Spoering & Lewis 2001). This is most likely due to slow growth and the presence of microbial persister cells. Antibiotic exposure kills the majority of the biofilm and planktonic cells, leaving a proportion that are protected by being in the biofilm state, as the concentration of antibiotic declines persister cells grow and repopulate the biofilm.

There may be a genetic basis for biofilm recalcitrance to antimicrobials. Whiteley *et al* used microarrays to compare gene expression in planktonic and biofilm cells of *P. aeruginosa.* Approximately 1% of all genes were differentially expressed in the biofilm (Whiteley *et al.* 2001). Exposure of the biofilm to tobramycin resulted in differential expression of 20 genes, several of which are known to have a role in increased aminoglycoside resistance. Thus it is likely that during biofilm growth alteration in gene expression induces resistance to many antibiotic classes. The *P. aeruginosa nvdB* gene is

required for synthesis of periplasmic glucans which may prevent tobramycin from exerting an effect via sequestration of the antibiotic. *P. aeruginosa* containing a mutant *ndvB* gene, resulting in less glucan production has been identified that can form biofilms but does not develop high level antibiotic resistance (Mah *et al.* 2003).

1.4.8 Genetic Diversity in Biofilms

Biofilm communities consist of distinct subpopulations. The diversity of biofilms increases the range of environmental conditions that the bacteria can survive. This is known as the 'insurance hypothesis' (Boles, Thoendel, & Singh 2004). Boles *et al* demonstrated that heritable variation in colony morphology, swimming capacity, biofilm formation, detachment, metabolism and hydrogen peroxide resistance occurs during biofilm growth of *P. aeruginosa.* A proportion of biofilm bacteria overproduced pyomelanin, which has a protective function against radiation and oxidants (Nosanchuk & Casadevall 2003). This variation is likely to be dependent on RecA, which can produce genetic change in the chromosome by recombination and can induce error prone DNA polymerases (Little & Mount 1982). Inactivation of RecA reduced colony variation.

Bacteria are continuously exposed to changing environmental conditions. Biofilms serve to buffer bacteria from these changes by offering protection from antimicrobial killing and the host immune system. These protected communities of cells select for genetic diversity. Increased diversity will not necessarily result in altered fitness of any one subpopulation as fitness will depend on the prevailing conditions. The existence of these subpopulations may enable a proportion of the population to survive environmental change.

1.5 Fluoroquinolone Antibiotics

1.5.1 Fluoroquinolones

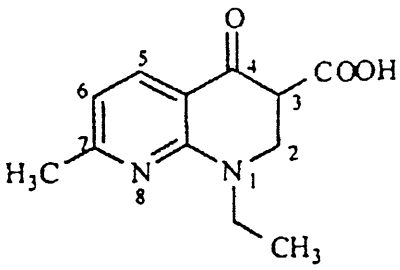
The fluoroquinolone (FQ) class of antibiotics contains synthetic broad spectrum antibacterial compounds that have been widely used to treat human infections, especially respiratory and urinary tract bacterial infections (Hooper 1998). FQs are a widely prescribed class of antibiotic, and this use continues to increase. Between 2003 and 2005, dispensed prescriptions of ciprofloxacin, the most widely used FQ, rose by 10% (www.publications.doh.gov/uk/prescriptionstatistics/index). The FQs are also commonly used for veterinary treatment and in animal husbandry (Chiu *et al.* 2002; Nelson *et al.* 2007).

FQ compounds contain a 4-quinolone ring in their structure and exert bactericidal activity by inhibition of DNA synthesis, through action on DNA gyrase (topoisomerase II) and topoisomerase IV (Drlica & Zhou 1997; Drlica & Hooper 2003). The topoisomerases are essential in resolving topological problems encountered by the cell and allow DNA to be packaged within cells. Most bacteria have two types of topoisomerases, type I and II. Type II enzymes mediate alterations in topology by introducing double stranded DNA breaks and allow subsequent re-joining. The type I enzymes cause single stranded breaks only. There is little conservation in amino acid sequence between these two types of topoisomerase. These enzymes are not present in human cells which are therefore not affected by FQs. In Gram-negative bacteria the primary target is gyrase and in Gram-positive bacteria it is topoisomerase IV (Drlica & Zhou 1997).

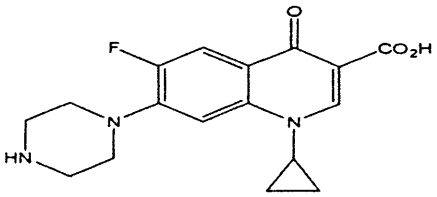
1.5.2 History of the Fluoroquinolones

The quinolones have been classified into four generations on the basis of their activity. An antibacterial compound derived from a preparation of the antimalarial agent chloroquine was used to develop the first quinolone, nalidixic acid, which was synthesized in 1962. The use of this compound was limited due to low plasma levels, limited oral absorbance, high toxicity and a narrow spectrum of activity (Goss, Deitz, & Cook 1964). Resistance develops quickly to nalidixic acid. However there are now many derivatives of this compound, see figure 1.2. Following the addition of a fluorine atom at the carbon 6 position to produce the agent flumequine the resulting second generation compounds had increased activity. Additional of a piperazinyl side chain at position 7 improved activity against Gram-negative bacteria. The resulting compound, pipemidic acid had activity against *Pseudomonas* and can penetrate cells more efficiently. The second generation compound, ciprofloxacin has a fluorine atom added at the C-6 position, an N1 cyclopropyl group, a piperazinyl ring at the C-7 position and a nitrogen atom at the C-8 position. The introduction of ciprofloxacin increased the therapeutic value of the FQs and there are now many derivatives of this compound. The combination of a fluorine atom at position 6 with a piperazine ring at position 7 to produce norfloxacin formed a cornerstone for the development of recent FQs. The majority of subsequent compounds contain a 4-oxo-1, 8-naphthyridin-3-carboxylic acid moiety. Compounds containing this structure are known as 4-quinolones due to the exocyclic oxygen at position 4. Two types of ring structures exist; a naphthyridone nucleus, containing a nitrogen atom at position 1 and 8 or a quinolone nucleus, containing only one nitrogen atom at position 1. Third generation compounds include clinafloxacin, moxofloxacin, gatifloxacin, levofloxacin and sparfloxacin. Trovafloxacin is a fourth generation compound and has increased streptococcal and

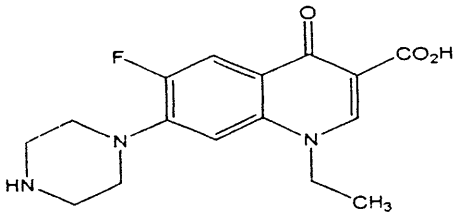
anaerobe coverage. Most licensed quinolones contain a quinolone nucleus while gemifloxacin, trovafloxacin, enofloxacin and sufloxacin contain a naphthyridone nucleus. Modification of the nucleus effects pharmokinetics, activity, drug interactions and adverse reactions. Subsequent compounds have a broader spectrum of activity are easily absorbed orally and can be used to treat systemic infections (Appelbaum & Hunter 2000).



Nalidixic acid
FIRST GENERATION

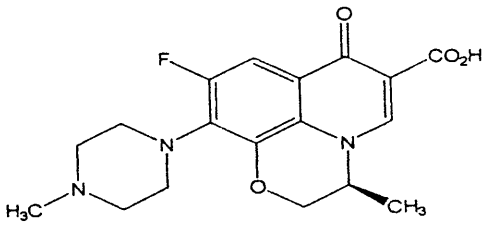


Ciprofloxacin

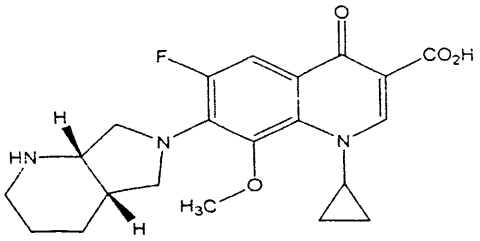


Norfloxacin

SECOND GENERATION



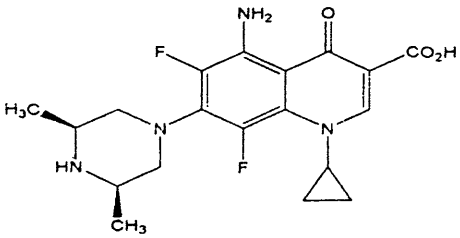
Levofloxacin



Sparfloxacin

floxacin

THIRD GENERATION



FOURTH GENERATION

Moxifloxacin

Figure 1.2 Development of 4th generation fluoroquinolones

The recent third and fourth generation FQ compounds have greater activity against Gram-positive cocci, anaerobes and atypical pathogens than the preceding compounds. Sitafloxacin and clinafloxacin, in particular, have improved activity against anaerobic bacteria (Tanlan 2001). However these compounds are not as active as ciprofloxacin against Gram-negative non fermenters. FQs can be administered orally once or twice daily and therefore are an attractive choice for treatment of infections in the hospital and the community. New FQs continue to be developed and moxifloxacin and gatifloxacin, two of the most recent, are used for treatment of respiratory tract infection, as they have activity against Gram-positive cocci, in particular *S. pneumoniae* (Tanlan 2001).

1.5.3 Mechanism of Action

FQs act by forming complexes with DNA gyrase and DNA, which consequently block cell growth. DNA gyrase passes a region of DNA through another and in doing so traps a quinolone/DNA/drug reaction intermediate. This blocks replication and produces double stranded breaks that are lethal to bacteria (Drlica 2003). DNA gyrase produces negative supercoils so that initiation proteins can bind to the origin of replication. It also relaxes positive supercoils that occur as the replication fork progresses. DNA gyrase is essential for the cell to efficiently process DNA. FQs bind to the DNA/gyrase complex and stabilise the strand breaks in the DNA. DNA gyrase relieves topological stresses encountered during DNA replication and supercoils DNA so that the genetic material can be packed into the bacterial cell by twisting DNA in the opposite direction to the normal turn of the molecule. DNA gyrase was discovered and characterised in 1976 (Gellert *et al.* 1976) and is a type II DNA topoisomerase that is composed of two A and two B subunits *gyrA* and *gyrB* respectively (Wang 1985). The A subunit is responsible for breakage and rejoining

of DNA while the B subunit is the site of ATP hydrolysis (Gellert *et al.* 1976). The Tyr-122 residue in the N terminal of GyrA is considered the active site of the quinolone resistance determining region. The protein can also catenate and decatenate covalently closed circular DNA (Drlica & Zhou 1997).

The primary function of topoisomerase IV is to decatenate daughter chromosomes following DNA replication. Topoisomerase IV, which is involved in chromosomal segregation shares significant homology with DNA gyrase and consists of 2 ParC and 2 ParE subunits, encoded by *parC* and *parE* which are homologous to *gyrA* and *gyrB* respectively. In *E. coli* the *gyrA* gene shares 60 % amino acid similarity with *parC* while *gyrB* has 62 % similarity with *parC* (Huang 1996; Kato *et al.* 1990; Peng & Marians 1993). Most bacteria contain genes encoding DNA gyrase and topoisomerase IV. However some bacteria e.g. *Treponema pallidum* lack genes encoding topoisomerase IV (Fraser *et al.* 1998).

1.5.4 Induction of the SOS Response

The SOS response is a post replication DNA repair system that is induced by DNA damage. The SOS response increases the dissemination of antibiotic genes. Beaber *et al* demonstrated that in *E. coli* induction of this response increases transfer of the *Vibrio cholerae* derived intregating conjugative factor SXT conferring resistance to chloramphenicol, sulphamethoxazole, trimethoprim and streptomycin and that this was also induced by exposure to ciprofloxacin (Beaber, Hochhut, & Waldor 2004).

Microarray technology has demonstrated that SOS genes are induced during exposure to fluoroquinolones in *E. coli*, *H. influenzae* and *S. typhimurium* (Piddock, Walters, & Diver 1990; Shaw *et al.* 2003; Ysern *et al.* 1990). Piddock *et al* have also used 2D gel electrophoresis to investigate the response of *S. enterica* exposed to fluoroquinolone at 0.5 and 2 x MIC. Exposure to FQs increased expression of the efflux pump AcrAB/TolC (Coldham *et al.* 2006).

1.5.5 Resistance Mechanisms

There has been a large increase in fluoroquinolone resistance since these drugs were introduced in the late 1980s due to extensive use in many organisms including *S. pneumonie*, *S. typhimurium* and *C. jejuni* (Aguiar *et al.* 1992; Chen *et al* 1999; Hakanen *et al* 1999; Kresken & Wiedemann 1988; Linares, de la Campa & Palla 1999; Tanaka *et al.* 2000) and this now restricts their use.

1.5.5.1 Target Alteration

Alteration in target genes and alterations that affect access of the drugs to targets e.g. increased expression of efflux pumps in Gram-negative bacteria, are the primary mechanisms of resistance to fluoroquinolones (Hooper 2003; Ruiz 2003). Bacteria become increasingly resistant to fluoroquinolones by accumulation of mutations in topoisomerase genes. This reduces the affinity of the drug for the targets and decreases susceptibility. These mutations occur in a stepwise manner, increasing the level of resistance with each successive mutation (Chen & Lo 2003; Hooper 2003). The result of this gradual increase in resistance may cause isolates that contain resistance mutations but are classed as

susceptible to be missed by resistance surveillance studies. Mutations in the quinolone resistance determining regions (QRDR) within topoisomerase II (GyrA and GyrB) and topoisomerase IV (ParC and ParE) confer resistance to FQs (Everett *et al.* 1996). In *E. coli* the primary target of FQs is DNA gyrase (Gellert *et al.* 1977). Additional mutations in *gyrA* and *parC* are required for high level fluoroquinolone resistance in *E. coli*, *A. baumannii, P. aeruginosa* and *S. maltophilia* (Mouneimne *et al.* 1999; Valdezate *et al.* 2002; Vila *et al.* 1995). Mutations in *parE* and *gyrB,* conferring a decrease in susceptibility, occur less commonly (Akasaka *et al.* 2001; Lee *et al.* 2005; Oh *et al.* 2003). Double mutations in topoisomerase II genes are associated with higher level resistance than individual mutations. The primary target of fluoroquinolones in *S. aureus* and other Gram-positive bacteria is topoisomerase IV (Drlica 2003; Ferrero *et al.* 1994; Ferrero, Cameron, & Crouzet 1995; Gellert *et al.* 1976; Gellert *et al.* 1977).

1.5.5.1.1 GyrA

Of the *gyrA* mutations that cause fluoroquinolone resistance *in vitro* in Gram-negative bacteria those that occur at codons 83 and 87 occur most frequently (Dessus-Babus *et al.* 1998; Mouneimne *et al.* 1999). Codon 83 and 87, found within the QRDR, encode amino acids which form an α helical domain in the DNA gyrase protein. These codons encode the DNA binding region of gyrase and therefore fluoroquinolones are less able to form a complex with the DNA and gyrase, when mutated. Double mutants containing two *gyrA* mutations have been described in *E. coli* (McDonald *et al.* 2001). There is a 10 fold reduction in affinity of the gyrase/DNA complex for ciprofloxacin in isolates which contain Ser83Ala Asp87Asn double mutation within *gyrA* (Barnard & Maxwell 2001). Other mutations between codon 67-106 can also confer resistance. Resistance mutations in

gyrA have been found outside the QRDR, such as Ala51Val but are less common (Yoshida *et al.* 1990). Alteration at codon 106 has been reported in *E. coli* and *P. aeruginosa* (Hallett & Maxwell 1991; Lee *et al.* 2005; Ruiz 2003). Mutations in *gyrA* are found more frequently than those in *gyrB* (Zhou *et al.* 2000). This can be explained by the larger MIC increases that result following *gyrA* mutation (Hooper 2003).

Mutations in *gyrA* have been detected by single strand conformation polymorphism (SSCP) analysis in a number of organisms (Takenouchi, Sakagawa, & Sugawara 1999; Vila *et al.* 1995). This technique utilises the altered migration in a nondenaturing polyacrylamide gel of isolates with changes in DNA conformation by nucleotide substitution to detect mutations (McHugh, 2000).

1.5.5.1.2 GyrB

Alterations in *gyrA* are reported more often than alterations in *gyrB*, this may be because *gyrB* mutations confer lower levels of resistance than *gyrA*. In *E. coli* common mutations are Asp426Val and Lys447Glu (Drlica & Malik 2003). Both result in decreased susceptibility to nalidixic acid but differ in extent of resistance conferred to FQ containing a piperazinyl moiety at position 7 (e.g. ciprofloxacin, norfloxacin). This may be due to electrostatic interactions between *gyrB* and drugs either containing or not containing the positively charged piperazinyl moiety (Yoshida *et al.* 1991). In *P. aeruginosa* substitution of Ser467 and Glu469 have been documented in norfloxacin resistant mutants selected *in vitro* (Kugelberg *et al* 2005).

1.5.5.1.3 ParC

Mutations in *parC* and *parE* in isolation do not confer resistance but contribute to further elevations in MIC when *gyrA* and *gyrB* mutations are also present (Ruiz 2003).

Common mutations are Ser80Phe/Tyr and Glu84Lys/Leu *E. coli* (Drlica 2003). ParC has a secondary role in fluoroquinolone resistance and mutations in *parC* or *parE* only cause resistance in *E. coli* in the presence of mutant *gyrA*. ParC mutations have been found in high level resistance in combination with mutations in *gyrA* in many Gram-negative bacteria including *E. coli* (Vila *et al.* 1996), *P. aeruginosa* (Kugelberg *et al.* 2005; Lee *et al.* 2005), *S. enterica* (Eaves *et al.* 2004), *Klebsiella pneumoniae* (Brisse *et al.* 1999), *Klebsiella oxytoca* (Brisse *et al.* 1999) and *Enterobacter aerogenes* (Brisse *et al.* 1999).

1.5.5.1.4 ParE

Mutations in *parE* have been found less commonly in clinical resistant isolates and mutants selected *in vitro* than mutations selected in the other topoisomerase genes but have not been evaluated as extensively as other mutations. Most mutations that have been reported are in Gram-positive bacteria. Few cases of *parE* mutations have been described in Gram-negative bacteria but include Ala425Val in *P. aeruginosa* (Kugelberg *et al.* 2005) and Leu445His in *E. coli* (Breines *et al.* 1997).

1.5.5.2 Efflux

Efflux is a major mechanism of fluoroquinolone resistance. Efflux mediated fluoroquinolone resistance confers resistance to other classes of antimicrobial with different structures e.g. chloramphenicol (Poole 2000).

1.5.5.3 Multiple Antimicrobial Resistance Phenotype (MAR)

Mutations in genes other than topoisomerase genes can have a role in the development of FQ resistance (Kern *et al.* 2000). For example mutations in regulatory genes can result in expression of the Multiple Antimicrobial Resistance Phenotype (MAR). This involves expression of porins and efflux pumps to confer chromosomally encoded low level cross resistance to FQs and other classes of antibiotics. These include β-lactams, tetracyclines, chloramphenicol (George & Levy 1983), agents causing oxidative stress (Ariza *et al.* 1994), disinfectants (McMurry, Oethinger, & Levy 1998; Moken, McMurry, & Levy 1997) and organic solvents (Asako *et al.* 1997). Mutation in *marA* increases expression of *micF*, which decreases expression of the OmpF porin and decreases permeability of the cell (Cohen, McMurry, & Levy 1988). The Mar operon has been identified in *E. coli* (Hachler, Cohen, & Levy 1991), *Klebsiella*, *Citrobacter*, *Hafnia*, *Enterobacter* (Cohen, Yan, & Levy 1993) and *S. enterica* (Randall & Woodward 2001).

Mutations in *acrR*, the repressor gene of the AcrAB multidrug efflux pump, also have a role in fluoroquinolone resistance in *E. coli* (Wang *et al.* 2001). If the *acrAB* is inactivated in *E. coli* strains that also contain topoisomerase mutations then all strains become susceptible (Oethinger *et al.* 2000).

1.5.5.4 Plasmid Mediated Resistance

The major mechanisms of FQ resistance are target alteration and overexpression of efflux pumps however plasmid mediated low level quinolone resistance has been described and was first documented in *K. pneumoniae* (Martinez-Martinez, Pascual, & Jacoby 1998). This gene, *qnr*, encoding a member of the pentapeptide repeat family, prevents fluoroquinolones from inhibiting DNA gyrase by binding to GyrA and GyrB before formation of the gyrase/DNA/quinolone complex. This has been hypothesised to reduce binding of gyrase to DNA thereby reducing the number of targets available to the fluoroquinolone molecules (Tran, Jacoby, & Hooper 2005). The mechanism by which this occurs is not known. The Qnr protein has similarity to gyrase protecting proteins, which protect gyrase from self produced microcin B17 (Garrido *et al.* 1988). Microcins are small inhibitory molecules that target DNA gyrase (Lomovskaya, Kawai, & Matin 1996). Before 2003 the *qnr* gene was thought to be a rare and had been found in one *E. coli* isolate, four *K. pneumoniae* isolates and one *Klebsiella* spp. isolate despite screening of 350 isolates of Gram-negative bacteria in the United States (Jacoby, Chow, & Waites 2003; Wang *et al.* 2003). However the *qnr* gene has been found in 78 unrelated strains of *E. coli* in a Shanghai hospital and subsequently in further *K. pneumoniae* strains in the United States (Wang *et al.* 2003; Wang *et al.* 2004). It has been postulated that these strains are now spreading across the United States (Wang *et al.* 2004). Plasmid mediated resistance may complement topoisomerase mutations and efflux mediated resistance by increasing the level at which resistance mutations can be selected. Other plasmid mediated quinolone resistance genes, *qnrB* and *qnrS*, have also been found in *K. pneumoniae* and *Shigella flexneri* respectively (Hata *et al.* 2005; Jacoby *et al.* 2006).

1.5.5.5 Inactivation

Degradation of quinolones by the brown rot fungus *Gloeophyllum striatum*, has been described (Wetzstein, Schmeer, & Karl 1997). In 2006 the first evidence for FQ inactivation by bacterial enzymes was presented. Robicsek *et al* unexpectedly found that a variant of a previously reported plasmid mediated aminoglycoside modifying enzyme could also modify ciprofloxacin and norfloxacin by N-acetylation of the amino nitrogen on the piperazinyl substituent. This conferred a small increase in resistance. Other FQ antibiotics were not affected (Robicsek *et al.* 2006).

1.6 The *Burkholderia cepacia* complex

1.6.1 Taxonomy of *Burkholderia*

The genus *Burkholderia* consists of non spore-forming, motile, aerobic, Gram-negative bacilli that are pathogens of animals, plants and humans. In 1992 *Pseudomonas cepacia* and six other pseudomonads (*Pseudomonas pseudomallei*, *Pseudomonas mallei*, *Pseudomonas solanacearum*, *Pseudomonas gladioli*, *Pseudomonas picketti* and *Pseudomonas caryophylli*) were transferred to the new genus of *Burkholderia* in the β-subdivision of the phylum *Proteobacteria* (Yabuuchi *et al.* 1992).

Following increased scientific interest, the genus *Burkholderia* now contains at least 40 species. The taxonomy of this group of organisms is shown in figure 1.3.

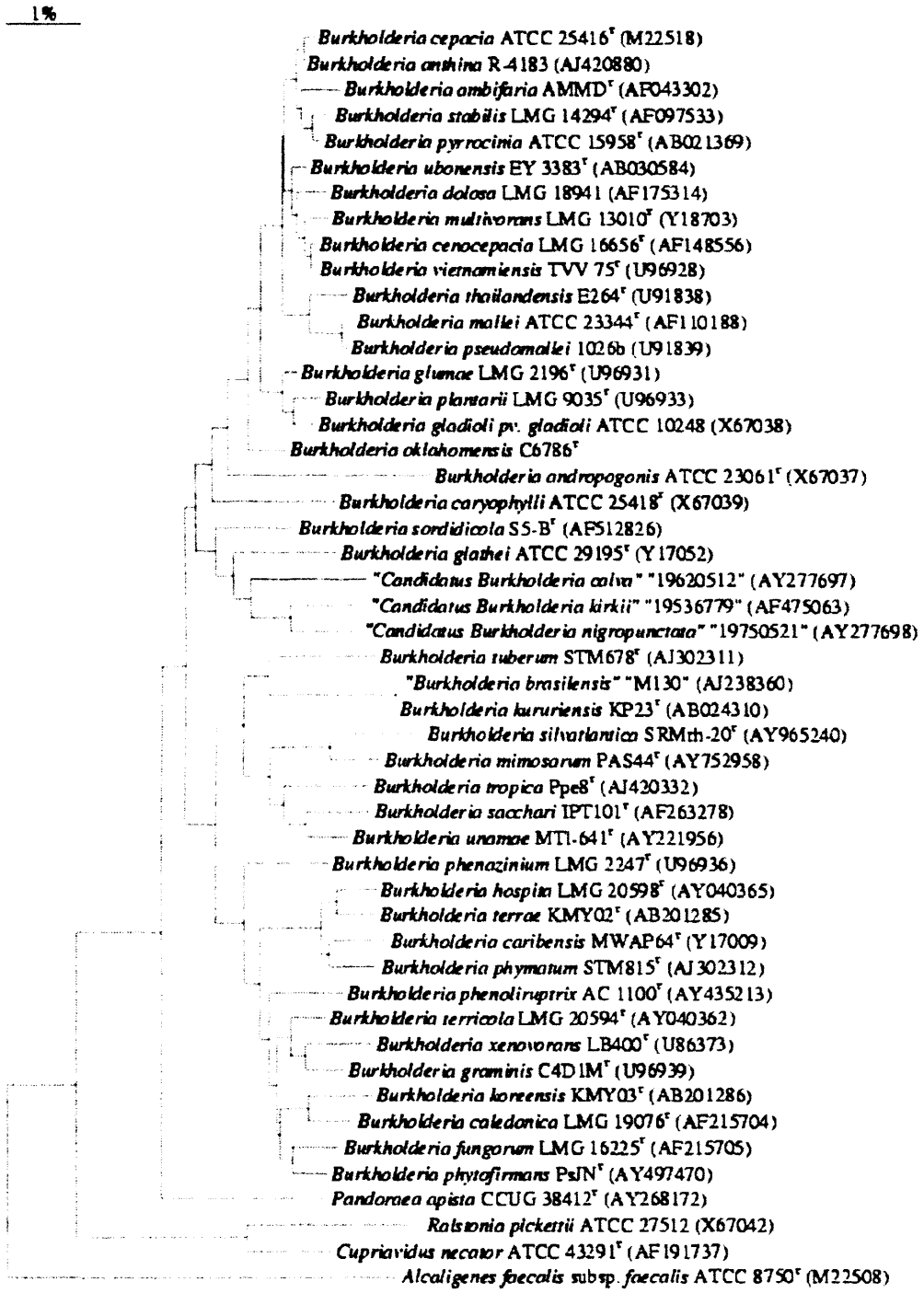


Figure 1.3 Phylogenetic tree of *Burkholderia* species. Based on 16S rRNA gene sequences. Scale bar represents dissimilarity. Species names in quotation marks have not been validated (Vandamme, Govan & LiPuma 2007).

1.6.2 The *Burkholderia cepacia* complex (Bcc)

The *Burkholderia cepacia* complex (Bcc) consists of at least 10 species (previously known as genomovars) that were originally thought to be a single species. However different groups of bacteria within this complex are genotypically heterogenous but are hard to differentiate on the basis of their phenotypic properties. In 1997, Vandamme *et al* proposed that these strains comprised five distinct genomovars (Coenye *et al.* 2001; Vandamme *et al.* 1997) that can be identified using genotypic and phenotypic methods (Vandamme *et al.* 1997). The complex now consists of 10 species; *B. cepacia* (genomovar I; Vandamme *et al.* 1997; Yabuuchi *et al.* 1992), *B. multivorans* (genomovar II; Vandamme *et al.* 1997), *B. cenocepacia* (genomovar III; Vandamme *et al.* 2003), *B. stabilis* (genomovar IV; Vandamme *et al.* 2000), *B. vietnamensis* (genomovar V; Gillis *et al.* 1995; Vandamme *et al.* 1997), *B. dolosa* (genomovar VI; Vermis *et al.* 2004), *B. ambifaria* (genomovar VII; Coenye *et al.* 2001), *B. anthinia* (genomovar VIII; Vandamme *et al.* 2002), *B. pyrrocinia* (genomovar IX; Vandamme *et al.* 2002) and *B. ubonensis* (genomovars X; Yabuuchi *et al* 2000). Genomovar I contains the type strain (the species chosen as the one that best represents the complex) and retains the name *B. cepacia.*

B. cepacia (formerly *Pseudomonas cepacia*) was first described by Burkholder as the causative agent of soft rot in onions (Burkholder 1950). In 1970 the similarity of a pseudomonad with metabolic versatility known as *P. multivorans* (Stanier & Doudoroff 1966) to the previously described *P. cepacia* was noted (Ballard R.W. *et al.* 1970). Other names that have been assigned in the past are the eugenic oxidider group I, *Pseudomonas kingii* (Jonsson 1970) and *Pseudomonas multivorans* (Stanier & Doudoroff 1966).

Vandamme *et al* proposed that within a species there is a high degree of DNA relatedness (70% or higher), low but significant DNA relatedness below the species level and no significant DNA relatedness (30%) or less (Vandamme *et al.* 1996) between unrelated species. This is demonstrated within *Burkholderia*. Different species within the Bcc have DNA-DNA hybridization values of 30-60%, isolates within the same species have a value of greater than 70%. Other *Burkholderia spp.* have a value of less than 30% (Coenye *et al.* 1999; Coenye *et al.* 2001; Gillis *et al.* 1995; Vandamme *et al.* 1997).

1.6.3 *Burkholderia pseudomallei* and *Burkholderia mallei*

Many species of the genus *Burkholderia* are primarily plant pathogens or saprophytes. However two species; *Burkholderia pseudomallei* and *Burkholderia mallei* can cause life threatening human disease in patients that are not immunocompromised. *B. pseudomallei* is a soil saprophyte, causing meliodosis in humans and is endemic in the Far East and northern Australia. Transmission occurs via exposure of wounds to contaminated water or soil or by inhalation. The most common clinical manifestation is septicaemia with abscess formation, although it is also a major cause of pneumonia (Chaowagul *et al.* 1989). Asymptomatic infections occur and progression to disease depends on the immune status of the host and can manifest many years after exposure (Kingston 1971).

B. mallei is the causative agent of glanders in equines and can cause disease in humans exposed to infected animals. *B. pseudomallei* and *B. mallei* are closely related and no differences were found in the 16S rRNA genes of the two species (Gee *et al.* 2003). Both *B. mallei* and *B. pseudomallei* are considered potential biological weapons (USA CDC, Category B; Rotz *et al.* 2002) due to their mode of transmission and worldwide availability of these agents in the environment.

1.6.4 Clinical Significance

1.6.4.1 Cystic Fibrosis (CF)

CF is an autosomal recessive disorder and is one of the most common genetic disorders in humans, affecting 1 in 2,500 live births in the Caucasian population (Ratjen & Doring 2003). CF results from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, found on the long arm of chromosome 7. This protein is a chloride ion channel protein and patients homozygous for the mutant allele have defective electrolyte transport across the epithelial cell membrane, resulting in the production of thick, dehydrated mucus in organs, especially in ducts of the male genital tract, pancreas and in the airways. These secretions impair mucocillary clearance leading to the formation of mucus plugs in the lungs and pancreas and progressive decline in organ function. This thick mucus layer provides a protected niche within the lungs, for bacteria such as Bcc and *P. aeruginosa.*

Over 1000 CFTR mutations have been identified that result in CF (Govan & Deretic 1996). These mutations may occur at different loci, resulting in a variation of CF phenotypes (Hart & Winstanley 2002). Over 70% of patients have a codon alteration resulting in loss of a phenylalanine residue at position 508 (ΔF-508) (Govan & Deretic 1996; Hart & Winstanley 2002). CF genotype appears to affect the type and frequency of bacterial infection. For example in patients with the ΔF508 mutation, there is increased likelihood of infection with *P. aeruginosa* and the Bcc. Patients wih the R117H mutation are less likely to be colonisied with these organisms (McManus *et al.* 2005).

Death often results following lung infection by a number of bacterial species. These include *P. aeruginosa*, *S. aureus*, *H. influenzae* and the Bcc. As patients are surviving

longer, other non-traditional pathogens are emerging. These include pathogens *Aspergillus* spp., *S. maltophilia*, MRSA, *Alcaligenes xylosoxidans, Achromobacter xylosoxidans*, *Klebsiella spp.*, *Pandoraea*, *Scedosprium apiospermum* (Nagano *et al.* 2007) and mycobacteria other than tuberculosis (MOTT; Devine *et al.* 2004; Jordan *et al.* 2007; Hart & Winstanley 2002). In a retrospective study of 54 adult CF patients *P. aeruginiosa* was present in 48%, *H. influenzae* in 34%, *Aspergillus* sp. in 7%, mycobacteria other than tuberculosis in 11%, *S. maltophilia* in 9%, *Acinetobacter* sp. in 7% and *B. cepacia* complex in 2% (Paschoal *et al.* 2007).

There is a continuing improvement in the survival of CF patients; a child born in 1990 with CF has a life expectancy of 40 years whereas a child born in 2000 with CF will have a life expectancy of approximately 50 years (Colten 1990; Dodge *et al.* 2007). Approximately 80% of CF patients now survive into adulthood (Elborn, Shale, & Britton 1991).

Members of the *Burkolderia cepacia* complex are opportunistic pathogens and rarely cause disease in immunocompetent hosts. Immunocompromised hosts, especially patients with CF are at risk from *B. cepacia* lung infection (Isles *et al.* 1984) via person to person transmission (Govan *et al.* 1993). Nosocomial outbreaks of Bcc bacteraemia have also been described in non CF patients (Holmes *et al* 1999; Bressler *et al* 2007). In CF patients the most common clinical scenarios are asymptomatic carriage following colonization of the airways, or chronic infection resulting in decline in lung function. However in approximately 20% of colonized individuals a rapidly progressing necrotizing pneumonia occurs that often leads to death (Govan & Deretic 1996). This outcome has been termed 'cepacia syndrome' (Isles *et al.* 1984) and is not observed with *P. aeruginosa* infection.

Eradication of *B. cepacia* occurs very rarely once a CF patient becomes colonized. Bcc bacteria are acquired late in the disease course, following colonization by *S. aureus*, non encapsulated *H. influenzae* and *P. aeruginosa. B. cepacia* complex infection in CF has been associated with lower life expectancy, longer hospital stay and a poor prognosis (Isles *et al.* 1984; Mahenthiralingam *et al.* 2001; Tablan *et al.* 1987b). Long term colonisation can occur in some CF patients with no decline in lung function (Gilligan 1991).

Colonisation of the CF lung by the Bcc is considered a contraindication to lung transplant by many transplant centres (Barlow *et al.* 2000). However lung transplant may be the final option for CF patients and can be beneficial. The survival rates for CF patients who have undergone lung transplants are 81%, 58% and 49% after 1, 3 and 5 years respectively (Aris *et al.* 2001). These survival rates are higher than those for CF patients with end stage disease that do not receive a lung transplant (Hosenpud *et al.* 1998). Reviews of the outcome following lung transplantation in CF patients demonstrated high mortality following lung transplantation in Bcc colonised CF patients (Chaparro *et al.* 2001; Snell *et al.* 1993). In contrast Egan *et al* reported that no significant difference in survival rates were observed between Bcc colonised and non colonised CF patients following transplantation (Egan *et al.* 1994). This group suggested that colonisation with Bcc alone was not sufficient to refuse lung transplantation.

1.6.4.2 Bcc species Distribution

B. multivorans and *B. cenocepacia* are the most commonly isolated species from CF patients, although *B. multivorans* is now the most prevalent in the UK (Mahenthiralingam,

Baldwin, & Vandamme 2002; Mahenthiralingam, Urban, & Goldberg 2005; Vandamme *et al.* 1997; Baldwin *et al* 2008). In contrast the prevalence of *B. cepacia*, *B. stabilis*, *B. anthina* and *B. pyrrocinia* (Mahenthiralingam, Baldwin, & Vandamme 2002; Vandamme *et al.* 2002) among CF patients is low, while *B. vietnamensis* and *B. ambifaria* are rarely found (Mahenthiralingam, Baldwin, & Vandamme 2002; Mahenthiralingam, Urban, & Goldberg 2005). Further studies to assess incidence of each species among CF patients have supported the initial observations regarding species prevalence (Agodi *et al.* 2001; LiPuma *et al.* 2001; Speert 2002). For example Speert *et al* examined 905 isolates from 447 CF patients in Canada and found that 83% of isolates were *B. cenocepacia* and 9.6% were *B. multivorans*. LiPuma *et al* demonstrated that 50% of Bcc isolates from CF patients in treatment centres across the United States were *B. cenocepacia*, 38% were *B. multivorans* and 5% were *B. vietnamensis*. The remaining 5% was comprised of all other species (LiPuma *et al.* 2001). Infection with *B. cenocepacia* is associated with a worse outcome than other species (Jones *et al* 2004) although the effect of the less commonly isolated species on prognosis is unclear.

1.6.4.3 Chronic Granulomatous Disease

Patients with chronic granulomatous disease are also at risk from infection with Bcc bacteria. These patients suffer recurrent, life threatening infections with catalase positive bacteria and fungi due to defects in oxidative killing in polymorphonuclear lymphocytes (Speert *et al.* 1994). Infection with Bcc is the second most common cause of death in this patient group, causing pneumonia and sepsis (Johnston, Jr. 2001).

1.6.5 Transmission

1.6.5.1 Environmental Transmission

Outbreaks of Bcc have been attributed to contamination of disinfectants, antiseptics, dyes, nebulizers and medical devices (Hutchinson *et al.* 1996; Kaitwatcharachai *et al.* 2000; Oie & Kamiya 1996; van Laer *et al.* 1998). The ability of bacteria to survive drying allows their maintenance on environmental surfaces (Smith, Eng, & Padberg, Jr. 1996) and the possibility of transmission between hosts. *B. cepacia* complex bacteria can survive for extended periods in respiratory droplets on surfaces at room temperature (Drabick *et al.* 1996). Bcc organisms were detected in air from patient rooms following physiotherapy and therefore this represents a risk for transmission to patients from the environment (Moore *et al* 2002).

1.6.5.2 Person to Person Transmission

Person to person infection was first documented in 1990 (LiPuma *et al.* 1990). However retrospective analysis of stored strains has shown that the first recorded case of patient to patient transmission of *Burkholderia cepacia* was in fact caused by a strain of *B. dolosa* (LiPuma *et al* 1990). Contact with respiratory secretions from CF patients infected with Bcc represents a risk for transmission (Moore *et al.* 2004). Highly transmissible epidemic lineages have been documented that have caused outbreaks. Social contact between CF patients has been associated with transmission at clinics and at Summer camp (Govan *et al.* 1993; Pegues *et al.* 1994). Segregation of colonised individuals ensued and CF Summer camps were closed. This has had an adverse psychological and social impact effect on CF patients and their families (Govan & Nelson 1993).

1.6.5.3 Transmissible Strains of the Bcc

Epidemic *B. cepacia*, *B. cenocepacia* and *B. dolosa* have been described (Biddick *et al.* 2003). However most cases of documented cases of transmission occur with *B. cenocepacia* (Mahenthiralingam, Baldwin, & Vandamme 2002). Infection with *B. cenocepacia* can replace *B. multivorans* during CF infection (Mahenthiralingam *et al.* 2001). Vandamme *et al* have demonstrated by analysis of the *recA* gene that there are 4 distinct *B. cenocepacia* lineages (Vandamme *et al* 2003).

B. cenocepacia ET-12 is the most studied transmissible Bcc strain which caused epidemics across Canada and the UK in the 1980s and 1990s. It is believed to have spread by person to person transmission (Clode *et al.* 2000; Ledson *et al.* 2002; Sun *et al.* 1995). This lineage was isolated from 50% of UK CF centres and from 38% of Bcc colonised patients (Pitt *et al.* 1996).

The enhanced capacity for spread of *B. cenocepacia* ET-12 is not fully understood. However this is the only strain that contains all three putative transmissibility markers, cable pili (Sajjan *et al.* 1995), the 22 kDa adhesin (Sajjan & Forstner 1993) and the *B. cepacia* epidemic strain marker (BCESM; Mahenthiralingam, Simpson, & Speert 1997). Cable pili are encoded by the *cblA* gene and mediate adherence to respiratory mucin (Sun *et al.* 1995). Although associated with epidemic strains of *B. cenocepacia* a variant form has been documented in *B. cepacia* (LiPuma *et al.* 2001). This lineage is highly resistant to antibacterials and is resistant to meropenem (Nzula, Vandamme, & Govan 2002).

The BCESM, otherwise known as *esmR*, is a 1.4 kb putative ORF with homology to negative transcriptional regulators (Mahenthiralingam, Simpson, & Speert 1997). The role of BCESM in transmissibility is unclear. Other epidemic *B. cenocepacia* lineages that are causing outbreaks in CF patients include the PDHC clone and the Midwest clone. The PDHC clone causes infection in the Mid Atlantic region of the USA (Chen *et al.* 2001) while the Midwest clone causes infection of CF patients in the Midwest region of the USA (LiPuma *et al.* 1988). These isolates described do not contain BCESM or the *cblA* gene (Chen *et al.* 2001). Isolates of the PHDC clone have been isolated form soil, suggesting that transmission of epidemic clones from the environment can occur (LiPuma *et al.* 2002).

Clinical outcomes following infection of CF patients by PHDC isolates and strains of the ET-12 lineage are variable. While a proportion of patients develop cepacia syndrome others are chronically infected with little effect on clinical status.

1.6.6 Identification

In the clinical laboratory, the Bcc are identified using a combination of selective media and biochemical tests. Bcc bacteria can be difficult to isolate in the clinical laboratory as these bacteria may be overgrown by other bacteria in a patient sample. The taxonomy of the Bcc is still changing and the potential of some of the newly recognized species to cause disease is not clear. There are variations in protocol for identification of Bcc between laboratories. Prior to the routine use of selective media only 32% of laboratories correctly identified *B. cepacia* from mock CF sputum (Tablan *et al.* 1987a). Members of the Bcc have been misidentified as a number of organisms including *Alcaligenes* spp,

Pseudomonas spp., *S. maltophilia*, *Flavobacterium* spp. and *Chryseobacterium* (Kiska *et al.* 1996). As some of these organisms can also cause disease in CF patients, the effects of these misidentifications can be serious. Identification of *B. cepacia* may be complicated as phenotypic changes can occur following long term colonization. Auxotrophic isolates of *P. aeruginosa* (Barth & Pitt 1995a), *S. aureus* (Gilligan *et al.* 1987) and *B. cepacia* (Barth & Pitt 1995b) have been found in the lungs of CF patients but not from non CF patients. Auxotrophic bacteria cannot synthesize compounds required for growth therefore these must be provided by the medium. Within the CF lung auxotrophic *B. cepacia* obtain necessary nutrients from sputum and are selected from the prototrophic population (Barth & Pitt 1995b). Colonies will grow on nutrient or blood agar and vary in appearance from grey, white and yellow to brown, red or purple.

Selective media are used to isolate Bcc bacteria from specimens as other bacteria are also likely to be present in greater numbers. The selective media employed make use of the high intrinsic resistance of the Bcc as other less resistant bacteria are killed. A number of selective agars are available that can be used to isolate *B. cepacia* from respiratory specimens. Oxidation-fermentation polymixin bacitracin lactose agar (OFPBL) contains lactose, polymixin and bacitracin. *B. cepacia* selective agar (BCSA) contains lactose, sucrose in a base of casein and yeast extract containing polymixin, gentamicin and vancomycin. Henry *et al* reported that this medium was superior to commercially available plates and the oxidation fermentation agar (Henry *et al.* 1997). *Pseudomonas cepacia* medium (PCA) contains polymixin and ticaricillin. However other Gram negative bacteria that are polymixin resistant are also able to grow on selective media. Further identification tests should follow.

Most species cannot be identified phenotypically. A number of Polymerase Chain Reaction (PCR) based assays were developed, that amplified the 16S gene, to identify Bcc. However the majority of these assays were developed before it was recognized that organisms identified as *B. cepacia*, consisted of a complex of species and utilized sequence data that may not have been representative of the whole complex (Campbell, III *et al.* 1995; Karpati & Jonasson 1996; O'Callaghan, Tanner, & Boulnois 1994). The usefulness of 16S rDNA gene sequencing for species identification is limited because there is a high degree of sequence similarity between 16s rRNA genes of Bcc and discrimination of all members of the Bcc is difficult (LiPuma *et al.* 1999). Amplification of the 16S rRNA gene followed by restriction enzyme mediated fragmentation produces a *Burkholderia* species specific RFLP banding pattern (Segonds *et al.* 1999). Mahenthiralingam used species specific primers that amplified the *recA* gene and a *recA* based RFLP approach, to identify the Bcc (Mahenthiralingam *et al.* 2000).

Other techniques that have been used to identify members of the Bcc include sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of whole cell proteins (Vandamme *et al.* 1997), amplified fragment length polymorphism (AFLP) fingerprint typing (Coenye *et al.* 1999) and whole cell fatty acid analysis (Vandamme *et al.* 1997).

Isolates survive well when stored in frozen tap water or as glycerol suspensions on beads. However viability is not maintained on plates or refrigerated slopes. The oxidase reaction is slow, nitrate is not reduced and members of the complex are lysine and ornithine decarboxylase positive.

1.6.7 Treatment

Bcc bacteria are highly resistant to many individual antibacterial agents and as a result are difficult to eradicate. Prognosis is improved if transmission can be prevented. These bacteria are intrinsically resistant to aminoglycosides due to low permeability of the outer membrane as a result of an unusual structure of the core oligosaccharide of LPS (Cox & Wilkinson 1991). Lipopolysaccharide is unusual in that the core oligosaccharide contains less phosphate or 3-deoxy-D-manno-oct-2-ulosonic acid than other Gram-negative bacteria (Cox & Wilkinson 1991). Strains also exhibit high levels of resistance to β-lactam antibiotics due to altered penicillin binding proteins and inducible chromosomal β-lactamases (Hancock 1998). The ability of *B. cepacia* to use pencillin G as a carbon source is a well recognised example of its intrinsic resistance (Beckman & Lessie 1979). Semisynthetic penicillins, carbapenems, fluoroquinolones, ceftazidime and trimethoprim sulfamethoxazole can have activity against Bcc (Lewin, Doherty, & Govan 1993; LiPuma 1998). Nzula *et al* investigated the antibiotic susceptibility patterns of 65 Bcc isolates including the *B. cepacia* complex strain panel (Mahenthiralingam *et al.* 2000) and *B. cenocepacia* isolates cultured from soil (Nzula, Vandamme, & Govan 2002). All strains were resistant to polymixin and colistin; this is unsurprising as incorporation of these antibiotics into agar plates is used to allow selective growth of Bcc bacteria isolated from clinical samples. Most strains were resistant to tetracycline and all strains were susceptible to meropenem. Most strains were susceptible to ciprofloxacin (88%) and ceftazidime (97%; Nzula, Vandamme, & Govan 2002).

CLSI (Clinical and Laboratory Standards Institute) recommends susceptibility testing to ticaricillin plus clavulanic acid, ceftazidine, levofloxacin, minocycline, meropenem and trimethoprim-sulfamethoxazole (CLSI, 2006) as these antibacterials can have activity

against the Bcc. Treatment options include meropenem, ciprofloxacin, minocycline, chloramphenicol and trimethoprim-sulfamethoxazole.

Two agents are recommended for exacerbations of CF (Gibson, Burns, & Ramsey 2003). Double antibiotic combinations have more effect and are therefore used as treatment (LiPuma 1998). Combinations including meropenem are particularly effective but combinations including co-trimoxazole, tetracyclines and chloramphenicol also exhibit activity.

1.6.8 Immunity

In the lungs of cystic fibrosis patients inflammation and deterioration is characteristic due to infiltration of inflammatory cells, primarily neutrophils and production of cytokines found in bronchial lavage samples of these patients. Compared to non CF patients lung inflammation is increased with an elevated neutrophil response in CF patients (Hendry *et al.* 1999). The response to infection with Bcc bacteria within the CF lung is undefined. However certain virulence factors have been implicated. These include LPS and flagellin.

Differences in the abilities of Bcc isolates to cause inflammation have been observed. *B. cenocepacia* isolates have the greatest propensity to cause inflammation through release of TNF and IL-1. Within isolates of the ET lineage, the potential of whole cell lysates to induce cytokine production from human monocytic cells has been shown to vary between strains (De Soyza *et al* 2004).

It has been demonstrated that the supernatants from strains of *B. cepacia*, stimulate release of interleukin-8 (IL-8) from lung epithelial cells and peripheral blood monocytes (Palfreyman *et al.* 1997). This has been shown to be due to flagellin of *B. cepacia* interacting through a Toll-like receptor to initiate a signalling cascade that results in IL-8 secretion and results in inflammation of the lung (Urban *et al.* 2004).

B. cenocepacia LPS produces increased amounts of TNFα and IL-6 compared to *B. multivorans* (Shaw *et al* 1995). Differences within the lipid A portion of LPS are likely to account for the differences in cytokine production as this component is the main determinant of induction of inflammation (Khan *et al* 1998). Cytokine induction from *B. cepacia* LPS is nine times greater than from *P. aeruginosa* LPS.

The genomes of *Burkholderia* spp. contain large numbers of these CpG motifs because of the large genome size (Coenye & Vandamme 2005). It has been shown that bacterial DNA containing unmethylated CpG motifs stimulate lymphocytes to produce an inflammatory response (Schwartz *et al.* 1997). It is likely, therefore, that CpG rich *Burkholderia* DNA can initiate an inflammatory response in the host, although it is not clear how this contributes to inflammation observed in CF patients.

1.6.9 Resistance to human antimicrobial peptides

Cationic antimicrobial peptides are produced by all organisms. β defensins 1 and 2 have an important role in the innate immune system and have antibacterial action against Gram-negative bacteria but not against Gram-positive bacteria (Raj & Dentino 2002; Schroder 1999). Genes encoding β defensins 1 and 2 are expressed throughout the respiratory

epithelia. However the bactericidal activity of these defensins is thought to be inactivated by the high salt concentrations that are found in the CF lung (Goldman *et al.* 1997). Human β defensin (hBD-3) is active against both Gram-negative and Gram-positive bacteria and is not inactivated by high concentrations of salt. However *Burkholderia* spp. are unusual in that isolates are resistant to β defensins 1, 2 and 3 (Baird *et al.* 1999; Sahly *et al.* 2003).

The protegrins are also antimicrobial peptides. Protegin-1 is bactericidal, is not inactivated by high salt concentration and has a broad spectrum of activity (Harwig *et al.* 1996; Steinberg *et al.* 1997). Five isolates of *B. cenocepacia* investigated were more resistant to the bactericidal activity of protegrin-1 than *P. aeruginosa*. The increased resistance may be due to the reduced number of protegrin binding sites on the lipid A moiety of the LPS (Albrecht *et al.* 2002).

1.6.10 Genome

Members of the Bcc complex have large and unusual genomes which allow ecological and nutritional diversity. The genome sizes range from 6MB to 9MB and have a GC content of approximately 67%, a similar proportion to *Pseudomonas* spp. (Yabuuchi *et al.* 1992). The genomes are unusual in that they contain more than one chromosome and contain many insertion sequences and genomic islands (Mahenthiralingam, Urban, & Goldberg 2005). The number of chromosomes and genome size can vary between strains of the same species (Parke 2001). Nine *Burkholderia* genomes have been sequenced. These completed genomes represent strains of *B. pseudomallei*, *B. mallei*, *B. thailandensis*, *B. xenovorans*, *B. cenocepacia*, *B. cepacia* complex Group K and *B. dolosa*. The genome

sequences of strains of *B. pseudomallei*, *B. mallei* (Holden *et al.* 2004; Nierman *et al.* 2004) have been published. *B. cenocepacia* J2315 is currently being annotated.

Bacteriophages with interspecies host ranges have been found in soil and could contribute to genetic transfer within the Bcc (Langley *et al.* 2003).

1.6.11 Virulence of the Bcc

Members of the Bcc exhibit a number of virulence determinants. These include siderophore production (Sokol *et al.* 1999; Visser *et al.* 2004), expression of flagellar proteins (Hales *et al.* 1998; Urban *et al.* 2004), biofilm formation (Conway, Venu, & Speert 2002), quorum sensing (Venturi *et al.* 2004; Sokol *et al* 2003), type III secretion (Glendinning *et al.* 2004; Tomich *et al.* 2003) and production of extracellular proteases (Corbett *et al.* 2003).

1.6.11.1 Invasion

There is evidence that *B. cepacia* can invade respiratory epithelial cells (Burns *et al.* 1996) and pulmonary macrophages to evade the immune response of immunocompromised patients. Using a modified protection assay, Burns *et al* demonstrated that invasion of a human alveolar epithelial carcinoma cell line (A549) monolayer occurs (Burns *et al.* 1996).

The ET strain J2315 causes apoptosis via production of a haemolytic toxin in macrophages (Hutchison, Poxton, & Govan 1998). Bcc bacteria express one of two types of flagellin which can be distinguished by size (55 kDa and 45 kDa) and by RFLP analysis

of the *fliC* gene (Hales *et al.* 1998). Non motile J2315 strains are deficient in the ability to adhere to and invade epithelial cells *in vitro* (Tomich *et al.* 2003).

1.6.11.2 Quorum Sensing

At present members of the *B. cepacia* complex are known to contain at least two quorum sensing systems. The first pathway identified was the *CepIR* system, a homologue of the lasIR/systems of *P. aeruginosa* (Lewenza *et al.* 1999) and is widely distributed throughout species of the Bcc (Lutter *et al.* 2001). Analysis of gene expression of this system controls expression of virulence factors including production of chitinase, proteases and siderophores, swarming motility and maturation of biofilm (Aguilar *et al.* 2003; Huber *et al.* 2001; Lewenza *et al.* 1999; Lewenza & Sokol 2001). Mutations in these genes cause decreased virulence in infection models including killing of *Caenorhabditis elegans* (Kothe *et al.* 2003; Lewenza *et al.* 1999; Sokol *et al.* 2003), infection of rats and maceration of onion tissue (Aguilar, Bertani, & Venturi 2003). The autoinducer synthase is encoded the *cepI* gene and is responsible for the production of the two signalling molecules, *N*-hexanoyl-acylhomoserine lactone (C_6-HSL) and *N*-octanoyl-acylhomoserine lactone (C_8-HSL). The increase in cell density and signal concentration allows CepR to control expression of target genes. The second quorum sensing system was found within a pathogenicity island carried by epidemic strains of *B. cenocepacia*, designated *cciIR*, during random polymorphic DNA typing (Baldwin *et al.* 2004). The *cciI* gene is responsible for production of the same two signalling molecules as the *cepIR* system, *N*-hexanoyl-acylhomoserine lactone (C_6-HSL) and small amounts of *N*-octanoyl-acylhomoserine lactone (C_8-HS). Mutants in *cciI* demonstrated reduced protease production and swarming motility (Malott *et al.* 2005). In general Bcc bacteria produce two signalling molecules, N-octanoylhomoserinelactone (C_8-HSL) and N-

hexanoylhomoserine lactone (C_6-HSL). Some strains of *B. vietnamiensis* produce other long chain AHL molecules. The most common of the long chain AHL molecules was confirmed as N-decanoylhomoserine lactone (C_{10}-HSL).

1.6.11.3 Exopolysaccharide

Production of exopolysaccharide (EPS) has a role in biofilm formation in *P. aeruginosa* (Hoyle, Williams, & Costerton 1993). Overproduction of the polysaccharide alginate in the lungs affects long term survival of CF patients infected by *P. aeruginosa* (Govan & Deretic 1996). Approximately 80-90 % of CF Bcc isolates at a Portuguese centre were found to produce the polysaccharide cepacian (Richau *et al.* 2000), suggesting a role in the pathogenesis of Bcc in the CF lung. Cepacian consists of a branched acetylated heptasaccharide repeating unit made up of D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid (Sist *et al.* 2003). Random plasposon insertion mutagenesis was used to create mutants defective in EPS production and these mutants were unable to form thick biofilms. It is likely that cepacian is required for development of mature biofilms (Cunha *et al.* 2004).

1.6.11.4 Proteases

Between 69 and 88 % of clinical Bcc isolates produce extracellular proteases (Gessner & Mortensen 1990; Gilligan 1991; McKevitt & Woods 1984; Nakazawa, Yamada, & Ishibashi 1987). Gotschlich *et al* have reported that strains of *B. cepacia*, *B. cenocepacia* and *B. stabilis* have been shown to have extracellular protease activity while *B. multivorans*, *B. dolosa* and *B. vietnamensis* do not (Gotschlich *et al.* 2001). Two metalloproteases have been described in *B. cepacia* (McKevitt *et al.* 1989).

The 36 kDa protease, described in *B. cenocepacia*, has been identified as a zinc metalloprotease (ZmpA; McKevitt *et al.* 1989). This protease had been shown to not cleave elastin (McKevitt & Woods 1984), unlike *P. aeruginosa* which can cleave elastin. Purified protease causes bronchopneumonia in rats with polymorphonuclear leukocyte infiltration and airway exudate. Sokol *et al* demonstrated that zinc metalloprotease peptides given as vaccines offer some protection against lung damage in lungs of rats (Sokol *et al.* 2000). At 10^9 CFU or higher, the ZymA protease is lethal in mice (Gonzalez & Vidaver 1979). Nonproteolytic strains colonised skin of burned mice and persisted at 10^7 - 10^9 CFU for 3 weeks after injection of 10^5 CFU. No organ invasion was observed (Stover, Drake, & Montie 1983). A second metalloprotease (ZmpB) has recently been identified in Bcc that can cleave immunoglobulins, lactoferrins and transferrin while ZmpA can not (Kooi *et al.* 2006).

1.6.11.5 Type III Secretion

Type III secretion is a mechanism by which Gram-negative bacteria translocate proteins from the bacterial cytoplasm into the host cell cytoplasm. When inside the host cell, these effector proteins subvert the host cell machinery to the advantage of the bacterium. Type III secretion dependent delivery of effector proteins is associated with invasion with *S. typhimurium* (Collazo & Galan 1996), lesion formation by enteropathogenic *E. coli* (Jarvis *et al.* 1995), cytotoxicity caused by *P. aeruginosa* (Finck-Barbancon *et al.* 1997), evasion of phagocytosis by *Yersinia* (Cornelis & Wolf-Watz 1997) and intracellular survival by *S. enterica* (Cirillo et al. 1998).

Burkholderia spp. possess TTS machinery. *B. pseudomallei* (Rainbow, Hart, & Winstanley 2002; Stevens *et al.* 2002) and *B. mallei* (Ulrich & DeShazer 2004) contain putative TTS gene clusters. TTS systems are present in all Bcc species, except *B. cepacia* (Parsons *et al.* 2001). A TTS mutant of *B. cenocepacia* J2315 was attenuated in a murine model, it is therefore likely that TTS have a role in virulence of the Bcc (Tomich *et al.* 2003).

1.6.11.6 Siderophores

Iron is required as a co-factor for redox dependent enzymes in bacteria. However in many environments the concentration of soluble iron is insufficient to allow bacterial growth (Ratledge & Dover 2000). Siderophores are small molecules with high affinity for iron that compete with the host proteins to chelate iron molecules. Siderophore chelated iron can then be recognized by specific cell receptors and taken up by the cell, where iron is released. Bcc bacteria produce four types of siderophores; pyochelin, salicyclic acid, cepabactin and ornibactin (Meyer, Hohnadel, & Halle 1989; Sokol 1986; Sokol, Lewis, & Dennis 1992; Stephan *et al.* 1993; Visca *et al.* 1993). In a study by Sokol *et al* pyochelin producers were associated with severe pulmonary disease while pyochelin negative strains were associated with mild disease (Sokol 1986). Addition of pyochelin to a pyochelin negative strain increased virulence in the rat lung model (Sokol & Woods 1988). Ornibactins were found to be the most frequently produced siderophore in a study of 61 Bcc isolates (Darling *et al.* 1998).

1.6.11.7 Virulence Models

Animal models have been used to elucidate roles of virulence factors. However alternative models are required as using animal models is time consuming, labour intensive and expensive. Alternative model systems that have been described in other species, include *Arabidopsis thaliana* (Rahme *et al.* 1995; Rahme *et al.* 1997; Rahme *et al.* 2000), *C. elegans* (Tan & Ausubel 2000; Tan, Mahajan-Miklos, & Ausubel 1999), *Drosophila melanogaster* (D'Argenio *et al.* 2001) and *Galleria mellonella* (Jander, Rahme, & Ausubel 2000). Alfalfa has been used successfully to measure virulence in Bcc (Bernier *et al.* 2003).

One of the most commonly used animal models used for studying Bcc lung infections is the mouse agar bead model. This involves intratracheal inoculation of mice with an agar bead containing 10^5 CFU organisms (Cieri *et al.* 2002). Intranasal or intraperitoneal administration of Bcc to immunosupressed mice are also used (Chu *et al.* 2002; Speert *et al.* 1999).

1.6.12 Use in Agriculture

Bcc bacteria are some of the most common culturable microorganisms in the plant rhizosphere (Tsuchiya *et al.* 1995). Some members of the genus are also bio-degraders of chlororganic pesticides and polychlorinated biphenyls. *Burkholderia* species have been used in agriculture as biodegraders and plant-growth-promoting rhizobacteria. The risks of transmission to immunocompromised patients are as yet unclear (Govan & Vandamme 1998). The production of antibiotics can control soil borne plant pathogens. Bcc bacteria

have been used to prevent damping off disease caused by *Phythium* sp., *Rhizoctania solani* and *Fusarium* sp. (Parke & Gurian-Sherman 2001). This offers an alternative to treatment to fungicides, which have adverse effects on the environment and human health. Strains of *B. vietnamensis* and *B. ambifaria* are favoured as biopesticides as these species are not commonly isolated from CF patients (Parke & Gurian-Sherman 2001). Isolates of *B. vietnamensis* are more susceptible to ceftazidime compared to isolates of other species (Nzula, Vandamme, & Govan 2002). *B. vietnamensis*, therefore, may be the most appropriate species for use as a bio-control agent. However the risk to CF patients of using strains in this way is unclear.

1.7 Aims of thesis

The aims of this thesis were to investigate the evolution of fluoroquinolone resistance in *Burkholderia cepacia* complex bacteria. Fluoroquinolone antibiotic use is increasing and Bcc bacteria can be susceptible to this drug class. A method for estimation of mutation rate in topoisomerase genes was standardised and is described in chapter 3. Fluoroquinolone resistant *B. cepacia*, containing single and double topoisomerase mutations, were selected *in vitro* and characterised. Acquisition of resistance mutations may or may not incur a fitness cost and the extent of this cost may affect the ability of resistant bacteria to survive in the bacterial population. Fitness costs may be ameliorated by reversions or compensatory mutations that restore reproduction potential. Models, relevant to the transmission of *B. cepacia*, were used to assess the fitness cost of these characterised topoisomerase mutations and described in chapter 5. A method for quantifying biofilm formation is described in chapter 4. Application of tools developed in this thesis, have been used to investigate clinical isolates in chapter 6. Methods of detecting hypermutability of clinical *B. cepacia* complex bacteria isolated from CF patients isolates are described, also in chapter 6.

Chapter 2 Materials and Methods

2.0 General Materials and Methods

2.1 Culture Conditions

To ensure that strains did not undergo further mutation all strains and antibiotic resistant mutants were stored at -70°C using the Microbank system, consisting of cryovials containing beads and cryopreservative solution (Pro-lab Diagnostics, Neston, UK). All FQ resistant mutants were derived from the NCTC 10661 *B. cepacia* strain. All clinical isolates were isolated from adult cystic fibrosis patients with well characterised infection attending a CF clinic at Belfast City Hospital, Northern Ireland (kindly provided by Dr J.E. Moore; Table 2.1).

To culture the strain, a bead was inoculated onto a Columbia blood agar plate (Oxoid, Basingstoke, UK), spread with a disposable loop and incubated at 37°C for 18 hours.

Isolate	Source
NCTC 10661	National Type Culture Collection, Health Protection Agency, Colindale
BCH 1	Belfast City Hospital
BCH 2	Belfast City Hospital
BCH 3	Belfast City Hospital
BCH 4	Belfast City Hospital
BCH 5	Belfast City Hospital
BCH 6	Belfast City Hospital
BCH 7	Belfast City Hospital
BCH 8	Belfast City Hospital

Table 2.1 Sources of *B. cepacia* isolates

2.2 Preparation of Media

2.2.1 Muller Hinton broth

22 g Muller Hinton broth powder (BD, Le Pont de Claix, France) was dissolved in 1 L distilled water and autoclaved, according to the manufacturer's instructions.

2.2.2 Luria-Bertani (LB) broth

25 g of Luria Bertani broth powder was dissolved in distilled water and autoclaved, according to the manufacturer's instructions.

2.2.3 Muller Hinton agar

38 g Muller Hinton agar powder (BD, Le Pont de Claix, France) was dissolved in 1 L distilled water and autoclaved, according to the manufacturer's instructions. The agar was allowed to equilibrate to 50°C in a water bath. 20 mL of liquid media was poured into sterile disposable Petri dishes (Sterilin, Supplied by Western Laboratory Service, Aldershot, Hampshire, UK) using sterile technique. Plates were allowed to set and stored upside down in plastic bags at 4°C. Plates were dried before use at 37°C for 15 min.

2.2.4 Commercially Available Agar Plates

Ready prepared Nutrient agar, Columbia agar with horse blood and Isosensitest agar plates (Oxoid, Hampshire, UK) were used.

2.3 Preparation of Buffers and Solutions

2.3.1 1M Tris

121.1 g Tris base (Promega, Hampshire, UK), 42 mL of concentrated HCL stock was dissolved in 1 L of distilled water and adjusted to pH 8.0.

2.3.2 0.5 M EDTA

1.86 g EDTA disodium salt was dissolved in 800 mL distilled water and adjusted to pH 8.0 with NaOH (Sigma Aldrich, Steinheim, Germany) and stirred vigorously.

2.3.3 Tris-Borate EDTA (TBE) buffer

A 5 x solution was prepared by mixing of 54 g Tris base (Promega, Hampshire, UK), 27.5 g boric acid (BDH, Leicestershire, UK) and 20 mL 0.5M EDTA pH 8.0 in 1 L of distilled water. This was dissolved using a magnetic hot plate stirrer and flea.

2.3.4 5M NaCl

146.1 g sodium chloride (VWR International Ltd., Poole, UK) was dissolved in 500 mL distilled water.

2.3.5 Phosphate Buffered Saline (PBS)

1 x PBS solution was prepared by dissolving 1 PBS tablet (BDH, Leicestershire, UK) in 100 mL distilled water. PBS was then autoclaved.

2.3.6 Ciprofloxacin

0.025 g ciprofloxacin powder (98.4 % purity) (CellGro, Herndon, Virginia, USA) was dissolved in 24.61 mL sterile distilled water (SDW) to produce a 1000 mg/L stock solution. 1 mL aliquots were stored for later use at -70°C for no more than 4 weeks.

2.3.7 Clinafloxacin

0.025 g clinafloxacin powder (98%) (Sequoia Research Products, Pangbourne, UK) was dissolved in 24.5 mL SDW to produce a 1000 mg/L stock solution. 1 mL aliquots were stored for later use at -70°C for no more than 4 weeks.

2.4 Growth Curve

A 25 mL conical flask containing 5 mL of Muller Hinton broth was inoculated with 100 µl of an overnight Muller Hinton broth culture and sealed with a cotton wool bung. This was incubated at 37°C in an orbital shaker (200 r.p.m) (Barloworld Scientific, Staffordshire, UK). Samples (0.5 mL) were removed aseptically at 30 min. intervals

2.5 Miles and Misra Viable Cell Count (Miles & Misra 1938)

Muller Hinton agar plates were dried at 37°C for 15 min. prior to inoculation. 100 µl of Muller Hinton broth culture was diluted in 900 µl PBS, this was then vortexed and used to produce a bacterial dilution series (10^{-1} to 10^{-6}). Each dilution was vortexed

briefly and three replicate 20 μL volumes of diluted broth culture were spotted onto three segments of blood agar plates from approximately 1 cm above the surface of the plate. Plates were then incubated at room temperature for 30 min. to allow the drops to soak into the agar and incubated at 37°C overnight. Colonies were counted using the dilution that yielded between approximately 20 and 40 colonies. The mean number of colonies used to calculate the number of colony forming units per mL in the neat broth culture.

2.6 Determination of Minimum Inhibitory Concentration (MIC)

2.6.1 E-test

Minimum Inhibitory Concentrations (MIC) of parent and mutant strains were determined by E-test (AB Biodisk, Solna, Sweden), following the manufacturer's guidelines. Organisms were suspended in 3 mL sterile distilled water to a turbidity of 0.5 MacFarland. A cotton wool swab was immersed into this suspension and excess fluid removed and was swabbed three ways across an Isosensitest plate (Oxoid, Basingstoke, UK) an E-test strip was applied and the plate incubated (37°C, 18 hours) to obtain semi-confluent growth. Results were interpreted by recording the point of intersection between the ellipse of inhibition and the strip.

2.6.2 Agar Dilution

MIC was determined according to the CLSI guidelines for susceptibility testing of aerobic organisms (CLSI, 2006).

For the agar incorporation method 1 mL of ciprofloxacin of a range of concentrations was added to 19 mL of molten agar. Approximately 10^4 organisms were spotted onto the surface of an agar plate using a multi prong inoculator. The lowest dilution that completely inhibited growth was recorded as the MIC.

2.6.3 Method for determination of mutation rate by the method of the median.

Isolates were removed from the -70°C freezer and one bead was used to sub culture *B. cepacia* onto a blood agar plate and incubated aerobically at 37°C. One colony of Bcc was suspended in 5 mL of Muller Hinton Broth in a 25 mL conical flask. This was sealed with a cotton wool bung and incubated at 37°C on a rotary shaker (200 r.p.m.) (Barloworld Scientific, Staffordshire, UK) for 2.5 hours until an optical density (OD_{600}) of approximately 0.1 OD units was reached (ensured that 100 µL of the 10^{-3} dilution of this culture would contain approximately 10^3 cells. Serial dilutions of this broth culture were performed in PBS (neat to 10^{-6}). A 100 µL aliquot of the 10^{-3} dilution (containing approximately 10^3 cells) was added to each of 28 microcentrifuge tubes, containing Muller Hinton Broth (1 mL). These cultures were incubated at 37°C, 200 r.p.m 18 hours (Barloworld Scientific, Staffordshire, UK). Muller Hinton Agar plates (Oxoid, Basingstoke) containing 2 x MIC of ciprofloxacin were prepared and inoculated the following day. Antibiotic free agar plates were also prepared (section 2.2.3). Before inoculation, plates were allowed to dry at 37°C with the lids removed for 20 min. This ensured that drops were absorbed by the agar. At the end of the incubation period, a Miles and Misra plate count was performed on 3 randomly selected broths, section 2.5 (Miles 1938). Dilutions of 10^{-1} to 10^{-6} were prepared. Twenty microlitres of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spotted in triplicate onto duplicate drug free Muller Hinton plates.

The microcentrifuge tubes were centrifuged at 4000 *g*, 3 min. and the supernatant discarded. Using a fine tipped pastette, the deposit was resuspended in residual broth (approximately 40 µL). The total volume was then inoculated onto ciprofloxacin containing Muller Hinton plates, spread with a plastic spreader and allowed to dry. All plates were incubated (37°C, 48 hours). Colony forming units per mL were calculated from the Miles and Misra drug free plates. The number of visible colonies on each plate were counted by eye and recorded. The median number of colonies was determined. The number of mutations per culture was calculated using the following equation 4.

$$\bar{r}/m\text{-}\ln(m) - 1.24 = 0 \qquad \text{[Eq 4]}$$

The mutation rate (μ) was calculated using Equation 5.

$$\mu = \frac{m}{\text{average cfu/mL}} \qquad \text{[Eq 5]}$$

This experiment was repeated 4 times to give a median mutation rate.

2.6.4 Detection of Efflux

The ciprofloxacin MIC of the fluoroquinolone resistant strains was determined in the absence and presence of reserpine (25 mg/L) in Muller Hinton agar (Beyer *et al.* 2000). Reserpine (25 mg) was dissolved in 250 µl chloroform and immediately added to 1 L molten agar. Molten agar containing reserpine was mixed by agitation. Isolates were considered positive for reserpine inhibited efflux if there was a fourfold or greater decrease in the ciprofloxacin MIC in the presence of reserpine (Sigma

Chemical Co., St. Louis, Mo., USA). The addition of the 250 µl chloroform alone did not affect MIC.

2.7 DNA Extraction

2.7.1 Crude Extraction

A loopful of *B. cepacia* was emulsified in 0.5 mL PCR grade water and heated at 95°C for 10 min. Cellular debris was removed by centrifugation (3 min., 8000 *g*). Supernatant was removed, using a fine tipped pastette, and transferred to a clean microcentrifuge tube.

2.7.2 DNA Extraction

DNA was extracted using the Promega Wizard DNA extraction kit (Promega, Hampshire, UK), following the manufacturers' guidelines. 1 mL of an overnight culture was centrifuged in a microcentrifuge tubes at 16,000 *g* for 2 min. and the supernatant discarded. Cells were resuspended in 600 µL of lysis buffer and mixed by pipetting. Tubes were incubated at 80°C for 5 min. and cooled to room temperature. 3 µL of RNAse solution was added and the tubes were incubated at 37°C for 15-30 min. and cooled to room temperature. 200 µL of protein precipitation solution was added and the tubes vortexed for 20 seconds and incubated on ice for 5 min. Cell debris was precipitated by centrifugation at 16,000 *g* for 3 min. and the supernatant transferred to a fresh microcentrifuge tube containing 600 µL of room temperature isopropanol (BDH, Leicestershire, UK). The suspension was mixed by gentle inversion until strands of DNA could be seen. Microcentrifuge tubes were centrifuged for 10 min. and the pellet washed in 70 % ethanol and dried for 15 min. at room temperature. 100

μL of DNA rehydration solution was added and the tubes were incubated at 65°C in a water bath for 60 min, mixing by inversion every 20 min. Extracted DNA was stored at 4°C for no longer than 1 week.

2.8 Polymerase Chain Reaction and sequence analysis

2.8.1 Polymerase chain reaction (PCR)

PCR primers were rehydrated and diluted with PCR quality water (Royal Free Hospital Pharmacy) to 100 mM stock solutions and stored at -70°C. PCR water was exposed to UV light, in 1 mL aliquots, for 10 min. to eliminate DNA contamination. Working primer stocks were diluted to 10 mM and stored at 4°C. Bacterial DNA was diluted 1:10 in PCR quality water and 10 μL was added to each PCR reaction. This represented approximately 40 ng of DNA. The PCR mastermix contained 1.5 mM MgCl in KCL buffer (Bioline, London, UK), 10 μL of each primer at a 1 μM concentration (Sigma Aldrich, Steinham, Germany), 10 μM of deoxynucleoside triphosphates (Promega, Hampshire, UK) and 1 U of *Taq* polymerase (Bioline, London, UK). Cycling conditions consisted of 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. This was followed by strand elongation at 72°C for 7 min (GeneAmp PCR system 9700).

2.8.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise PCR products. 1.5 % agarose gels were prepared by addition of 0.45 g agarose powder (Bioline, London UK) to

30 mL Tris Borate EDTA (TBE) buffer. This was mixed, melted by heating until boiling in a microwave oven and allowed to cool. Ethidium bromide (Sigma Aldrich, Steinheim, Germany) was then added to give a final concentration of 0.05 μg/mL. On each gel, 5 μL of 100 base pair ladder (Invitrogen, Paisley, UK) was loaded in order to determine size of PCR amplicons.

2.8.3 Gel Photography

Gels were photographed with a digital camera (DC120 Kodak Digital) on a UV transilluminator (UVL Inc, California, USA) using camera software version 2.0 (Kodak, California, USA). Images were stored as JPEG files.

2.8.4 PCR Product Purification

PCR products were purified using the MinElute Purification Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. PCR products were then quantified by agarose gel electrophoresis. Intensity of the band was compared to lambda DNA of known concentrations to quantify the amount of DNA present.

2.8.5 Cycle Sequencing

Forward and reverse cycle sequencing reactions were performed in duplicate using the Big Dye Terminator Cycle sequencing ready reaction DNA sequencing kit v 2.0. (Applied Biosystems, Inc., Foster City, California, USA) according to the manufacturer's instructions. Purified PCR products (approximately 40 ng), were added per cycle sequencing reaction. Each reaction consisted of 1 μL ready reaction mix, 10.8 μL PCR quality water and 3.2 μL mmol primer. PCR products were sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster

City, CA, USA). Sequences were analysed using Bionumerics Version 2.0 (Applied Maths, Kortrijk, Belgium) and compared to the QRDR sequences of the susceptible parent.

2.8.6 Ethanol Precipitation

Labelled DNA was precipitated by the addition of 62.5 µL of 95 % ethanol (Royal Free Hospital Pharmacy), 3 µL sodium acetate (pH 4.6, 2.3 mol/L) (Sigma Aldrich, Steinheim, Germany) and 14.5 µL PCR quality water (RFH Pharmacy). The solution was vortexed and centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was discarded using a fine tipped pastette (Western Laboratories, Hampshire, UK). Ethanol (200 µL, 70 %; RFH Pharmacy) was added to clean the DNA pellet and the sample was centrifuged at 13,000 g for 5 min. at 4°C. The supernatant was removed with a fine tipped pastette and the tubes were dried at 37 °C for 30 min. Formamide (4 µL) and loading buffer (1 µL) was added to each sample (Sigma Aldrich, Steinheim, Germany) and 1.7 µL of the resulting mixture was loaded into each well. Samples were sequenced using an ABI 377 Applied Biosystems sequencer (Applied Biosystems, Foster City, California, USA).

2.9 Fitness Assays

2.9.1 Biofilm Growth

2.9.1.1Constant Depth Film Fermenter

2.9.1.1.1 Conditions

The constant depth film fermenter (CDFF) was maintained at a constant temperature of 37°C by housing it in an incubator. An aerobic atmosphere was maintained by exposure to the environment via a filtered air inlet in the top plate. Muller Hinton Broth was delivered to the fermenter at a flow rate of 0.5 litres/day.

2.9.1.1.2 Inoculum

The inoculum used was 10 mL of an overnight, shaken culture (200 r.p.m.) of *B. cepacia* grown in Muller Hinton Broth (37°C) taken from a single colony from a blood agar plate. A 10 mL volume of the overnight culture was aseptically added to 1 L of autoclaved Muller Hinton Broth. This inoculum was pumped into the CDFF overnight via sterile tubing at the sampling port. Time point zero was taken as the time that this pump was activated.

2.9.1.1.3 Sampling

At 4 h and then every subsequent 24 h, from time point zero, a pan was sacrificed from the CDFF, using a sterile sampling tool. A Miles and Misra plate count was performed on Muller Hinton agar plates on three plugs and each dilution was plated in triplicate (section 2.5). To avoid contamination a surgical face mask was used during sampling and the port was ethanol flamed, using a portable Bunsen burner. A growth curve for the wildtype isolate and images of the developing biofilm were obtained.

2.9.1.1.4 Confocal Laser Scanning Microscopy (CLSM)

Biofilms were analysed by CLSM using a modification of methods described previously (Hope & Wilson 2003). The discs were placed onto a petri dish 5 cm in diameter (held in place by vacuum grease) biofilm side up, then carefully submerged in *Bac*Light™ LIVE/DEAD stain (Molecular Probes, Oregon, USA) which comprised of 2 µL each of component A and B in 8 mL of SDW, allowing minimum disruption and desiccation of the biofilms. This stain contains the two dyes SYTO 9 and propidium iodide. Viable cells appear green while non-viable cells appear red, due to damaged membranes. After incubation in the dark for 10 min., the biofilm was examined on a fixed stage microscope (BX51 stereomicroscope, Olympus UK limited, Southall, UK) with a Radiance 3000 laser scan head (Biorad, Jena, Germany) mounted on a vibration free platform. Lasers used were Helium Neon (543 nm) and Argon (488 nm). Image acquisition was performed using Biorad LASERSHARP 2000.

The procedure was optimised for viewing of the cells. The settings selected, using the x 60 water objective were zoom 1.8, speed 166 lps, pixels x lines 1024 x 1024. Filter set 2 was used for visualisation via the microscope eye piece. The prism refractor was removed in order to scan and an empty filter case used. Captured images were visualised and a 3D representation of the spatial visualisation of the biofilm produced using the java based ImageJ software (National Institute of Health). The 3D images were created from live and dead colour channels using the 3D project option. These were combined to create a single RGB stack using the RGB Merge function.

2.9.1.2 Crystal Violet Assay

Quantification of biofilm growth was achieved by spectrophotometric measurement of crystal violet binding using a previously published method (O'Toole & Kolter 1998). Briefly 100 µL of a 1:100 overnight LB broth (BD, Le Pont de Claix, France) culture containing 0.5 % casamino acids (BD, Le Pont de Claix, France) was used to inoculate 8 independent wells of a 96 well polyvinylchloride microtitre plate (Falcon 3911 Microtest III flexible assay plate, Becton Dickinson LabWare, Becton Dickinson, Oxford, UK). Negative control wells contained broth only. This was incubated for 24 hours at 30°C in a humid atmosphere. The medium was gently removed and the wells washed 3 times with 200 µL SDW using a multi channel pipette. The microtitre plate wells were stained with 200 µL of 1 % (w/v) crystal violet for 15 min. at room temperature (Sigma Chemical Co., St. Louis, Mo., USA). Unbound crystal violet was removed by repeated washing with water. Bound crystal violet was solubilised with 2 x 200 µL of 95% ethanol, transferred to a microcentrifuge tube and the volume made up to 1 mL. The resulting absorbance was determined at wavelength 590 nm using a spectrophotometer (Pharmacia Biotech Ultraspec 2000). Each experiment was repeated in triplicate.

2.9.2 Planktonic Growth

The method of Youmans and Youmans (Youmans & Youmans 1949) was modified to determine growth rate.

The Bactec 9240 continuous, blood culture system with standard aerobic medium (Plus Aerobic/F) was used (Becton Dickinson, Oxford, UK). Aliquots of 100 µL of

diluted exponentially growing culture (1/10 and 1/1000), were removed using a 0.5 mL syringe and a needle and were aseptically inoculated into duplicate culture vials. The vials were then loaded immediately into the system. The length of time to detection (time to positivity) was measured for all strains. Gram stain and a purity plate were performed to confirm absence of contaminants. The growth rate constant k can be determined using the following equation (equation 6), where *A* is the largest inoculum employed, *B* is the smallest inoculum and t is the difference in time to positivity in hours. Generation time can then be determined by dividing log of 2 by the growth rate constant (equation 7). This experiment was repeated in triplicate.

$$k = \frac{\log A - \log B}{t} \quad \text{[Eq.6]}$$

$$G = \frac{\log 2}{k} \quad \text{[Eq.7]}$$

2.9.3 Competition Assays

The optical densities of wild type and mutant overnight 1 μL Muller Hinton broth cultures were adjusted to the same value (1.0 OD_{600} units) using a spectrophotometer (Pharmacia Biotech Ultraspec 2000). Then 250 μL of each culture was inoculated into 15 mL of LB broth, in the absence of antibiotics and this mixed culture was incubated for 10 hours, (200 r.p.m; Barloworld Scientific, Staffordshire, UK). The culture was then plated onto both drug free Muller Hinton plates and Muller Hinton plates containing twice the MIC of the susceptible parent. The number of parent susceptible cells was determined by subtracting the number of viable cells growing on drug containing plates from the total number of viable cells on drug free plates.

The fitness of the strains was determined by calculation of the number of generations grown by the parent and resistant strains using the equation 8 (Gillespie, Voelker, & Dickens 2002):

$$G = \frac{\log B - \log A}{\log 2} \quad \text{[Eq. 8]}$$

where G is the number of generations, *A* is the number of CFU at time 0 and *B* is the number of CFU per mL after the incubation period. The relative fitness of each strain was calculated from the ratio of the number of generations grown by the resistant to susceptible strains. Five independent pair wise cultures were performed for each mutant.

2.9.4 Survival in water

Survival in water was assessed using the method employed by Sanchez *et al* (Sanchez *et al.* 2002). Overnight Muller Hinton 1 mL broth cultures were pelleted by centrifugation (13,000 g, 3 min.), washed three times in PBS (1 mL) and an inoculum of approximately 1 x 10^8 CFU was added to duplicate duran bottles, each containing 19 mL of autoclaved tap water. Aliquots were aseptically removed at time intervals (Day 0, 1, 2, 5, 7, 8, 9 and 13) and the viable CFU/mL determined, using the Miles and Misra technique (section 2.5). Each experiment was repeated in triplicate.

2.9.5 Survival on dry surfaces

Survival on dry surfaces was assessed using the method employed by Sanchez *et al* (Sanchez *et al.* 2000). Overnight Muller Hinton 1 mL broth cultures were pelleted by centrifugation, washed three times in 1 mL PBS and concentrated 10 fold to a final cell concentration of approximately 1x 10^9. Using a pipette aliquots (10 µL) of the concentrated culture were spotted into duplicate wells of a 96 well flat bottomed microtitre plate (Western Laboratories, Hampshire, UK) and allowed to dry at room temperature. Survival was determined by sampling at hourly intervals for 7 hours by the addition of 100 µL of PBS containing 0.25 % v/v Triton x 100 (BDH, Leicestershire, UK) to the wells, followed by mixing to form a bacterial suspension. The 100 µL was then added to 900 µL of PBS, vortexed, and numbers of viable bacteria were determined by the Miles and Misra technique (section 2.5). The addition of Triton x 100 to PBS did not reduce numbers of viable cells. Each experiment was repeated in triplicate.

Chapter 3: Estimation of mutation rate in topoisomerase genes of *B. cepacia*

3.0 Introduction

3.1 Fluoroquinolone resistance

3.1.1 Fluoroquinolone Resistance in *B. cepacia*

The molecular mechanisms of fluoroquinolone resistance in *B. cepacia* have not previously been reported. Amino acid substitutions in the quinolone resistance determining regions (QRDR) of topoisomerase genes have been described in other Gram-negative bacteria including *Proteus mirabilis*, *S. enterica*, *Chlamydia trachomatis*, *E. coli* and *P. aeruginosa* (Chen & Lo 2003; Dessus-Babus *et al.* 1998; Eaves *et al.* 2004; Rafii, Park, & Novak 2005; Weigel, Anderson, & Tenover 2002).

Evidence from related organisms suggests that mutation in *gyrA* is most likely to be the primary resistance mechanism in *B. cepacia*. Amino acid substitutions at codon 83 in *gyrA* have been identified in oxolinic acid resistant strains of *Burkholderia glumae* (Maeda *et al.* 2004). Mutations at codon 83 and 87 of *gyrA* were identified in nalidixic acid resistant *B. vietnamensis* mutants (Miche & Balandreau 2001) and mutation in *gyrA* is the most primary cause of fluoroquinolone resistance in *P. aeruginosa* (Cambau *et al.* 1995; Kugelberg *et al.* 2005; Yonezawa *et al.* 1995).

3.1.2 Double mutation in topoisomerase genes

Single mutations in subunit A of DNA gyrase are the most common cause of low to moderate increases in resistance in Gram negative organisms (Chen & Lo 2003; Hooper 2003). To obtain high level FQ resistance a second mutation is required in *parC* or *gyrA*.

Double mutations in DNA gyrase/topoisomerase IV have been characterised in clinical and *in vitro* isolates of *E. coli* (Truong *et al.* 1997; Vila *et al.* 1994) and in isolates of *P. aeruginosa* and *S. pneumoniae* that have been selected *in vitro* (Gillespie *et al.* 2003; Gillespie, Voelker, & Dickens 2002; Kugelberg *et al.* 2005). A limited number of amino acid substitutions conferring resistance, have been reported and in Gram-negative bacteria the most common occurs at codon 83 (Drlica 2003; Ruiz 2003; Vila *et al.* 1995) and the second most common at codon 87 (Everett *et al.* 1996; Vila *et al.* 1994). Substitutions at these codons are also found in Gram-positive bacteria, including *S. aureus* and *S. pneumoniae* (Jones *et al.* 2000; Schmitz *et al.* 1998). Substitutions in *gyrB* conferring resistance in *E. coli* that have been described occur at codons 426 and 447 (Nakamura *et al.* 1989; Yoshida *et al.* 1990a).

3.1.3 Applications of Mutation Rate Estimation Experiments

Oliver *et al* used the Crane method, (see section 1.2.9) (Crane, Thomas, & Jones 1996) to show that antibiotic resistant isolates of *P. aeruginosa* were present prior to antibiotic therapy due to the existence of hypermutable bacteria (Oliver *et al.* 2004). Mutation rate experiments have been used in *S. pneumoniae* to show that mutations in the *gyrA* gene occur at a lower rate than *parC* mutations and that mutation in either gene predisposes to further mutation (Gillespie *et al*, 2003). There is a pressing need to develop or adapt methods to investigate mutation rate in Bcc.

3.2 Aims of Chapter

The aims of this chapter were to generate FQ resistant mutants, optimise a method to estimate the mutation rate to fluoroquinolone resistance in *B. cepacia* and to characterise the genetic basis of resistance. The presence of mutations conferring resistance to fluoroquinolones was confirmed by MIC determination and sequence analysis of topoisomerase genes. Resistant mutants were screened for reserpine inhibited fluoroquinolone efflux to assess involvement of an efflux pump in observed resistance. Reserpine is a plant alkaloid that inhibits active efflux and has been used in assays to determine the contribution of efflux pumps to antibiotic resistance (Baranova & Neyfakh 1997; Beyer *et al.* 2000; Brenwald, Gill, & Wise 1997; Markham 1999).

3.3 Materials and Methods

3.3.1 Bacterial Strain

All optimisation experiments were performed using *B. cepacia* NCTC 10661 which was obtained from the National Collection of Type Cultures, Centre for Infections, HPA, Colindale, UK. This isolate had a ciprofloxacin MIC of 1.0 mg/L. The bacteria were stored at -70°C in a tube containing storage media and beads (Prolab, Preston, UK). The inoculum for each mutation rate experiment was prepared by inoculation of one bead onto Columbia Blood Agar (Oxoid, Basingstoke, UK), which was spread and incubated at 37°C for 18 hours.

3.3.2 Choice of Selective Antibiotic

Ciprofloxacin was chosen as the selective antibiotic as fluoroquinolone resistance mainly occurs due to point mutations in the subunit A of gyrase in Gram-negative bacteria and because the type strain used was sensitive to this drug.

3.3.3 MIC Determination

The MIC of *B. cepacia* 10661 was determined by E-test (2.6.1) and by the agar dilution method, according to the CLSI guidelines for susceptibility testing of aerobic organisms (section 2.6.2).

3.3.4 Sequence Analysis of the QRDR of Fluoroquinolone Resistant Mutants

DNA was extracted from wild type and fluoroquinolone resistant mutants using either a crude extraction method (section 2.7.1) or the Promega Extraction Kit (section 2.7.2). Mutants were characterised by sequence analysis of the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*. Primers were designed using the sequenced genome of *Burkholderia cenocepacia* AU 1054 chromosome 1 (accession number CP000378) (Markowitz *et al.* 2006) and the primer design programme, Primer3, (Rozen & Skaletsky 2000) to amplify *gyrA*, *gyrB*, *parC* and *parE* and nucleotide sequences of the primers are shown in Table 3.1. In *E. coli* the QRDR comprises codons 67-106 of *gyrA*, therefore the primers were designed to amplify codons 38-122 (Yoshida *et al.* 1990a). Mutations conferring resistance in *E. coli*, within *gyrB* are commonly found at codon positions 426 and 447 (Yoshida *et al.* 1990b; Yoshida *et al.* 1991). Therefore primers were designed to amplify codons 400-500. PCR reaction

composition, cycling conditions and amount of DNA added to reactions were optimised (data not shown). PCR and sequencing of topoisomerase genes were performed (section 2.8)

Gene	Primer Position (*B. cenocepacia* numbering)	Sequence (5'-3')	Amplicon Size (b.p.)
gyrA	62-81	5' ATCTCGATTACGCGATGAGC	449
	493-511	5' GCCGTTGATCAGCAGGTT	
gyrB	1127-1146	5' GAGGAAGTTGTGGCGAAGG	400
	1502-1520	5' AGTCTTCCTTGCCGATGC	
parC	98-118	5' ATTGGTCAGGGTCGTGAAGA	229
	295-315	5' GTAGCGCAGCGAGAAATCCT	
parE	1178-1198	5' CAGGGCAAGGTAGTCGAAAA	380
	1557-1577	5' GTGAGCAGCAAGGTCTGGAT	

Table 3.1. Primers used to amplify the Quinolone Resistance Determining Region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* of *B. cepacia.*

3.3.5 Detection of Efflux

The ciprofloxacin MIC of the fluoroquinolone resistant mutants was determined in the absence and presence of reserpine in Muller Hinton agar, (section 2.6.4) (Beyer *et al.* 2000).

3.4 Results

3.4.1 Development of Methodology

3.4.1.1 Inoculum

3.4.1.1.1 Cell Number

The assumptions of all mutation rate estimations require that in each parallel culture the final cell number (N_f) should be the same and that the number of cells at time zero (N_0) should always be negligible compared to N_f (section 1.2.6) and so an inoculum size of 10^2

cells was used initially in this study. The number of colony forming units per mL was determined using the Miles and Misra plate count technique (section 2.5) (Miles & Misra 1938). The 10^{-4} dilution was found to contain 10^2 CFU per 100 µL and this was therefore used as the initial inoculum. An inoculum of 10^2 cells was added to 1 mL aliquots of Muller Hinton Broth (Oxoid, Basingstoke, UK) and incubated for 18 hours using an orbital shaker (37°C, 200 r.p.m,) (Barloworld Scientific, Staffordshire, UK). This resulted in a final number of cells of approximately 1-2 x 10^8 CFU/mL. Using 10^8 cells as the final cell number the proportion of cultures with mutations was below the level for the p_0 to be considered valid i.e. the proportion of cultures without mutants was above the valid range of 0.1 and 0.7. In five experiments, using this inoculum, the average number of plates with ciprofloxacin resistant mutants was zero or one. The initial inoculum was subsequently changed to an inoculum of 10^3 cells. This was achieved by the addition of 100 µL of the 10^{-3} dilution (of the original culture) to each mutation rate estimation culture aliquot. Upon selection at 2 x and 4 x MIC all plates contained ciprofloxacin resistant colonies. However no topoisomerase mutations (0/45 colonies sequenced) were found in colonies growing on plates containing 2 x MIC. At 4 x MIC colonies from one plate only (2/55) contain topoisomerase mutations (section 3.4.3). At 6 x MIC all colonies contained confirmed mutations in *gyrA* (section 3.4.3). In conclusion the final inoculum used was 10^3 cells.

3.4.1.1.2 Growth phase

A second assumption requires that cells added to a mutation rate estimation experiment should be in exponential phase. A growth curve experiment was performed to determine the time period that the initial broth should be incubated before inoculation of the mutation rate estimation to ensure cells were in exponential phase (figure 3.1). It was apparent that cells

will be in mid exponential phase at 2-3 hours, following incubation of a 5 mL Muller Hinton broth (3 hour, 200 rpm), inoculated with a single colony.

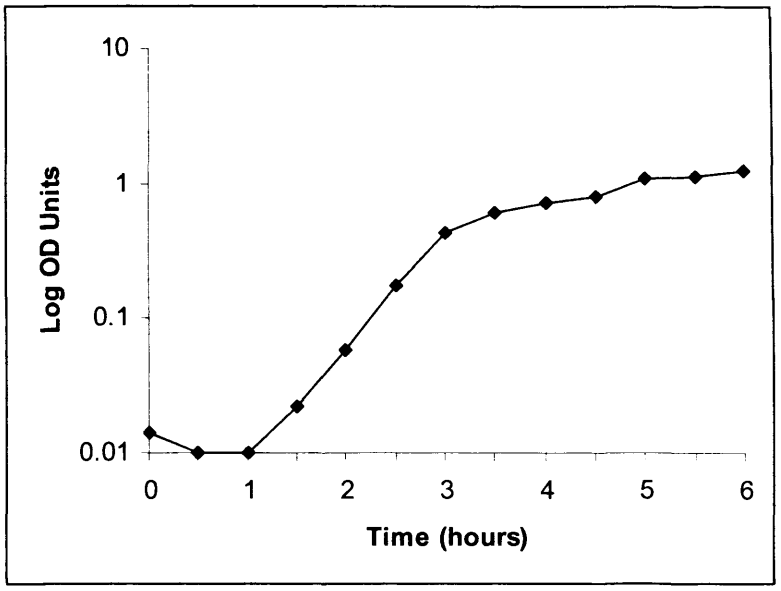


Figure 3.1 Growth curve of *B. cepacia*

In order for inoculated cells to be in exponential phase the initial inoculum was incubated for 2.5 hours to an OD_{600} of approximately 0.1 OD units (Pharmacia Biotech Ultraspec 2000).

3.4.1.1.3 Incubation Period

The final number of cells (N_t) was optimised so that the proportion of cultures with mutants (plates with resistant colonies) was high enough for the p_0 method to be valid. This involved optimisation of the size of the initial inoculum and of the incubation conditions. Initially aliquots were incubated for 18 hours. Subsequently this was changed to 22 hours, increasing the final number of cells (N_t) and therefore increasing the proportion of cultures with mutants. Following the increase in incubation time, the average final number of cells, in a

culture aliquot, increased from approximately 2 x 10^8 CFU to approximately 6 x 10^9 CFU. In summary the incubation period was increased to 22 hours.

3.4.1.2 Selective Antibiotic Concentration

The selective antibiotic concentration is an important parameter because it influences the numbers of observed resistant colonies and therefore will govern which method is most appropriate. Ciprofloxacin stock solution was prepared as described in section 2.3.6. Selective agar plates were used containing 2 x and 4 x the MIC of ciprofloxacin. The nucleotide sequences of the topoisomerase QRDR regions were determined (section 3.3.4). Following selection at 4 x MIC only one plate contained colonies. These colonies were confirmed as having a gyrase mutation in the QRDR, an Asp87Asn mutation. At 4 x MIC 53/55 colonies did not contain QRDR mutations. No colonies (0/45) selected at 2 x MIC contained mutations within the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*. The observed increase in resistance of these colonies was due to increased FQ efflux (see section 3.4.5).

The MIC of each mutant was determined. The Asp87Asn gyrase mutant, selected at 4 x MIC, has an MIC of 12 mg/L compared to approximately 5 mg/L for the bacterial colonies not containing mutations in topoisomerase genes. The mutation rate calculated using a low concentration of selective antibiotic (2 x – 4 x MIC) is not a measure of the rate of mutations that occur in topoisomerase genes only because other mechanisms may be contributing to the observed resistance. To address this, the selective antibiotic concentration was increased to 6 x and 8 x MIC. As the majority of plates did not exhibit resistant colonies upon selection 6 x and 8 x MIC then the p_0 method was the most appropriate method. To be valid the p_0 method requires that >25 % of plates must contain resistant colonies, at 6 x MIC this criteria was met (42 % of plates contained colonies) and

therefore this method was used to determine the mutation rate. Upon selection at 8 x MIC 1/25 plates contained resistant colonies, 5/5 colonies sequenced contained a Thr83Ile mutation. All of the 50 mutants selected at 6 x MIC and sequenced contained the same Thr83Ile mutation.

The final estimate is a mean of four replicate mutation rate experiments. Each replicate was performed from different initial broth inoculums. The isolated mutant colonies were stored at -70°C (section 2.1) for measurement of fitness costs associated with topoisomerase mutations (chapter 5).

To ensure that colonies growing would be as a result of mutations that occurred prior to plating, plates were incubated for 24 hours at 37°C. A longer incubation may allow the occurrence of post plating mutation which would confound the calculation of the true mutation rate.

In conclusion resistant mutants were selected on 6 x MIC of ciprofloxacin.

3.4.1.3 Choice of mutation rate calculation method

3.4.1.3.1 Lea and Coulsons Method of the Median

Upon selection at 2 x and 4 x MIC each plate contained between 30 and 400 colonies. These colonies varied in size and colour. Confirmation as *B. cepacia* was performed on 24 representative colonies (2 colonies from each of 12 plates) by Gram stain and API 20 NE, using the manufacturer's instructions. This excluded the possibility of contaminants that could have been mistaken as *B. cepacia* colonies. At 24 hours it was difficult to distinguish and count colonies by eye and so colonies were counted at 48 hours. A median method was

used to estimate the mutation rate (Lea & Coulson 1949) because all plates contained mutants, see section 2.6.3 for method. From each plate at least 2 colonies were picked, sub cultured and stored at -70°C (section 2.1). The QRDR sequence of *gyrA*, *gyrB*, *parC* and *parE* was sequenced for the mutant colonies to confirm their identity as topoisomerase mutants. However at 2 x and 4 x MIC the majority of resistant mutants did not contain topoisomerase mutations (section 3.4.3). Estimated mutation rates using the method of the median, from three rounds of selection at 4 x MIC, are shown in table 3.2. At 2 x MIC there were too many colonies to enumerate and data is not shown. Selection at 6 x MIC using the p_0 method was therefore used. The Method of the Median could not be used at 6 x MIC because approximately half of the plates did not contain mutants.

Replicate	Estimated Mutation rate (mutations/division)
1	2×10^{-8}
2	6.1×10^{-7}
3	5.9×10^{-7}
Median	**6.1×10^{-7}**

Table 3.2 Estimated mutation rates in *B. cepacia* using the method of the median. Each replicate consisted of 25 culture aliquots. Mutation rate was estimated by the Method of the Median at 4 x MIC.

3.4.1.3.2 p_0 method

For the purpose of these experiments the method used to estimate the mutation rate is the p_0 method (section 1.2.5.1). Estimated mutation rates were the median value of four replicate experiments (see section 3.4.1.4 for optimised method).

3.4.1.4 Optimised Method for Determination of Mutation Rate using the p_0 method

Isolates were removed from the -70°C freezer and one bead was used to sub culture *B. cepacia* onto a blood agar plate and was incubated aerobically at 37°C. One colony of *B. cepacia* was suspended in 5 mL of Muller Hinton Broth in a 25 mL conical flask. This was sealed with a cotton wool bung and incubated at 37°C on an orbital shaker (200 r.p.m.) (Barloworld Scientific, Staffordshire, UK) for 2.5 hours until an optical density (OD_{460}) of approximately 0.1 OD units was reached. Serial dilutions of this broth culture were performed in PBS (neat to 10^{-6}). A 100 µL aliquot of the 10^{-3} dilution (containing approximately 10^3 cells) was added to each of 28 bijoux tubes, containing Muller Hinton Broth (3 mL). These cultures were incubated at 37°C, 200 r.p.m., 22 hours (Barloworld Scientific, Staffordshire, UK). Muller Hinton Agar plates (Oxoid, Basingstoke, UK) containing 6 x MIC of ciprofloxacin were prepared and inoculated the following day. Antibiotic free agar plates were also prepared, (section 2.2.3). Before inoculation, plates were allowed to dry at 37°C with the lid removed for 20 min. This ensured that drops were absorbed by the agar. At the end of the incubation period, a Miles and Misra plate count was performed on 3 randomly selected broths (Miles & Misra 1938), (section 2.5). Dilutions of 10^{-1} to 10^{-6} were prepared. Twenty microlitres of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spotted in triplicate onto duplicate drug free Muller Hinton agar plates. The contents of the remaining bijoux tubes were transferred to 10 mL centrifuge tubes and centrifuged (MSE centrifuge) at 4000 *g*. for 10 min. and the supernatant discarded. Using a fine tipped pastette the deposit was resuspended in 300 mL PBS. The total volume was then inoculated onto ciprofloxacin containing Muller Hinton plates, spread with a plastic spreader and allowed to dry.

All plates were incubated (37°C, 24 hours) and colony forming units per mL were calculated from the Miles and Misra drug free plates. The proportion of cultures with no mutants (no growth on ciprofloxacin) was calculated (p_0) using equation 9.

$$p_0 = \frac{\text{number of cultures with no mutants}}{\text{total number of culture aliquots}} \qquad \text{[Eq 9]}$$

The number of mutations per culture was calculated using equation 10

$$m = -\ln(p_0) \qquad \text{[Eq. 10]}$$

The mutation rate (μ) was calculated using equation 11

$$\mu = \frac{m}{\text{average cfu/mL}} \qquad \text{[Eq. 11]}$$

This was repeated 4 times to produce a median mutation rate

3.4.1.5 Selection of Second Step Mutants

Second step fluoroquinolone resistant mutants were selected by inoculating first step mutants onto plates containing 2 x MIC of the mutants in a mutation rate experiment (section 3.4.1.4). All mutants were stored as previously described (section 2.1).

3.4.2 Estimated Mutation Rate

Estimated mutation rates of *B. cepacia* to ciprofloxacin resistance at 6 x MIC were determined by the p_0 method (section 3.4.1.4) (Table 3.3). The mutation rate to ciprofloxacin resistance by mutation in *gyrA* was estimated by selecting mutants on 6 mg/L and was determined as 9.6×10^{-11}.

Replicate	Estimated Mutation rate (mutations/division)
1	7.4×10^{-11}
2	1.0×10^{-10}
3	9.2×10^{-11}
4	1.0×10^{-11}
Median	**9.6×10^{-11}**

Table 3.3 Estimated mutation rates in *B. cepacia* using p_0 method. Each replicate consisted of 25 culture aliquots. Mutation rate was estimated by the p_0 method at 6 x MIC.

3.4.3 Characterization of Ciprofloxacin Resistant Mutants

Ciprofloxacin resistant colonies were isolated using selection at 2 x, 4 x, 6 x and 8 x MIC and characterised. During three independent rounds of selection at 2 x MIC 45 colonies were isolated and characterised, at 4 x MIC 55 colonies were isolated and characterised and during three rounds of selection at 6 x MIC 50 colonies were isolated and characterised. At 8 x MIC 5 colonies were isolated and characterised.

Mutants containing second step mutations were selected at twice the MIC of the single step topoisomerase mutation. All FQ resistant mutants that were selected had an MIC of at least five-fold higher than the susceptible parent. At 4 x MIC an Asp87Asn mutation, conferring a 12 fold increase in MIC, was found in one mutant on one plate only. All other mutants selected at this concentration contained no mutations in QRDRs of *gyrA*, *gyrB*, *parC* or *parE*. These colonies had small increases in MIC (less than five-fold differences as compared to the parent strain). At 4 x MIC only 2/53 colonies contained a Thr83Ile mutation in *gyrA*. All mutants (50/50) selected at 6 x MIC contained the Thr83Ile mutation in the QRDR of *gyrA*. All mutants selected (5/5) at 8 x MIC contained the Thr83Ile

mutation. No mutations in the QRDRs of *gyrB*, *parC* or *parE* were identified and no silent mutations were observed. Mutations, MIC and selection step information is shown in Table 3.4. Mutation rates for second step mutations (F3 and F4) were higher than for the first step mutations and are shown in table 3.4. A map of mutations observed is shown in figure 3.2.

				QRDR Sequence			
Mutant	**MIC (mg/L)**	**Selection**	**Step**	***gyrA***	***gyrB***	***parC***	***parE***
Wildtype	1						
F1	12	1st		Asp87Asn	wt	wt	wt
F2	64	1st		Thr83Ile	wt	wt	wt
F3	>256	2nd		Asp87Asn	wt	Ser80Leu	wt
F4	>256	2nd		Thr83Ile	wt	Ser80Leu	wt

Table 3.4 Mutations, MIC and selection step of fluoroquinolone resistant mutants. Strain F1 isolated on 4 mg/L ciprofloxacin (4 x MIC) using the wildtype as the starting point, F2 isolated on 6 mg/L (6 x MIC) using the wildtype as the starting point, F3 isolated on 24 mg/L ciprofloxacin using F1 as the starting point, F4 isolated on 128 mg/L ciprofloxacin using F2 as the starting point.

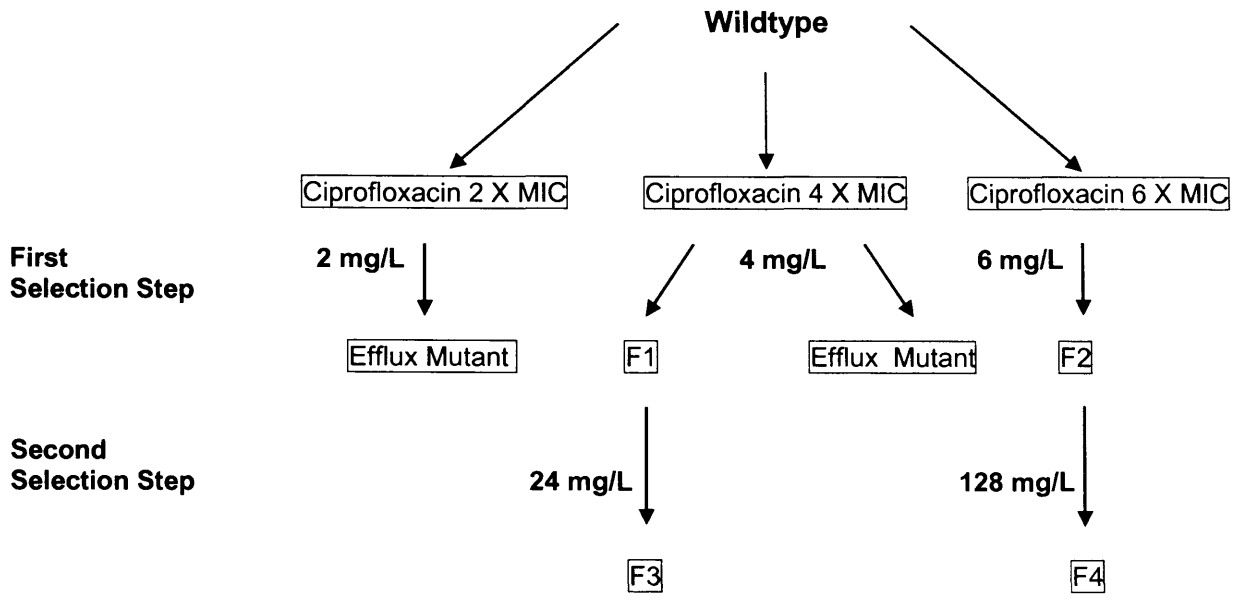


Figure 3.2 Relationship of *B. cepacia* mutants selected stepwise with ciprofloxacin. Antibiotic concentration used in each selection step is shown

Isolate	Median Mutation Rate per cell division (Range)
Wildtype	9.6×10^{-11} (9.2×10^{-11} - 1×10^{-10})
F1	6.8×10^{-10} (9.2×10^{-10} -1×10^{-9})
F2	1.1×10^{-10} (1.1×10^{-11} -1×10^{-9})

Table 3.5 Mutation rates of fluoroquinolone resistance. Median mutation rates, estimated by the p_0 method of first step mutations of wildtype to FQ resistance and second step mutations from Asp87Asn (F1) and Thr83Ile (F2) to additional Ser80Leu mutation in *parC*, using ciprofloxacin as the selective agent. Median mutation rates represent four p_0 replicate experiments.

3.4.4 Confirmation of QRDR mutation

The QRDRs of *gyrA, gyrB, parC and parE* were sequenced. The QRDR of 45 resistant mutants selected at 2 x MIC were sequenced but no isolates selected at this concentration contained a *gyrA* mutation. At 4 x MIC 2/55 colonies contained an Asp87Asn mutation. All fluoroquinolone resistant mutants selected at 6 x (50) and 8 x (5) MIC contained a *gyrA* mutation (Thr83Ile). Throughout the most commonly selected mutant was Thr83Ile, see figure 3.3. The consensus nucleotide sequence of the quinolone resistance determining region of gyrase A of the susceptible *B. cepacia* parent was determined. The translated amino acid sequence from this consensus is shown in figure 3.4.

```
195      -----AATCGCCGCGTATCGTCGGTGACGTGATCGGTAAGTACCATCCTCACGGCG Parent
190      ACAAGAATCGGCGCGTATCGTCGGTGACGTGATCGGTAAGTACCATCCTCACGGCG Asp87Asn
190      ACAAGAATCGGCGCGTATCGTCGGTGACGTGATCGGTAAGTACCATCCTCACGGCG Thr83Ile
         **************************************************

247      ACACCGCGGTGTACGACACGATCGTGCGGATGGCGCAAGACTTCTCGCTGCGTTAC Parent
247      ACATCGCGGTGTACGACACGATCGTGCGGATGGCGCAAGACTTCTCGCTGCGTTAC Asp87Asn
247      ACACCGCGGTGTACAACACGATCGTGCGGATGGCGCAAGACTTCTCGCTGCGTTAC Thr83Ile
         **** *********** **************************************

349      ATGCTGATCGACGGGCAAGGCAACT---------------------------- Parent
349      ATGCTGATCGACGGGCAAGGCAACTTCGGCTCGATCGACGGCGACAATGCCGCGGC Asp87Asn
349      ATGCTGATCGACGGGCAAGGCAACTTCGGCTCGATCGACGGCGACAATGCCGCGGC Thr83Ile

         *************************
```

Figure 3.3 Alignment of susceptible parent and mutant *gyrA* nucleotide sequences. Mutations shown in boxes (Thr83Ile) and (Asp87Asn).

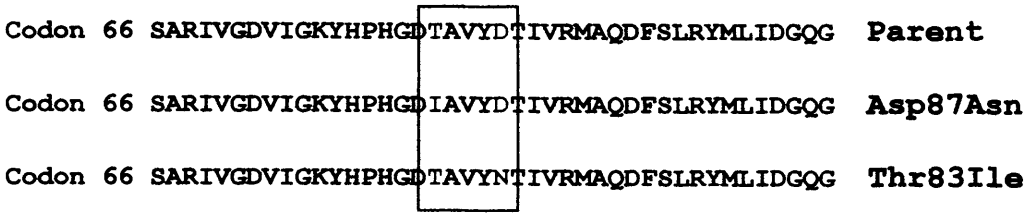


Figure 3.4 A comparison of the translated amino acid sequences of the QRDR of the characterised *B. cepacia gyrA* mutant and susceptible parent.

Only one mutant (2/55) selected at 4 x MIC contained a mutation in DNA gyrase subunit A. This mutant Asp87Asn had a corresponding MIC of 12 mg/L. All other colonies characterised at this ciprofloxacin concentration had MIC levels of 4-5 mg/L and no topoisomerase QRDR mutations were found by sequencing. The elevated MIC of these mutants was attributed to alteration in efflux activity, because incorporation of reserpine into the media reduced the MIC to wild type levels. Reserpine is an inhibitor of efflux and therefore the MIC of isolates exhibiting increased resistance to FQs will decrease in the presence of reserpine. However it is possible that topoisomerase mutations occurred outside the QRDR of the topoisomerase genes. Upon selection at 6 and 8 x MIC a change at position 83 from threonine to isoleucine was observed. The corresponding MIC for these mutants was 64 mg/L. No mutations were found in *gyrB*, *parC* and *parE* in the single step mutants. Second step mutants containing high level resistance additionally contained a Ser80Leu mutation in *parC*, see figure 3.5.

```
165   GGATGCCGATTCCAAGCACAAGAAGTCGGCGCGGCACCGTCGGCGACGTGCTCGGCAAGTTCC Parent
165   GGATGCCGATTCCAAGCACAAGAAGTCGGCGCGGCACCGTCGGCGACGTGCTCGGCAAGTTCC Ser80Leu
      ************************************************************

230   ACCCGCACGGCGACTCGGCCTGCTACGAGGCCATGGTGCTGATGGCGCAGCCGTTCTCTCCTA Parent
230   ACCCGCACGGCGACTTGGCCTGCTACGAGGCCATGGTGCTGATGGCGCAGCCGTTCTCTCCTA Ser80Leu
      *************** ********************************************

295   TCGCTAT                                                          Parent
295   TCG----                                                          Ser80Leu
      *******
```

Figure 3.5 Alignment of susceptible parent and mutant *parC* sequences. Mutation shown in box (Ser80Leu).

```
1180 GGCGCGCACGCGCGCCGGCCAGAAGGTCGAGAAGCGCAAGAGCTCGGGCGTCGCGGTGCTGCCCGGC Parent
1180 GGCGCGCACGCGCGCCGGCCAGAAGGTCGAGAAGCGCAAGAGCTCGGGCGTCGCGGTGCTGCCCGGC F1
     ************************************************************************

1250 AAGCTGACCGATTGCGAGACGGAAGATATCGCGCGCAACGAACTGTTCCTGGTCGAGGGCGACTCGG Parent
1250 AAGCTGACCGATTGCGAGACGGAAGATATCGCGCGCAACGAACTGTTCCTGGTCGAGGGCGACTCGG F1
     ************************************************************************

1300 CGGGCGGCTCCGCGAAGATGGGCCGCGACAAGGAATACCAGGCGATCCTGCCGCTGCGCGGCAAGGT Parent
1300 CGGGCGGCTCCGCGAAGATGGGCCGCGACAAGGAATACCAGGCGATCCTGCCGCTGCGCGGCAAGGT F1
     ************************************************************************

1350 GCTGAATACGTGGGAAACCGAGCGCGACCGCCTGTTCGCGAACAACGAGGTGCACGACATCTCGGTC Parent
1350 GCTGAATACGTGGGAAACGCGCGACCGCCTGTTCGCGAACAACGAGGTGCACGACATCTCGGTC F1
     ************************************************************************
```

Figure 3.6 Alignment of susceptible parent and resistant mutants for *gyrB* sequences. No mutations were found in the QRDR of *gyrB*. Presumptive QRDR (codon 426) shown in red.

```
1210    GTCGGTCGAACGTGAATCGGCGGAAATCGCCTTCGAACAGCGGGATCAGCAGTTCGCTGC    Parent
1210    -----TCGAACGTGAATCGGCGGAAATCGCCTTCGAACAGCGGGATCAGCAGTTCGCTGC    F1
        ************************************************************

1270    CCGTTGCTTTCGTCGAGCAGGCGCTGTAGCCGCGCAGGCCGTCTTCATACTTCCACGTCT    Parent
1270    CCGTTGCTTTCGTCGAGCAGGCGCTGTAGCCGCGCAGGCCGTCTTCATACTTCCACGTCT    F1
        ************************************************************


1330    GGCGCTTGCCGGTCTTCTCGTTGACGAGCACGACCTCGACGCCCGGCAGCAGCACGGCCT    Parent
1330    GGCGCTTGCCGGTCTTCTCGTTGACGAGCACGACCTCGACGCCCGGCAGCAGCACGGCCT    F1
        ************************************************************

1390    TCGAGCGCAGCAGGCGCTGCAGCTCGCCGAGCGGCAGGTTCGGCGAATCGAAGTACTTCG    Parent
1390    TCGAGCGCAGCAGGCGCTGCAGCTCGCCGAGCGGCAGGTTCGGCGAATCGAAGTACTTCG    F1
        ************************************************************

1450    GATTCGGCCACACCTGCACGCGCGTGC--                                   Parent
1450    GATTCGGCCACACCTGCACGCGCGTGCCG                                   F1
        ****************************
```

Figure 3.7 Alignment of susceptible parent and resistant mutants for *parE* sequences. No mutations were found in *parE*. Presumptive QRDR (codon 425 and 445) shown in red.

3.4.5 Detection of Efflux Pumps

The MICs of all mutants, selected at 2 x MIC and the majority selected at 4 x MIC, decreased five-fold in the presence of reserpine to the level of the wild type, shown in Table 3.6. Presence of reserpine did not affect the ciprofloxacin MIC of mutants containing topoisomerase mutations. It is likely therefore that these mutants exhibit increased expression of ciprofloxacin extruding efflux pumps.

Isolate	QRDR genotype *gyrA*	*parC*	MIC(mg/L)	MIC+ Reserpine
WT	wt	wt	1	1
E1	wt	wt	5	1
E2	wt	wt	5	1
E3	wt	wt	5	1
E4	wt	wt	5	1
E5	wt	wt	5	1
F1	Asp87Asn	wt	12	12
F2	Thr83Ile	wt	64	64
F3	Asp87Asn	Ser80Leu	>256	>256
F4	Thr83Ile	Ser80Leu	>256	>256

Table 3.6 MICs (mg/L) of ciprofloxacin in the presence and absence of reserpine (25 mg/L) for representative FQ resistant mutants. E1-E5 were selected at 2 x and 4 x MIC and contained no topoisomerase mutations.

3.5 Discussion

Most previous studies have measured mutation frequency as opposed to mutation rate (Bjorkholm *et al.* 2001; Gustafsson *et al.* 2003; Henderson-Begg, Livermore, & Hall 2006). This measure provides no information on when the mutation occurred or of the rate at which mutants occur. Mutations that occur early in the culture, known as 'Jackpot' mutations, produce large numbers of progeny and will cause large variance in results. Mutation rate methods, as used here, are more reproducible and can be used to predict how rapidly antibiotic resistance will develop.

Ideally mutation rates should be estimated using an antibiotic to which resistance arises via a single amino acid substitution caused by a change in a single base pair. This allows methods to be adapted between organisms. Antibiotics which are susceptible to point mutations that occur in chromosomal genes include the fluoroquinolones, rifampicin, pyrazinamide and isoniazid. Resistance to fluoroquinolones occurs mainly due to chromosomal mutations in target genes. Therefore the mutation rate contributes to the proportion of a bacterial population that are resistant to fluoroquinolones. It is unlikely that a mutation will occur in a population if the size of the population is less than the inverse of the mutation rate. As the population increases the probability of a resistance mutation occurring that increases the MIC to the antibiotic in question also increases. The mutation rate estimated here implies that a *B. cepacia* infection must involve at least 9.6×10^{11} cells for one cell to mutate to resistance.

Of the *gyrA* mutations that cause bacterial fluoroquinolone resistance *in vitro* those that occur at codons 83 and 87 in *gyrA* occur most frequently (Bachoual *et al.* 2001; Dessus-Babus *et al.* 1998; Mouneimne *et al.* 1999; Ruiz *et al.* 1998; Vila *et al.* 1995). These codons encode the amino acids that form the DNA binding region of gyrase and therefore changes here result in a reduced capacity for fluoroquinolones to form a complex with the DNA and gyrase. Of the two amino acid changes observed one was at codon 83 and the other at 87. At codon 83 the change was from a hydrophilic threonine residue to a hydrophobic isoleucine residue. Mutation of codon 83 to a hydrophobic amino acid generally confers more resistance than mutation at codon 87 (Drlica & Zhou 1997). This data shows substitution at position 83 confers a greater level of resistance than substitution at position 87 and therefore supports this interpretation. Most fluoroquinolone susceptible organisms have an aspartic

acid residue at codon 87. If the amino acid at this position is not negatively charged then there will be increased resistance to fluoroquinolones. The change at codon 83 conferred a 64 fold change in MIC while alteration at codon 87 conferred a 12 fold increase in MIC. The substitution Thr83Ile in *gyrA* was the mutation most commonly identified in the *in vitro* mutants. Previous studies have also found this mutation to be the most common in clinical isolates of *P. aeruginosa* (Akasaka *et al.* 2001; Mouneimne *et al.* 1999; Oh *et al.* 2003). A common double mutation Thr83Ile and Asp87Asn, previously reported in *E. coli* and *P. aeruginosa* (Akasaka *et al.* 2001; Oh *et al.* 2003; Saenz *et al.* 2003) was not detected in these *in vitro* isolates. Changes at both of these codons have previously been described in other Gram-negative bacteria but the Thr83Ile mutation has only been described in *P. aeruginosa* (Drlica 2003).

When selection with lower concentrations of fluoroquinolone (2 x MIC) was used no mutations were found in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*. It is possible that mutations occurred outside the QRDR. However the MIC of these mutants was reduced to levels comparable to that of the wildtype following incorporation of reserpine into the agar. Therefore it is likely that the observed resistance is due to altered expression of an efflux pump. Similar results have been obtained by Zhou *et al* who found that low concentrations of fluoroquinolone selected non gyrase mutants in *M. smegmatis* (Zhou *et al.* 2000). These isolates may contain other mutations that confer resistance to other antibiotics as these mutants additionally exhibit decreased susceptibility to chloramphenicol and ampicillin (Zhou *et al.* 2000). It has previously been reported that at low plating concentrations colonies growing usually do not contain mutations in the QRDR of mycobacteria (Sindelar *et al.* 2000). On exposure to higher drug concentrations mutants conferring low levels of

resistance are unable to grow. Mutations that interfere significantly with gyrase may cause cell death and so will not contribute to the observed mutation rate.

At higher selection concentrations of ciprofloxacin (4, 6 and 8 x MIC) mutations in the topoisomerase genes were found. Moderate level resistance (12-64 mg/L) was caused by single mutations in *gyrA* that occurred during the initial selection step at 6 x MIC. High level resistance (256 mg/L), achieved following second step selection, was associated with double mutation in *gyrA* and *parC*.

Second step mutations were selected, using Asp87Asn and Thr83Ile as the respective starting points, on high concentrations of ciprofloxacin. The second round of mutant selection resulted in the occurrence of a mutation at codon 80 of *parC*, irrespective of the starting point. This mutation has previously been implicated in conferring fluoroquinolone resistance in clinical isolates of *P. aeruginosa* (Akasaka *et al.* 2001; Mouneimne *et al.* 1999; Oh *et al.* 2003). This mutation has been also been described in *P. aeruginosa* resistant isolates, selected *in vitro* (Kugelberg *et al.* 2005). Of the *gyrA* mutations that cause bacterial fluoroquinolone resistance *in vitro* those that occur at codons 83 and 87 in *gyrA* occur most frequently in other bacterial species (Bachoual *et al.* 2001; Dessus-Babus *et al.* 1998; Mouneimne *et al.* 1999; Ruiz *et al.* 1998; Vila *et al.* 1995). There is evidence that single mutations in gyrase or topoisomerase IV may predispose the genome to further mutation. As described previously Gillespie *et al* investigated the mutation rates of *S. pneumoniae* using ciprofloxacin and gemifloxacin as the selective agents. The mutation rate of second step mutations in isolates already containing a *gyrA* or *parC* alteration was higher than the first step mutation rates (Gillespie *et al.* 2003). In Bcc mutation rates of second step mutants are higher than the mutation rate of the first step mutation confirming this original observation.

The rate of mutation occurring in *B. cepacia* topoisomerase genes estimated by the experimental procedures described here is 9.6×10^{-11}. This is a lower rate than those estimates already published for related organisms but is of the same order of magnitude. There are no reported mutation rates in *B. cepacia*. Published measurements of mutation rate in other organisms are higher and range from of 10^{-6} to 10^{-8}. Published estimates include those in *E. coli* (Boe *et al*, 1994), *S. pneumoniae* (Gillespie *et al*, 2003), *P. aeruginosa* (Oliver *et al.* 2000) and *M. tuberculosis* (Billington, McHugh & Gillespie, 1999).

Mutation frequencies are reported more commonly than mutation rates. Mutation frequencies of single step spontaneous mutations resistant to FQs are low in comparison to nalidixic acid, which are typically $> 10^{-9}$ when selected at 8 x MIC. Mutation frequencies of Gram-negative bacteria selected at 4 x and 8 x MIC ranged from $>10^{-10}$ to 5.3×10^{-8} for temafloxacin, lomefloxacin and flerofloxacin (Chin *et al.* 1988; Chin, Novelli, & Neu 1988). Mutation frequency to tosufloxacin resistance at 8 x MIC for *S. aureus*, *E. coli* and *P. aeruginosa* were $< 10^{-10}$ (Espinoza *et al.* 1988). Phenotypic mutation frequency to ciprofloxacin resistance in *C. jejuni* is approximately 1×10^{-8} (Gootz & Martin 1991).

Mutation rate experiments (estimated using alteration in phenotype from susceptible to resistance) have estimated a mutation rate of 1.1×10^{-9} using ciprofloxacin as the selective antibiotic and 1.6×10^{-11} using gemifloxacin (Gillespie *et al*, 2003) for *S. pneumoniae*. Similarly in *M. tuberculosis* the estimated mutation rate in *rpoB*, conferring rifampicin resistance, was found to be 1×10^{-10} divisions per generation (Billington, McHugh, & Gillespie 1999). However phenotypic increases in MIC can occur due to induction of efflux

pumps that reduce accumulation of antibiotic within the cell. These changes are not due to point mutations in topoisomerase genes. Thus an estimated phenotypic mutation rate may result from numerous genotypic changes in different genes and at different loci within these genes. A mutation rate calculated including confirmed mutations in a single target gene only, is likely to be lower than a phenotypic mutation rate. For example the mutation rate in *B. cepacia* in *gyrA*, using the Lea and Coulson method of the median (Lea & Coulson 1949) at 4 x MIC without genotypic confirmation gives an estimate of 2×10^{-8} compared to 9.6×10^{-11} as measured by the p_0 on agar containing 6 x MIC with genotypic confirmation. Genotypic mutation rate could not be determined at 4 x MIC because the number of cultures in which a topoisomerase mutation occurred was very small. This may be due to the presence of multiple target genes and non heritable changes. Both approaches are valid but are essentially different measures. Previous measurements of mutation rates vary depending on the selective antibiotic concentration and the fluoroquinolone agent tested.

Increase in efflux activity results in smaller elevations of MIC than mutations in topoisomerase genes. If incorporation of reserpine into agar reduces the MIC of a drug then it can be concluded that efflux pumps have a role in the observed increase in resistance. In this thesis it is hypothesised that the low level increase in antibiotic resistance in mutants not containing topoisomerase mutations is due to altered expression of efflux pumps. Bast *et al* considered strains to contain active efflux if there was a four fold or greater decrease in MIC to the drug in question in the presence of reserpine (Bast *et al.* 2000). The advantages of this assay are that it is convenient and does not rely on measurement of fluorescence. However reserpine is labile and should be used immediately once in solution (Brenwald, Gill, & Wise 1998). Neyfakh *et al* demonstrated that reserpine can be inactivated by incorporation into agar (Neyfakh, Bidnenko, & Chen 1991). However Brenwald *et al* found no difference in

the effect of reserpine on MIC between MICs determined in broth or by agar incorporation (Brenwald, Gill, & Wise 1998).

Exclusion of antibacterial agents from the cell by efflux pumps is a major cause of antibiotic resistance in bacteria. In Gram- negative bacteria multi drug efflux pumps can extrude antibiotics from multiple classes to cause multiple resistance (Nikaido 1996). Increase in fluoroquinolone accumulation in a resistant strain that was promoted following addition of energy inhibitor such as carbonyl cyanide m-chloro phenylhydrazone (CCCP) has been used to identify efflux. However increase in accumulation following CCCP treatment can occur in the absence of efflux mechanisms (Furet, Deshusses, & Pechere 1992). Therefore in order to demonstrate efflux reduced accumulation of fluoroquinolone must occur in the resistant cell compared to the susceptible cell.

The estimated mutation rate in topoisomerase genes in *B. cepacia* is low. This would mean that large numbers of bacteria will be required for a mutation to resistance to occur during infection. However this experimental protocol would tend to result in low estimates and higher values would have been observed if a phenotypic mutation rate estimating method was chosen instead. All of the available methods only give an estimate of mutation rate. Each method relies on a set of pragmatic assumptions that are made in order to make estimations possible and therefore each mutation rate is only an estimate and can not represent exactly what is happening *in vivo*. Mutation rates in the same system estimated by separate groups via different methods can give very different estimates. For example it was noted that estimated mutation rates of purine analogue resistance in a Chinese Hamster V79 cell line by two groups differed by more than 1000 times (Chu & Malling 1968; Harris 1971; Kendal & Frost 1988).

In conclusion a standardised protocol for estimation of mutation rate in *B. cepacia* has been developed. The mutation rate in topoisomerase genes in the Bcc is low, although the mutation rate to phenotypic resistance is higher and this most likely occurs due to fluoroquinolone efflux from the cell. These studies provide important data that assist in the understanding of resistance development in this important organism.

Chapter 4 Measurement of fitness using a biofilm assay

4.0 Introduction

4.1 Biofilms

4.1.1 Biofilms of *B. cepacia*

It is likely that the ability of bacteria of the Bcc complex to form biofilms limits eradication by antimicrobial therapy and promotes long term persistence in the CF lung. For example, in a study of 21 cystic fibrosis patients those patients that died were colonised by biofilm forming isolates of Bcc bacteria (Cunha *et al.* 2004). The extent of biofilm formation varies between genomovars of the Bcc. *B. multivorans* and *B. cenocepacia* are capable of greater biofilm formation than *B. cepacia*, *B. stabilis* and *B. vietnamensis* (Conway, Venu, & Speert 2002). However *B. dolosa* has since been demonstrated to be comparable to *B. cenocepacia* and *B. multivorans* in biofilm formation (Caraher *et al.* 2006).

B. cepacia has been shown to form biofilms *in vitro* and it is likely that this occurs in the cystic fibrosis lung as has been demonstrated for *P. aeruginosa* (Mathee *et al.* 1999).

4.1.2 Multispecies Biofilms

Most biofilms in nature are undoubtedly composed of more than one species (Stoodley *et al.* 2002). However single species biofilms are important clinically. Polymicrobial biofilms tend to more stable and thicker than single species biofilms (Allison, McBain, & Gilbert 2000). In the CF lung, *P. aeruginosa* colonisation of the cystic fibrosis lung occurs before colonisation by *B. cepacia* (Govan & Deretic 1996; Koch & Hoiby 1993) leading to the conclusion that Bcc bacteria are able to adhere to the *P. aeruginosa* biofilm.

P. aeruginosa colonisation occurs before Bcc colonisation. *B. cepacia* must therefore be able to adhere to a *P. aeruginosa* biofilm. Dual species biofilms can develop following continuous challenge irrespective of the species forming the primary biofilm. However *P. aeruginosa* is always dominant and this may be due to the production of a substance that is inhibitory to *B. cepacia* (Al Bakri, Gilbert, & Allison 2004). Communication between these organisms has been documented; *B. cepacia* can detect and respond to acyl homoserine lactones produced by *P. aeruginosa*. Addition of supernatant from stationary phase *P. aeruginosa* culture fluid to *B. cepacia* culture increased siderophore, lipase and protease production (McKenney, Brown, & Allison 1995). This communication is unidirectional as *P. aeruginosa* cannot respond to the *B. cepacia* signals (Riedel *et al.* 2001). As many Gram-negative bacteria produce acyl homoserine lactones and inhabit similar niches, it is likely that further interspecies communication occurs.

4.1.3 Biofilm Models

There are numerous systems that have been developed and used to model growth of bacterial biofilms. These include growth on sorborod filters (Hodgson *et al.* 1995), the constant depth film fermenter (Peters & Wimpenny 1987), microtitre plates (O'Toole & Kolter 1998), flow cells and rotating disc bioreactors (Zelver *et al.* 1999). No single model is ideal for all experimental scenarios as each has been designed for a specific purpose. For bacteria that grow as biofilms during infection, a biofilm quantification assay should be included as a measure of fitness. This is because the propensity to form biofilms is likely to affect fitness. For use as a fitness assay, the model of biofilm growth should be simple and reproducible with sufficient replicate biofilms to allow statistical analysis. However, other criteria are important in selection of a model. It is desirable that

the biofilm is grown in as steady a state as possible. Depending on the question being asked of the system, the conditions of the biofilm should mimic the environmental conditions of the *in vivo* biofilm, therefore the ability to control the environment should exist. Simplicity of operation is advantageous. Capacity to substitute the substrata and vary the depth of the biofilm allows flexibility. Aseptic conditions are required with a means of inoculating the biofilm without contamination. Other factors that should be considered while selecting a model include incubation temperature, presence of antibiotics, organism and cell numbers required, flow rate, sheer and presence of a conditioning film.

4.1.4 Quantification of biofilm growth

Growth within a biofilm can be measured by sacrificing cells from the biofilm by sonication and/or vortexing before determining viable cell numbers estimated by plate counting, although using this method biofilm specific characteristics may be lost. No difference in bacterial counts was observed between *P. fluorescens* and *B. subtilis* biofilms disrupted by vortexing, sonication or shaking with beads (Lindsay & von Holy 1997). Biofilm growth can also be visualised *in situ* with fluorescent probes and reporter genes (Geesey 2001) as well as using imaging software to estimate biofilm coverage of the surface. Bioluminescence is particularly useful *in vitro* and *in vivo* as it allows biofilms to be monitored in real time (Kadurugamuwa *et al.* 2003; Kadurugamuwa *et al.* 2003). Selected models used for quantifying biofilm formation, are shown in table 4.1.

Model	Organisms	Flow	Substratum	Method of quantifying biofilm	Reference
Constant Depth Film Fermenter (CDFF)	*B. cepacia*, *P. aeruginosa*, oral bacteria	Continuous	Variable	Vortex plug, viable count. Can be observed directly by SEM and CLSM	(Hengtrakool, Pearson, & Wilson 2006; Hope & Wilson 2006; Peters & Wimpenny 1987; Pratten, Barnett & Wilson 1998)
CDC Biofilm Reactor	Gram negative bacteria	Continuous	Plastic connectors	Sonicate, vortex, homogenise, viable count	(Murga, Miller, & Donlan 2001)
Modified Robbins device	*B. pseudomallei*, *P. aeruginosa*	Batch	Variable	Viable count	(Honraet & Nelis 2006; Mikuniya *et al.* 2005; Vorachit *et al.* 1993)
Calgary biofilm device	*P. aeruginosa*, *S. aureus*, *E. coli*	Batch	Plastic pegs	Sonicate peg, viable count	(Ceri *et al.* 1999)
Sorborads Filter	*S. aureus*, *P. aeruginosa*	Continuous	Filter paper	Vortex, viable count	(Hodgson *et al.* 1995)

Table 4.1 Models that have been used to quantify bacterial biofilm growth

4.1.5 Examples of biofilm models

4.1.5.1 Constant Depth Film Fermenter

A constant depth film fermenter (CDFF; University of Wales, Cardiff) was used to grow a *B. cepacia* biofilm. A photograph of the apparatus is shown below (figure 4.1). The CDFF was originally chosen for use as a fitness assay for a number of reasons. Firstly this model allows the growth of many replicate biofilms (up to 75) that can be maintained in a semi

steady state, the environmental conditions can be controlled and the fermenter can be incubated in an aerobic or anaerobic atmosphere. Media can be supplied at a constant rate, effluent removed and inoculum can be added aseptically. The CDFF consists of an autoclavable, enclosed, stainless steel turntable which rotates underneath polytetrafluoroethylene (PFTE) scraper blades (figure 4.2) (Peters & Wimpenny 1987). This action removes excess growth and media. This model has previously been used to study mixed biofilms of *B. cepacia* and *P. aeruginosa* (Al Bakri, Gilbert, & Allison 2004), *Candida albicans* (Lamfon *et al.* 2003) and oral bacteria (Hengtrakool, Pearson, & Wilson 2006; Hope & Wilson 2003; Hope & Wilson 2006; Pratten, Barnett & Wilson 1998).

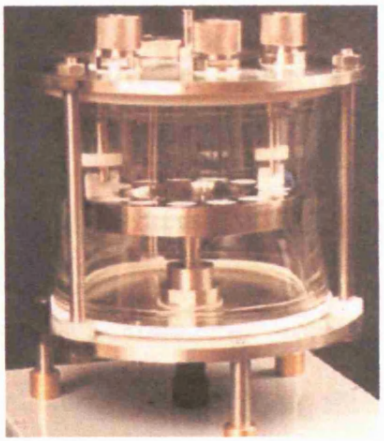


Figure 4.1 Photograph of a Constant Depth Film Fermenter

Figure 4.2 Photograph of PFTE Scraper Blades

The turntable contains 15 holes that contain PFTE pans, which can be removed via a sampling port. Each pan in turn contains 5 plugs (figure 4.3).

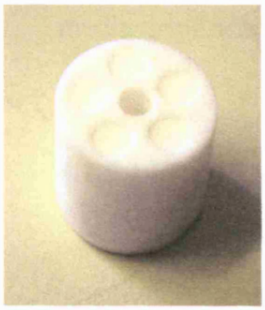


Figure 4.3 Photograph of Sample Pan

The depth of these plugs and therefore the depth of the biofilm can be set to a predetermined level. Media enters the apparatus through a port in the stainless steel top and is delivered via a peristaltic pump at a constant rate.

4.1.5.2 Sorborods Filter

The sorborods filter consist of a cylindrical paper sleeve encasing a concertina of cellulose fibres. Single filters are inserted into lengths of PVC and sterilized by autoclaving (Maira-Litran, Allison, & Gilbert 2000). Sorborods filters can be incubated and media is supplied at a constant rate via a peristaltic pump. In order to enumerate cell numbers, filters can be sacrificed and vortexed in saline and a plate count can be performed. This system has been used to model mixed biofilms of *B. cepacia* and *P. aeruginosa* (Al Bakri, Gilbert, & Allison 2004).

4.1.5.3 Drip Flow Reactors

This biofilm model consists of petri dishes containing stainless steel slides onto which media drips onto at a constant rate (Huang *et al.* 1998).

4.1.5.4 Flow Cells

Flow cells are small continuous flow systems that allow direct observation of the intact biofilm via a viewing port. Media may be recycled or may be 'once flow', meaning that media enters the system, passes through the cell, and is then collected as waste. Flow cells are often used for short term colonization studies because adhesion can be observed in real time.

4.1.5.5 Crystal Violet Microtitre Plate Method

The crystal violet microtitre plate biofilm assay is a simple and rapid method which quantifies biofilm formation indirectly via the extent of crystal violet binding to bacterial peptidoglycan to the wells of a microtitre plate. Bacteria are grown in wells of a microtitre plate that contain media. Wells are washed to remove planktonic cells and incubated with crystal violet. Unbound crystal violet is removed by repeated washing with water. Bound crystal violet is solubilised with ethanol and the resulting optical density determined. O'Toole *et al* initially used the assay to assess the impact of growth conditions and as a screen for mutants deficient in biofilm formation in *P. fluorescens* (O'Toole & Kolter 1998). Modifications of this assay have been used to study biofilm formation in a number of bacteria including *E. coli* (Pratt & Kolter 1998), *V. cholerae* (Watnick & Kolter 1999), *S. aureus* (Stepanovic *et al.* 2003) *Listeria monocytogenes* (Djordjevic, Wiedmann, &

McLandsborough 2002) and *Streptococcus gordonii* (Loo, Corliss, & Ganeshkumar 2000). This method has also been used to quantify biofilm growth in *B. cepacia* mutants that are defective in biofilm formation and of a panel of Bcc strains of different genomovars (Conway, Venu, & Speert 2002; Huber *et al.* 2001).

A novel assay, the BioFilm Ring Test, has been described that measures biofilm formation based on the immobilization of magnetic beads embedded in the bacterial aggregate. Biofilm formation has been measured in *L. monocytogenes*, *E. coli*, *Staphylococcus carnosus* and *S. xylosus* (Chavant *et al.* 2007). This method gives faster comparable results to the crystal violet assay and does not include any washing or staining steps, which can lead to poor reproducibility.

4.1.6 Effect of Incubation Conditions

Incubation atmosphere may affect biofilm formation. For example, *S. aureus* forms less biofilm in CO_2 than in aerobic incubation (Stepanovic *et al.* 2003). Different environmental conditions may stimulate different development pathways. O'Toole *et al* demonstrated that in *P. fluorescens* there are multiple biofilm development pathways that are regulated by nutrient status (O'Toole 2003).

4.2 Aims of chapter

Aims were to select and optimize a model of biofilm growth which could be used as a fitness assay to measure fitness costs associated with topoisomerase mutations, selected *in vitro* and characterized in chapter 3.

4.3 Materials and Methods

4.3.1 Constant Depth Film Fermenter

Biofilms were grown in a CDFF, (section 2.10.1.1.) Viable CFU counts were performed at 4, 24, 48, 72 and 120 hours, (section 2.10.1.1.3.) Biofilms and visualized using Confocal Scanning Laser Microscopy (CSLM), (section 2.10.1.1.4).

4.3.2 Crystal Violet Microtitre Plate Assay

Quantification of biofilm growth was facilitated by spectrophotometric measurement of crystal violet binding using a previously published method (O'Toole & Kolter 1998) and has previously been used to quantify biofilm growth in Bcc bacteria (Conway, Venu, & Speert 2002), (section 2.9.1.2). Microtitre plates were incubated for 0, 1, 4, 7, 24 and 48 hours in order to optimize incubation period, see figure 5.7. For each time point 8 replicate wells were used. Three replicate experiments were performed.

4.4 Results

4.4.1 Growth of *B. cepacia* biofilm

Viable CFU counts were used to construct a growth curve of the *B. cepacia* biofilm (figure 4.4).

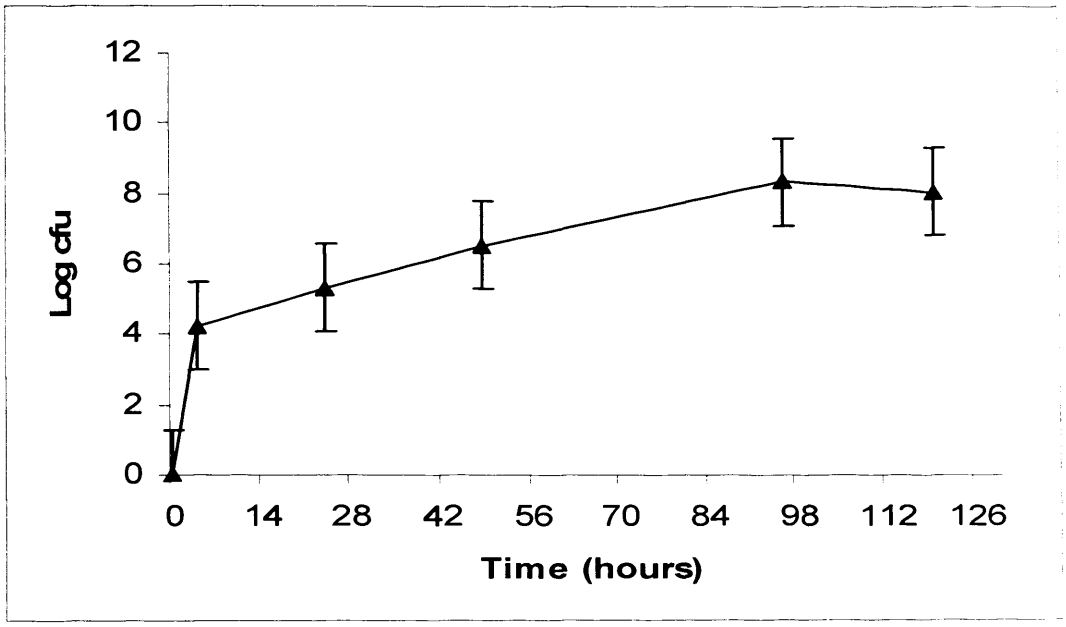


Figure 4.4 Growth curve of *B. cepacia* 10661. Mean colony forming units for four replicate runs. Error bars show 1 standard deviation at each sample point.

4.4.2 Confocal laser scanning microscopy of the *B. cepacia* biofilm

Sample plugs were removed at 4, 24, 48, 72 and 120 hours. Confocal images demonstrating development of the *B. cepacia* biofilm at 4 and 72 hours are shown below. Images at other time points are not shown. A single slice of a completed 3D project was saved as a TIFF file and these are shown below. These images show progression of the *B. cepacia* biofilm. At 4 hours there is approximately 5% coverage and at 24 hours 25% coverage. At 48 hours coverage was approximately 40%. However the biofilm was less dispersed than at 24 hours. At 72 hours coverage was approximately 90% and 100% at 120 hours. At 72 and 120 hours water channels throughout the biofilm were visible.

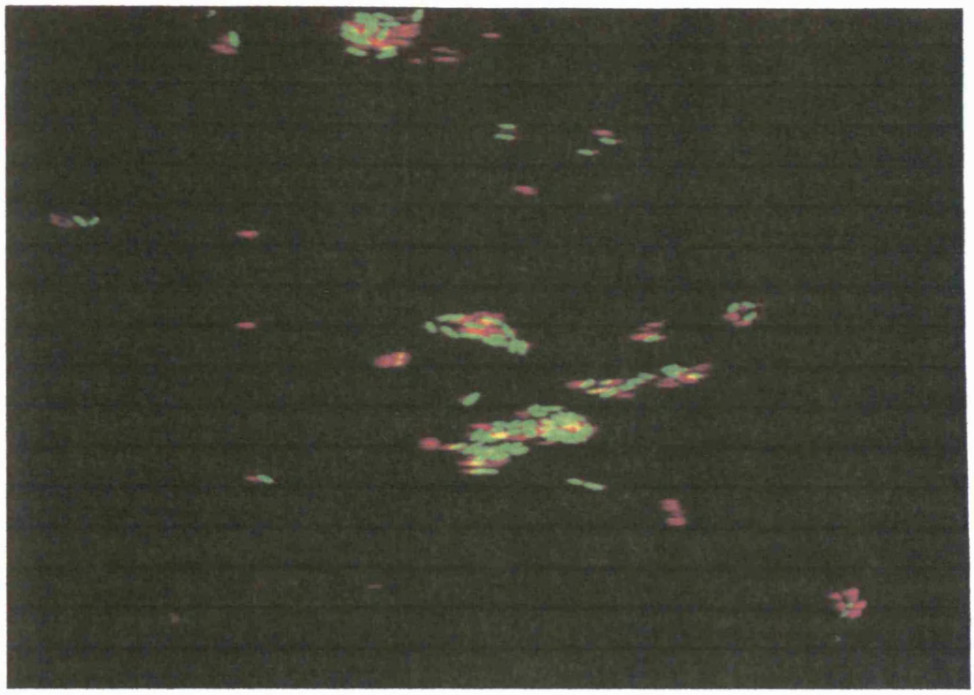


Figure 4.5 Image of biofilm at 4 hours. Single slice of a 3D representation of the biofilm, using ImageJ software. Viable cells appear green and non viable cells appear red.

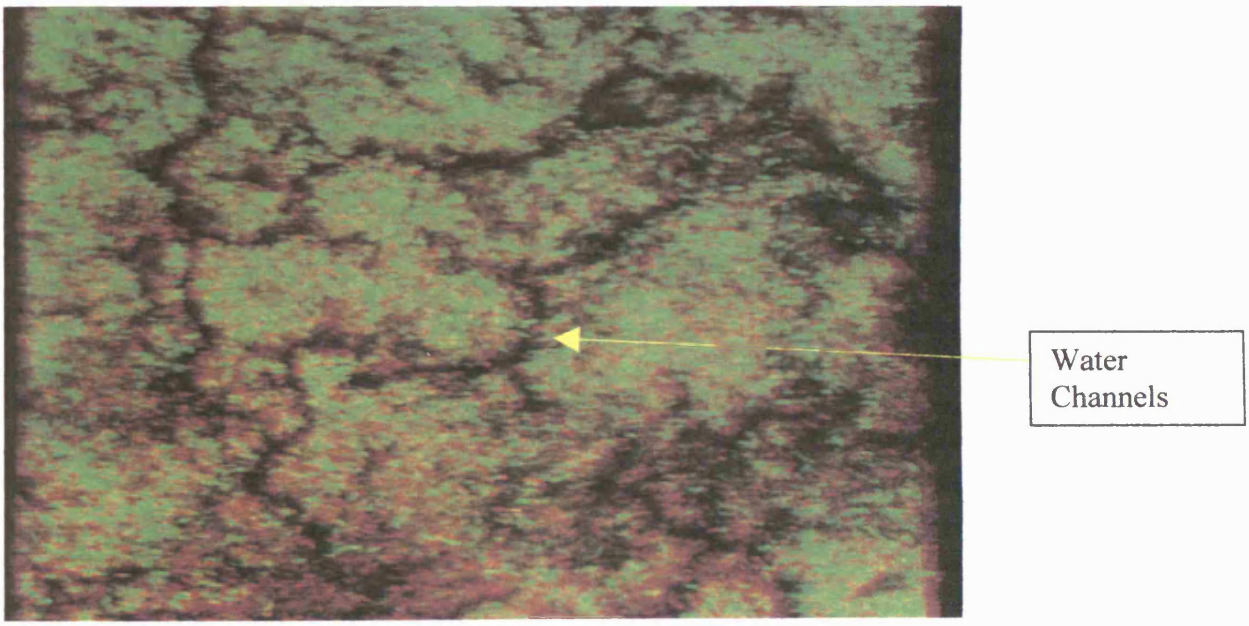


Figure 4.6 Image of biofilm at 72 hours

4.4.3 Effect of incubation period on biofilm formation

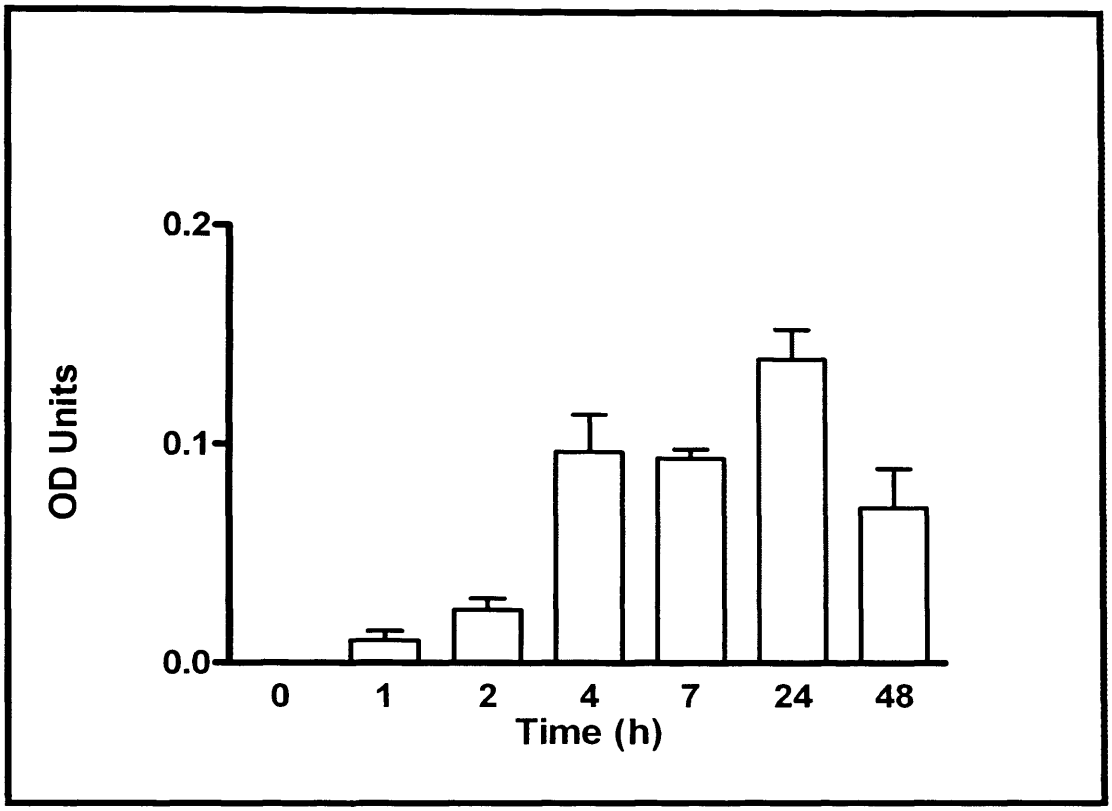


Figure 4.7 Biofilm growth at 0, 1, 2, 4, 7, 24 and 48 hours measured using the crystal violet microtitre plate method. Error bars represent SEM. Each experiment consisted of 8 replicare wells and the experiment was repeated three times.

4.5 Discussion

Although there is a limited understanding of biofilm biology, *P. aeruginosa*, *P. fluorescens*, *E. coli* and *V. cholerae* have been used extensively as models in biofilm research. For other bacterial species, less is known.

Scanning confocal laser microscopy (SCLM) is well suited to the study of biofilms (Shotton 1989) and has elucidated their three dimensional structure. The technique lacks resolution compared to electron microscopy but has the advantage of being able to

visualise living, hydrated biofilms. Biofilms of *B. cepacia*, visualized by SCLM, contained visible water channels between microcolonies (figure 4.6). These permeable water channels have been compared to primitive circulatory circuits and allow of exchange nutrients and toxic metabolites (Stoodley, Debeer & Lewandowski 1994). Biofilm architecture is affected by flow conditions (Stoodley *et al.* 1999). *P. aeruginosa* biofilms in turbulent flow form streamlined patches while in laminar flow monolayers interspersed with small circular microcolonies are formed (Purevdorj, Costerton, & Stoodley 2002).

Despite precautions taken, including ethanol flaming of the sampling port each time that a sample was taken, use of a surgical mask to prevent contamination during sampling and overnight inoculation through sterile tubing, contamination was a problem using the CDFF. Together with laborious and time consuming nature of the CDFF it does not appear to be suitable for use as a fitness assay in the context of these studies.

The crystal violet assay involves multiple washing steps resulting in loss of cells and variability was observed between wells using this assay. A fixation step has not been included in assays used to quantify biofilm formation in the Bcc (Conway, Venu, & Speert 2002; Huber et al. 2001) and was not included here. However a fixation step has been included in some crystal violet biofilm assays (Stepanovic *et al.* 2003) and this step may increase reproducibility between assays. Reflectance assays have been developed to measure biofilm formation, utilizing changes in optical reflectance. Various surfaces (opaque or non opaque) can be used and biofilm samples can be fixed and stored before taking a measurement (Broschat *et al.* 2005). The ability to form biofilms of 14 strains of *Enterococcus* was assessed using both the crystal violet assay and the reflectance assay.

Similar results were obtained independently with both assays as the same isolates were identified as the highest and lowest biofilm producers. However 2 isolates forming abundant biofilms in the crystal violet assay, did not form biofilms in the reflectance assay (Broschat *et al.* 2005).

Huber *et al* used the crystal violet assay to assess the ability of *B. cepacia* H111 to form biofilms containing *cepI* and *cepR* genes that had been inactivated by random transposon mutagenesis (Huber *et al.* 2001). Biofilm formation was not affected by composition of the media or incubation temperature (30°C or 37°C). Although biofilms grew on polystyrene microtitre plates, *B. cepacia* cells could not form a biofilm on polypropylene. The *cepI* and *cepR* mutants were defective in their ability to form AHLs and in biofilm formation. Addition of AHLs to the media restored biofilm formation (Huber *et al.* 2001). It is likely that the *cep* quorum sensing system has a role in biofilm formation in *B. cepacia.*

In this study LB broth (0.5% casamino acids) was used with incubation at 30°C for 24 hours. Following longer incubation, the amount of biofilm detected decreased, see figure 4.7. It is likely that this is due to dispersal of the cells from the biofilm following a reduction in available nutrients in the LB broth.

In this thesis the crystal violet binding assay was adopted as a biofilm model to measure fitness. This method was chosen as it is simple, rapid and allows investigation of large numbers of isolates.

Chapter 5: Fitness cost of fluoroquinolone resistance in *B. cepacia*

5.0 Introduction

5.1 Determination of fitness

5.1.1 Choosing appropriate fitness models

The probability of resistance mutations occurring in target genes and any fitness deficit associated with them are important as these factors affect the likelihood of resistance developing and being maintained in the population. Growth rate is an accepted measure of fitness to measure fitness deficits associated with antibiotic resistance (Billington, McHugh, & Gillespie 1999; Gillespie, Voelker, & Dickens 2002). Laboratory growth conditions usually involve growth in rich media and these conditions may vary dramatically from natural growth conditions in the environment or human body. Growth in rich media provides a selective advantage to fast growing strains and therefore faster growing strains may be selected for in the laboratory. No single measure of fitness is sufficient to describe the impact of resistance on the survival ability of bacterial strains. Hence, models have been selected that represent growth and survival conditions for *B. cepacia* to increase the clinical relevance (Conway, Venu, & Speert 2002; O'Toole & Kolter 1998; Sanchez *et al.* 2002). Fitness models can be adapted for use in many bacterial species by changing growth conditions.

P. aeruginosa is known to form biofilms within the human body, for example within the CF lung (Govan & Deretic 1996; Singh *et al.* 2000) and it is likely that Bcc bacteria also grow as biofilms in the CF lung. In these cases a biofilm model may be included when measuring fitness in order to reflect growth and environmental survival conditions of the species of interest. Similarly environmental survival e.g. resistance to drying or survival in water is

relevant for nosocomial pathogens which can be transmitted via contaminated surfaces. Bcc bacteria are introduced into the environment by cystic fibrosis patients during physiotherapy and this may allow cross infection to occur (Ensor *et al.* 1996). The risk of transmission remains unclear but the ability of bacteria to survive drying allows their maintenance on environmental surfaces (Smith, Eng, & Padberg, Jr. 1996) and also the possibility of transmission between hosts. Bcc bacteria can survive for extended periods in respiratory droplets on surfaces at room temperature (Drabick *et al.* 1996). These bacteria have also been identified in shower drains, soil (Nelson *et al.* 1991) and homes of CF patients (Butler *et al.* 1995).

5.1.2 Semi automated liquid culture system

Viable cell count estimation is subjective and dependent on enumeration of colonies that grow under the growth conditions provided, introducing sampling error. In using an automated system which is less time consuming and less prone to operator variation, it is possible to perform replicate growth rate estimations for large numbers of strains.

In this study the Bactec 9240 continuous, blood culture system with standard aerobic medium (Plus Aerobic/F) was used to determine growth rate. The Bactec 9000 (9240/9120/9050) series of automated blood culture systems are used to rapidly detect viable microorganisms in clinical specimens. Bactec Plus Aerobic vials, contain 25 mL of enriched soybean-caesin digest broth, 0.05% sodium polyanetholesulfonate (SPS), resins, CO_2, O_2 and a sensor. This sensor, within each vial, responds to changes in oxygen and carbon dioxide levels as a result of bacterial metabolism. These changes are measured by an increase in the fluorescence of

the sensor which is monitored every ten minutes. A positive fluorescence reading indicates an increase in CO_2 or decrease in oxygen and the presence of microorganisms in that vial.

5.2 Aims of chapter

B. cepacia was used as a model to assess the fitness costs of FQ resistance mutations as these bacteria are highly resistant to many antibacterial agents, form biofilms within the lungs and, due to the chronic nature of cystic fibrosis infection, are exposed to long term antibiotic treatment. The fitness costs of single and double topoisomerase mutations, selected *in vitro* and characterised in chapter 3 were assessed using fitness models relevant to transmission. These included models of biofilm and planktonic growth and environmental survival. A method using a semi-automated liquid based culture system to measure growth rates was developed.

5.3 Materials and Methods

5.3.1 Culture Conditions

The strain used in these studies was *B. cepacia* 10661 (National Collection of Type Cultures, HPA, Colindale, UK) and subsequent mutants of this strain. The ciprofloxacin MIC of this strain was 1.0 mg/L as determined by E-test (see section 2.6.1). To ensure uniformity of strains the isolate was stored at -70°C using Cryobank cryogenic beads (Pro-lab Diagnostics, Neston, UK). For each fitness assay the culture was initiated by inoculating a bead, from a stored culture, onto nutrient agar (Oxoid, Basingstoke, UK) then incubated at 37°C for 18 hours.

5.3.2 MIC Determination

The MIC of parent and mutant strains were determined by ciprofloxacin E-test (AB Biodisk, Solna, Sweden; section 2.6.1) and by the agar doubling dilution method, according to the CLSI guidelines for susceptibility testing of aerobic bacteria (CLSI, 2006; section 2.6.2).

5.3.3 Selection of Resistant Mutants

Fluoroquinolone resistant mutants of *B. cepacia*, containing single and double mutations were selected *in vitro* (see chapter 3). First step spontaneous ciprofloxacin resistant mutants were selected at 2, 4, 6 and 8 x MIC, as described in chapter 3. Colonies were stored at -70°C for subsequent study using Microbank beads (Prolab Diagnostics, Neston, UK).

5.3.4 Miles and Misra Viable Cell Count

The number of CFU in 20 µl drops of diluted broth culture was determined using the Miles and Misra method, as described previously in section 2.5 (Miles & Misra 1938).

5.3.5 Fitness Assays

5.3.5.1 Biofilm Growth

Quantification of biofilm growth was achieved by spectrophotometric measurement of crystal violet binding using a previously published method (O'Toole & Kolter 1998). This method has been used to quantify biofilm growth in Bcc bacteria (Conway, Venu, & Speert 2002) and is described in detail in section 2.9.1.2.

5.3.5.2 Planktonic Growth

The method of Youmans and Youmans was modified (Youmans & Youmans 1949) to determine growth rate (as described previously in section 2.9.2).

5.3.5.3 Competition Assays

Fitness of the fluoroquinolone resistant mutants, containing single and double topoisomerase mutations, was compared to the susceptible parent using a modification of a previously published method (section 2.9.3) (Billington, McHugh, & Gillespie 1999; Gillespie, Voelker, & Dickens 2002).

5.3.5.4 Environmental survival

The ability of the resistant mutants to survive in water and survive drying was compared to the susceptible parent, described in sections 2.9.4 and 2.9.5

5.3.5.4.1 Statistical Analysis

The statistical significance of differences in survival in water and during drying between the clinical isolates was assessed by one way ANOVA, using GraphPad software version 3.00 for Windows (GraphPad Software, California, USA). Differences in generation time were assessed by Student's t-test.

5.4 Results

5.4.1 Fitness Assays

Using the modified Youmans and Youmans method, relative fitness of Asp87Asn (F1) and Thr83Ile (F2) compared to the susceptible parent were 1.01 +/- 0.01 SEM and 1.01 +/- 0.152 SEM, respectively using paired competitive cultures. Differences in relative growth rates of the single *gyrA* mutants found during paired competition assays were not significant, as determined by Students t-test. Relative fitness of the double mutants F3 and F4 was 0.88 +/- 0.12 and 0.78 +/- 0.18 respectively. Double mutation in topoisomerase genes, therefore, did incur a fitness deficit as measured by competitive growth culture.

Strain	Generation Time (min) (+/- 95% confidence interval)	Generation Time P value (Students T test)	Relative Fitness (+/- S.E.M)	Relative fitness P value (Students T test)
WT	**38.0 (37.06 – 38.94)**			
F1 Asp87Asn	**37.0 (36.77 – 37.23)**	0.331	1.01 +/- 0.01	0.831
F2 Thr83Ile	**37.1 (36.90 – 37.3)**	0.377	1.01 +/- 0.152	0.868
F3 Asp87Asn Ser80Leu	**43.0 (41.85 – 44.15)**	0.004	0.88 +/- 0.12	0.003
F4 Thr83Ile Ser80Leu	**45.7 (44.2 – 47.2)**	0.001	0.78 +/- 0.18	0.002

Table 5.1 Generation times (+/- 95% confidence intervals) and relative fitness (+/- S.E.M) of the susceptible parent and topoisomerase mutants F1 and F2 are the single *gyrA* mutants, Asp87Asn and Thr83Ile respectively. F3 contains Asp87Asn and the additional Ser80Leu *parC* mutation. F4 contains Thr83Ile and Ser80Leu. Differences in growth rate between wild type, F1 and F2 are not significant, as determined by Students t test and therefore do not have a measurable fitness cost. However F3 and F4 exhibit significantly slower growth.

Mutation in *gyrA* and *parC* did not affect biofilm formation in *B. cepacia*, see figure 5.1. The transmissibility of *B. cepacia* isolates could be affected by the ability to survive in water and survive during drying. A decrease in viability over time was observed for the susceptible parent and the fluoroquinolone resistant mutants but there were no statistically significant differences between the mutants and the susceptible parent.

As shown in figure 5.2 and figure 5.3, no significant differences were found at any time point in the ability of the *gyrA* and *parC* mutants to survive in water or survive drying, as compared to the parent *B. cepacia* strain.

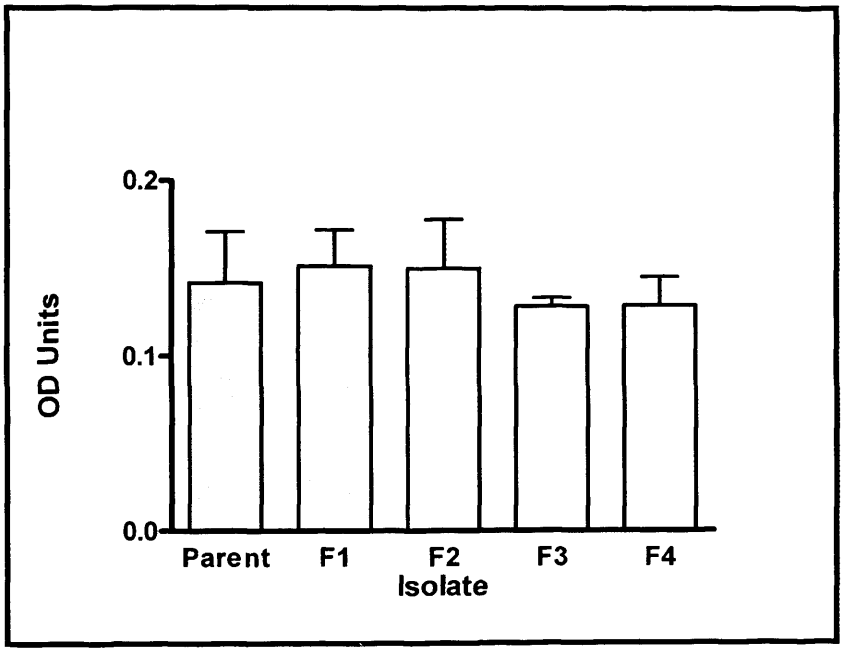


Figure 5.1 Effect of topoisomerase mutations on ability of *B. cepacia* to form biofilms. Mutation in *gyrA* and *parC* did not affect biofilm formation in *B. cepacia*. Error bars represent SEM. Differences are not significant, determined by one way ANOVA using the GraphPad Prism software.

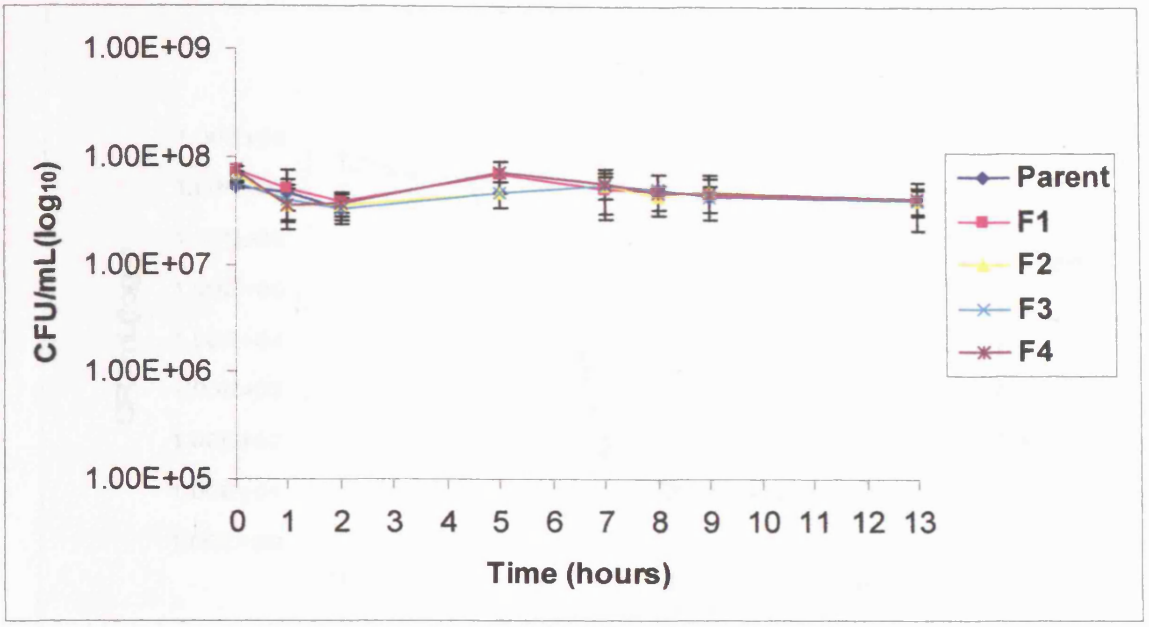


Figure 5.2 Effect of topoisomerase mutations on the survival of *B. cepacia* in water. Sampled at 0, 1, 2, 5, 7, 8, 9 and 13 days. Survival of *B. cepacia* in water was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars demonstrate SEM. Differences in survival are not significant.

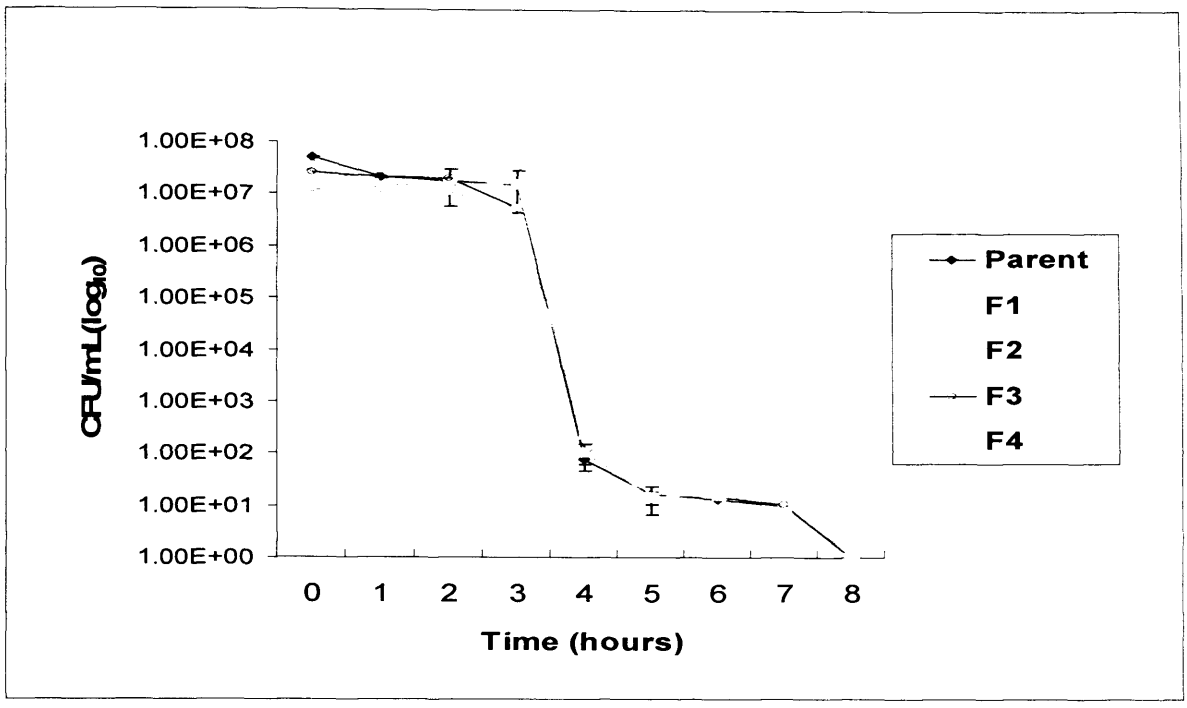


Figure 5.3 Effect of topoisomerase mutations on the survival of *B. cepacia* on dry surfaces. Survival of *B. cepacia* on dry surfaces was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars demonstrate the standard error of the mean. Error bars are not shown if obscured by the symbol. Differences in survival are not significant.

5.5 Discussion

No fitness cost was found in fluoroquinolone resistant mutants containing a single topoisomerase mutation as measured by growth rate, biofilm growth and environmental survival. A significant fitness cost was found in *gyrA* and *parC* double mutants, in growth rate and competitive culture only. Fitness was not impaired in any other assay (Pope *et al.* 2007).

The cost of mutations conferring fluoroquinolone resistance depends on the bacterial species, the number of resistance gene mutations and the level of resistance conferred as reported in *E. coli* (Bagel *et al.* 1999), *S. pneumoniae*, (Gillespie, Voelker, & Dickens 2002) *S. typhimurium* (Giraud *et al.* 2003) and *P. aeruginosa* (Kugelberg *et al.* 2005). Single topoisomerase mutations in *S. pneumoniae* incur no or low cost while double mutations in *gyrA* and *parC* incur a significant cost of approximately 0.85 relative to the susceptible parent (Gillespie, Voelker, & Dickens 2002). Topoisomerase mutations in *S. typhimurium* (Giraud *et al.* 2003), conferring small increases in resistance, are also associated with either no reduction in fitness or a small loss. In *E. coli* the cost varies between strains (Bagel *et al.* 1999). The fitness cost has been shown to vary between organisms and depends on the resistance mutations present.

Common mutations in *rpoB,* conferring resistance to rifampicin in clinical isolates of *M. tuberculosis,* incur a lower cost than mutations that occur more rarely (Billington, McHugh, & and Gillespie 1999; Gagneux *et al.* 2006; Mariam *et al.* 2004). The most common *rpoB* mutations, His481Asn and Ser529Leu, found in clinical isolates of *S. aureus* do not exhibit a fitness cost (O'Neill *et al.* 2006). Moderate level fluoroquinolone resistance in *P. aeruginosa*, conferred by single mutations in *gyrA* and *gyrB*, incurs a fitness cost depending on the location and identity of the mutation in *P. aeruginosa* (Kugelberg *et al.* 2005).

Multiple mutations in topoisomerase genes, associated with high levels of resistance exhibit reduced fitness in other organisms. High level fluoroquinolone resistant *P. aeruginosa* mutants (>256 mg/L) containing double mutations in topoisomerase genes or other genes, all demonstrate reduced fitness (relative fitness of approximately 0.5, compared to wildtype), as measured by growth rate (Kugelberg *et al.* 2005). In *E. coli* double mutations in *gyrA*

(Ser83Leu and Asp87Gly), had a decrease in fitness as measured by increase in generation time of 33% (Bagel *et al.* 1999). These measurements of fitness cost are greater than the fitness costs of *gyrA* and *parC* mutations in *B. cepacia* that have been measured in this study. Triple mutations in topoisomerase genes are required for high level fluoroquinolone resistance (256 mg/L) in *S. typhi* (Turner, Nair, & Wain 2006). The MIC of mutants containing Ser83Phe/Tyr, Asp87Asn and Glu84Lys mutations was 32-64 mg/L. However this is a lower level of resistance than conferred by the Asp87Asn Ser83Ile double mutation in *B. cepacia*. Mutations in *parC* did not affect MIC without existing mutation in *gyrA* (Turner, Nair, & Wain 2006). The fitness cost depends on the resistance conferring mutation, the genetic background of the strain and the presence of compensatory mutations.

In vitro resistance to antibacterial agents, other than the fluoroquinolones, occurs via stepwise accumulation of mutations in target genes. Chromosomal resistance to mupiricin in *S. aureus* is attributed to mutations in isoleucyl tRNA synthetase, the most common mutations being Val588Phe and Val631Phe (Hurdle, O'Neill, & Chopra 2004). In agreement with the results reported in this thesis, first step mutations confer no measurable cost while second step mutations confer a fitness cost *in vitro* and *in vivo*. Hurdle *et al* reported relative growth rates of second step mutations range from 0.24-0.63 compared to wildtype. Fitness was restored following compensatory mutation occurring during sub culture (Hurdle, O'Neill, & Chopra 2004).

Expression of many genes is dependent on appropriate supercoiling (Steck *et al.* 1993). It has been reported that the observed fitness cost of fluoroquinolone resistance mutations is associated with alterations in supercoiling efficiency (Bagel *et al.* 1999). Growth rates of resistant mutants may be reduced as positive coils may accumulate in front of the replication

fork due to the decreased supercoiling activity of the altered DNA gyrase (Drlica & Zhou 1997). Compensatory mutations may occur that restore supercoiling activity of gyrase and may restore growth rates to levels comparable to the susceptible parent. This has been demonstrated in *P. aeruginosa* (Kugelberg *et al.* 2005). Alterations in supercoiling were measured by separation of topoisomers on agarose gels containing chloroquine. Variations exist in the electrophoretic mobilities depending on the extent of supercoiling (Bagel *et al.* 1999).

No cost resistance mutations have been reported in a number of organisms, conferring resistance to various antimicrobial agents. These include; fluoroquinolone resistance in *S. pneumoniae* (Gillespie, Voelker, & Dickens 2002), glycopeptide resistance in Enterococci (Ramadhan & Hegedus 2005) and aminoglycoside and spectinomycin resistance in *Borrelia burgdorferi* (Criswell *et al.* 2006).

However fitness assays lack sensitivity, failing to detect fitness costs of <1% (Andersson & Levin 1999). Small but significant deficits may therefore not be detected. These 'no cost' mutations may vary in impact in different environments. It is possible that mutation in *gyrA* did have an associated fitness deficit that could not be measured by these methods. For example due to large values of error in the biofilm assay, only large fitness costs could be detected. During the paired competition assays relative fitness was assessed after 10 hours. Small differences in relative growth between two strains may have been detected if successive cycles of pairwise competitive cultures were performed. It is unlikely that compensatory mutations have occurred that ameliorate fitness costs as mutants were immediately stored at -70°C following selection.

Youmans and Youmans measured the difference in time to positivity of cultures of *M. tuberculosis* inoculated with serial dilutions of a culture to calculate the growth constant k and generation time (Youmans & Youmans 1949). A liquid based automated culture system has been used in this thesis to determine growth rate and generation time, avoiding time consuming and laborious standard growth curve techniques. This has proved to be a reliable, reproducible and simple method for measurement of growth rate (Pope *et al.* 2007). It is possible that differences in lag time result in a delayed time to positivity. Cells were therefore added when they were in exponential phase to minimise the affect of differences in lag time. Laurent *et al* have also used an automated liquid culture system (MS2 Research System, Abbott Laboratories, Dallas, Tx, USA) and paired competitive cultures to determine growth rate, as a measure of fitness in MRSA. Epidemic MRSA clones that are susceptible to gentamicin have been increasing in France and these clones have a fitness advantage over MRSA clones that are gentamicin resistant (Laurent *et al.* 2001). The growth rate differences provide an explanation for the predominance of nosocomial gentamicin susceptible MRSA clones in France.

Growth rates determined in the laboratory using rich media may not correspond to growth rates during infection. However it is reasonable to assume that if there is a measurable cost for bacteria in rich media then there is likely to be a cost *in vivo*. Gustavson *et al* demonstrated that fitness costs associated with antibiotic resistance in *Staphylococcus epidermidis* were similar for *in vitro* growth rates and the ability to survive on the skin of healthy human volunteers (Gustafsson, Cars, & Andersson 2003). Fitness measurements may differ for the same bacterium when measured in different conditions (Remold & Lenski 2001) and may be affected in stressful environments (Kishony & Leibler 2003).

Few studies have used human colonization or infection to measure fitness cost. Andersson *et al* have assessed the fitness costs conferred by *parC* and *fusR* mutations, conferring resistance to fluoroquinolones and fusidic acid respectively, in *S. epidermidis* using a human competition model (Gustafsson, Cars, & Andersson 2003). Susceptible and resistant bacteria were inoculated onto human skin and relative numbers monitored. No loss of fitness associated with *parC* mutation was found. However *fusA* mutations resulted in a considerable loss of fitness as compared to the susceptible isogenic strain during competition (Gustafsson, Cars, & Andersson 2003).

In this chapter assays to measure fitness costs in the Bcc were optimised and used to investigate the fitness cost of single and double topoisomerase mutations. No measurable cost was associated with single mutations while double mutation occuring in *gyrA* and *parC* conferred a fitness cost as measured by growth rate. These methods will be used to measure the fitness of clinical Bcc isolates.

Chapter 6 Characterisation of clinical isolates of *Burkholderia cepacia* complex

6.0 Introduction

6.1 Hypermutability

6.1.1 Hypermutability of *P. aeruginosa* in Lungs of Cystic Fibrosis Patients

Oliver *et al* have described a correlation has been found that links high mutation rate with increased antibiotic resistance in isolates of *P. aeruginosa* from lungs of CF patients (Oliver *et al.* 2000). Strains exhibiting the mutator phenotype were found in the lungs of 37% of CF patients and in these patients 43% of the total *P. aeruginosa* population were mutators. This group have also shown that hypermutable strains of *P. aeruginosa* exist in the CF lung before initiation of antimicrobial therapy (Oliver *et al.* 2004). Resistant mutants can readily occur in *P. aeruginosa* within the lungs of CF patients before or during treatment. This would imply that treatment of chronic infections of this type should include multiple antimicrobials rather than a single agent. This environment is indeed unstable characterised by changing host immune responses and varying antibiotic levels. Although conditions for *B. cepacia* growing in the lungs of cystic fibrosis patients are likely to be similar to those for *P. aeruginosa*, no evidence of hypermutable populations of *B. cepacia* has been reported to date.

6.1.2 Detection of Hypermutability by E-test

Associations have previously been demonstrated between high levels of antibiotic resistance and hypermutability (Oliver *et al.* 2000). These hypermutable strains have defects in DNA repair systems, most commonly in the mismatch repair system (Miller 1996), (section

1.3.13). Macia *et al.* used E-test and disc diffusion to detect hypermutable strains of *P. aeruginosa* isolated from sputum of CF patients (Macia *et al.* 2004). It is assumed that resistant mutant colonies which appear within the E-test inhibition ellipse are unlikely to occur in bacterial populations which are non hypermutable. This also allows MIC determination of both the susceptible and resistant subpopulations. Strains were defined as hypermutable if resistant colonies were observed to three or more antibiotics of ceftazidime, imipenem, meropenem, tobramycin and ciprofloxacin. Hypermutable strains were associated with higher MICs (Macia *et al.* 2004). E-tests are expensive but have the advantage of providing precise MICs, as compared to disc diffusion which do not. Estimation of mutation rate and mutation frequency are time consuming and labour intensive procedures and would not be possible to incorporate into diagnostic clinical microbiology laboratories.

6.2 Aims of chapter

Hypermutability has been demonstrated in a number of pathogenic bacteria but has not been described in *B. cepacia*. However hypermutability has been described in *P. aeruginosa* isolated from the CF lung. As both organisms have a similar niche and lifestyle it is likely that hypermutability occurs in Bcc bacteria. Clinical CF isolates of Bcc bacteria, were collected in order to determine if mutator bacteria exist in these populations. The mutation rate method, discussed in chapter 3, was modified because the clinical isolates were resistant to ciprofloxacin and an alternative selective agent was required. A mutation rate estimation method was therefore optimised using clinafloxacin as the selective agent as the Bcc isolates were susceptible to this agent and this method was used to estimate the mutation rate in these bacteria.

The fitness of these clinical isolates was determined as measured by growth rate, biofilm formation, survival in water and survival to drying. Antimicrobial susceptibility of these isolates was determined and the sequence of the *gyrA* QRDR determined to assess the role of *gyrA* mutations in the development of FQ resistance.

6.3 Materials and Methods

6.3.1 Clinical Strains

Clinical strains of Bcc complex bacteria including isolates of *B. multivorans* (genomovar II) and *B. cenocepacia* (genomovar III) were obtained from Dr. John Moore, Belfast City Hospital. These bacteria were isolated from sputum samples from CF patients (see section 6.3.2 for recovery of Bcc from sputum).

6.3.2 Isolation of Bcc from sputum

Sputum containing Bcc was diluted with Sputasol (Oxoid, Basingstoke, UK) and spread onto *B. cepacia* selective agar (Mast Diagnostic, Bootle, UK) containing ticarcillin and polymixin, to obtain single colonies. On this medium, *B. cepacia* grows as 1-2 mm colonies and turns the medium pink within 48 hours. Plates were incubated aerobically (48 hours at 37°C). Isolates were confirmed as *B. cepacia* by Gram stain and API 20 NE as it is reliable in identification of the Bcc (van Pelt *et al.* 1999). Isolates were previously identified as Bcc and the species status determined at Belfast City Hospital. In brief expectorated sputum (50:50 sputasol; sputum) was inoculated onto *B. cepacia* selective agar (Mast Diagnostic, Bootle, UK). Bcc confirmed by API 20 NE and species status was previously determined by *recA* sequence typing (Mahenthiralingam *et al* 2000).

Colonies were stored at -70°C on beads, as described previously (section 2.1).

6.3.3 MIC determination

MICs were determined using E-test, for the following antibiotics; amoxicillin-clavulanic acid, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, temocillin, tetracycline, tigecycline and trimethoprim sulphamethoxazole, using the manufacturer's instructions (section 2.6.1). CLSI interpretive breakpoints for non *Enterobacteriacea* were used if *Burkholderia* breakpoints were unavailable. Isolates were recorded as resistant, intermediate or susceptible.

6.3.4 Sequence analysis of the QRDR of *gyrA*

The QRDR of the subunit A of DNA gyrase was sequenced (section 2.7 and 2.8) using primers described in Table 3.1.

6.3.5 Detection of Hypermutability

The presence of hypermutability was measured using mutation rate (section 6.3.6.3). Alternatively, the presence of hypermutability was determined using the E-test method (section 2.6.1) which identifies hypermutators by the presence of resistant colonies observed within the inhibition ellipse to determine if resistant colonies are observed within the inhibition ellipse. The antibiotics used were ceftazidime, meropenem, ciprofloxacin, imipenem and gentamicin (Macia *et al.* 2004).

6.3.6 Assay development for determination of mutation rate of clinical Bcc isolates

6.3.6.1 Choice of selective antibiotic

The clinical Bcc isolates (BCH1-BCH8) exhibited variable susceptibility to ciprofloxacin. Isolates BCH2, BCH3, BCH6, BCH7 and BCH8 (5/8) had ciprofloxacin MIC values of >32 mg/L (Figure 6.4). An alternative agent was therefore required in order to estimate mutation rates from susceptibility to resistance in these isolates. The clinical Bcc isolates were tested for susceptibility to clinafloxacin. Clinafloxacin was more active against the Bcc isolates than ciprofloxacin (Table 6.4).

6.3.6.2 Selective Antibiotic Concentration

Using NCTC strain 10661 as the test isolate, at 4 x and 6 x MIC of clinafloxacin, no resistant mutants grew irrespective of incubation conditions and final cell numbers. A 1 mL culture, inoculated with 10^2 cells, that was spread onto plates containing 2 x MIC of clinafloxacin, gave rise to single colonies that were visible at 48 hours of plate incubation. When higher broth culture volumes were used a film of growth covered every antibiotic plate. Between 3 and 90 colonies were present on each plate. Therefore the Lea and Coulson method of the median was used to determine mutation rate (section 6.3.6.3). All mutants selected at 2 x MIC had an MIC of 0.5 mg/L. This represents a 2 fold increase in MIC. However mutants selected at 2 x MIC (25/25) did not contain mutations in *gyrA*. MIC was reduced in the presence of reserpine. Therefore the observed increase in resistance is likely to be due to an increase in efflux, as was the case with *in vitro* ciprofloxacin resistant mutants selected at 2 x MIC.

6.3.6.3 Method for determination of mutation rate for the clinical Bcc isolates by the method of the median using 2 x MIC clinafloxacin.

Isolates were removed from the -70°C freezer and one bead was used to sub culture Bcc onto a blood agar plate and incubated aerobically at 37°C. One colony of Bcc was suspended in 5 mL of Muller Hinton Broth in a 25 mL conical flask. This was sealed with a cotton wool bung and incubated at 37°C on a rotary shaker (200 r.p.m.) (Barloworld Scientific, Staffordshire, UK) for 2.5 hours until an optical density (OD_{600}) of approximately 0.1 OD units was reached. Serial dilutions of this broth culture were performed in PBS (neat to 10^{-6}). A 100 µL aliquot of the 10^{-3} dilution (containing approximately 10^3 cells) was added to each of 28 microcentrifuge tubes, containing Muller Hinton Broth (1 mL). These cultures were incubated at 37°C, 200 r.p.m 18 hours (Barloworld Scientific, Staffordshire, UK). Muller Hinton Agar plates (Oxoid, Basingstoke, UK) containing 2 x MIC of clinafloxacin were prepared and inoculated the following day. Antibiotic free agar plates were also prepared (section 2.2.3). Before inoculation, plates were allowed to dry at 37°C with the lids removed for 20 min. This ensured that drops were absorbed by the agar. At the end of the incubation period, a Miles and Misra plate count was performed on 3 randomly selected broths, section 2.5 (Miles 1938). Dilutions of 10^{-1} to 10^{-6} were prepared. Twenty microlitres of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spotted in triplicate onto duplicate drug free Muller Hinton plates. The microcentrifuge tubes were centrifuged at 4000 *g*, 3 min and the supernatant discarded. Using a fine tipped pastette, the deposit was resuspended in residual broth (approximately 40 µL). The total volume was then inoculated onto clinafloxacin containing Muller Hinton plates, spread with a plastic spreader and allowed to dry. All plates were incubated (37°C, 48 hours). Colony forming units per mL were calculated from the Miles and Misra drug free plates. The number of colonies on each plate were

counted and recorded. The median number of colonies was determined. The number of mutations per culture was calculated using equation 10:

$$\bar{r}/m\text{-}\ln(m) - 1.24 = 0 \text{ [Eq 10]}$$

The mutation rate (μ) was calculated using equation 11:

$$\mu = \frac{m}{\text{average cfu/mL}} \text{ [Eq 11]}$$

This was repeated 4 times to give a median mutation rate.

6.3.7 Fitness of Clinical Bcc Isolates

As previously, a comprehensive approach to measuring the fitness of clinical isolates was adopted and biofilm formation (section 2.10.1.2), growth rate (section 2.10.2), survival in water (section 2.10.4) and survival to desiccation (section 2.10.5), was determined for each isolate.

6.3.8 Statistical Analysis

6.3.8.1 Calculation of t ½

Data was entered into GraphPad software version 3.00 for Windows (GraphPad Software, California, USA) and statistical analysis was performed. The mean and standard error values were calculated from the three experiments for each clinical isolate. The t ½ (time at which half of the cells will have died) was calculated using non-linear regression analysis with a

monophasic exponential decay curve. Exponential decay curves were calculated for each clinical isolate.

The correlation coefficient (r^2) was calculated as an estimate of goodness of fit using GraphPad Prism by the following equation:

$$r^2 = \frac{\text{(sum of squares of the distance from the best fit curve)}}{\text{(sum of squares of distance from the mean Y value)}}$$

The Global comparison of fits was used to determine if the survival of the isolates to drying were significantly different to each other. A two way ANOVA, followed by the Bonferroni multiple comparison test, was performed in order to determine if survival in water between isolates was different at time points.

6.4 Results

6.4.1 Antibiotic Susceptibility Testing

	MIC mg/L							
Antibiotic	**BCH 1**	**BCH 2**	**BCH 3**	**BCH 4**	**BCH 5**	**BCH 6**	**BCH 7**	**BCH 8**
Amoxicillin-clavulanic acid	>256	>256	>256	>256	>256	>256	>256	>256
Ceftazidime	3	6	4	1	1	64	24	12
Ciprofloxacin	6	>32	>32	1.5	3	>32	>32	>32
Colistin	128	>1024	>1024	>1024	192	>1024	>1024	>1024
Gentamicin	24	96	96	16	32	>256	>256	>256
Meropenem	2	4	12	2	0.75	>32	12	>32
Piperacillin-Tazobactam	64	>256	3	16	16	>256	>256	>256
Temocillin	12	4	32	24	32	24	8	12
Tetracycline	>32	>32	>32	>32	>32	>32	>32	>32
Tigecycline	6	8	24	128	32	24	8	6
Trimethoprim sulfmethoxazole	>32	>32	>32	>32	>32	>32	>32	>32

Table 6.1 Antibiotic susceptibilities of clinical Bcc isolates. Isolates BCH 2, BCH 3, BCH 5, BCH 6, BCH 7 and BCH 8 are isolates of *B. cenocepacia*. BCH 1 and BCH 4 are isolates of *B. multivorans*. MICs to amoxicillin-clavulanic acid, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, temocillin, tetracycline, tigecycline and trimethoprim sulfamethoxazole of clinical Bcc isolates, as determined by E-test according to the manufacturer's instructions.

6.4.2 Biofilm Growth

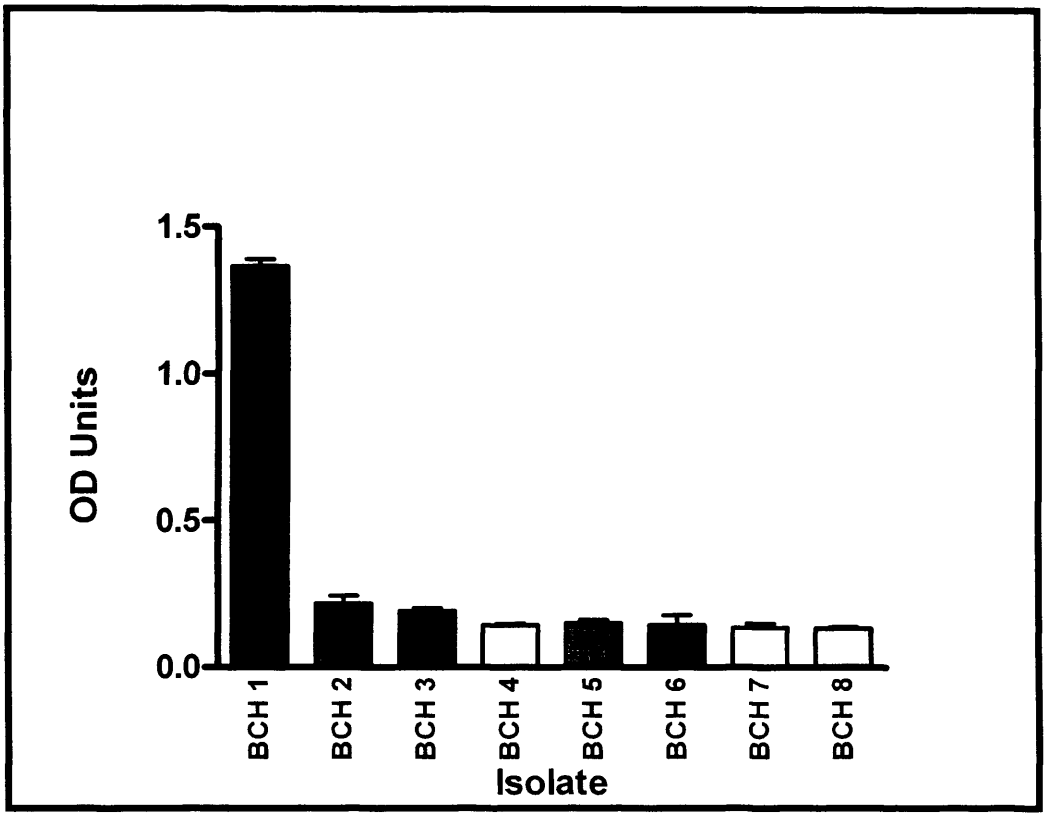


Figure 6.1 Comparison of biofilm formation between clinical isolates. Mean OD units obtained in the crystal violet assay are plotted to represent biofilm formation. Error bars represent SEM.

6.4.3 Environmental Survival

6.4.3.1 Survival during drying

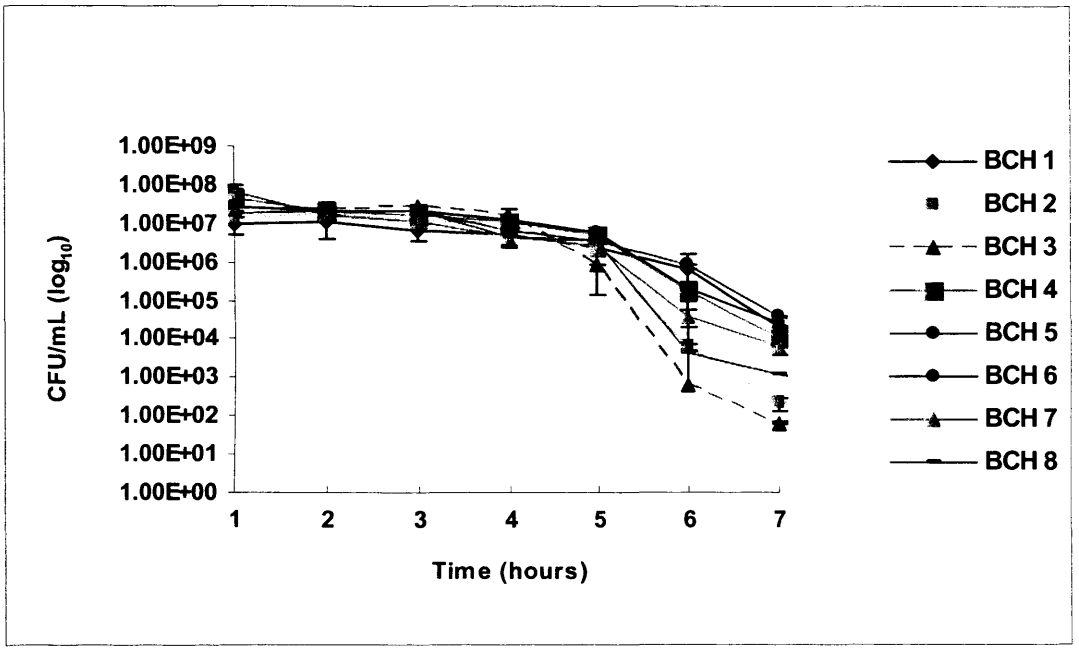


Figure 6.2 A comparison between the fitness of different Bcc isolates using survival during drying as a measure. The survival curve is obtained by plotting the mean viable count Sampled at 0, 1, 2, 3, 4, 5, 6 and 7 hours. Error bars represent the SEM. Error bars are not shown if obscured by the symbol. No significant differences found by F test of survival curves, using Graph Pad Prism.

To test the hypothesis that clinical isolates varied in their ability to survive drying, the t1/2 was calculated for each clinical isolate using non linear regression analysis. No significant difference was found in the ability of the clinical isolates to survive drying (global p value 0.2394). Because the viable cell count of Bcc isolates in water did not decline significantly after 3 days (figure 6.3) the t1/2 was not calculated for survival in water.

6.4.3.2 Survival in water

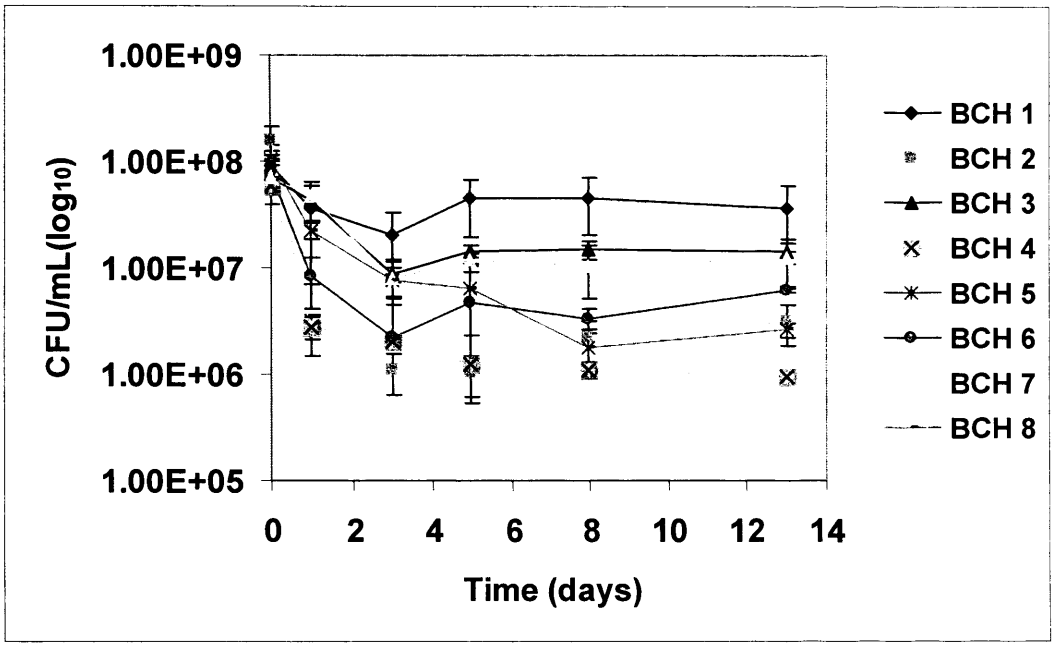


Figure 6.3 A comparison between the fitness of different Bcc isolates using survival in water as a measure. The survival curve is obtained by plotting the mean viable count. Survival in water of the clinical Bcc isolates. Sampled at 0, 1, 3, 5, 8 and 13 days. Error bars demonstrates SEM. Error bars are not shown if obscured by the symbol. For the survival in water data, there was a trend to declining CFU with time. However the difference in CFU between isolates at time points was found to be not significant (p value 0.0714) by two way ANOVA, using GraphPad Prism.

6.4.4.1 Growth Rate

Isolate	Generation Time (min +/- S.E.M)	Antibiotics to which resistant
BCH 1	54.8 +/- 0.61	5/10
BCH 2	76.4 +/- 0.93	8/10
BCH 3	83.1 +/- 0.48	8/10
BCH 4	54.7 +/- 1.12	6/10
BCH 5	54.6 +/- 0.63	5/10
BCH 6	120.7 +/- 1.13	10/10
BCH 7	71.6 +/- 0.76	10/10
BCH 8	85.07 +/-1.25	8/10

Table 6.2 Association between generation times of clinical Bcc isolates (min +/- S.E.M) and antibiotic resistance. Shows proportion of antibiotics tested (amoxicillin-clavulanic acid, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, temocillin, tetracycline, tigecycline and trimethoprim sulphamethoxazole) to which each strain is resistant.

6.4.4 Hypermutability

6.4.5.1 E-test

No resistant colonies were visible within the E-test ellipse for any clinical Bcc isolate tested.

6.4.5.2 Mutation rate

Isolate	Mutation rate (mutation per division)	Range
BCH 1	4.9×10^{-8}	3.4×10^{-8} - 5.5×10^{-8}
BCH 2	1.9×10^{-8}	1.8×10^{-8} - 7.0×10^{-8}
BCH 3	4.2×10^{-8}	2.6×10^{-8} - 6.9×10^{-8}
BCH 4	2.3×10^{-8}	1.7×10^{-7} - 2.6×10^{-8}
BCH 5	2.2×10^{-8}	2.2×10^{-8} - 8.2×10^{-8}
BCH 6	5.0×10^{-8}	2.3×10^{-8} - 5.8×10^{-8}
BCH 7	3.8×10^{-8}	2.7×10^{-8} - 4.2×10^{-8}
BCH 8	2.6×10^{-8}	1.1×10^{-8} - 2.7×10^{-8}

Table 6.3 Median mutation rates of Bcc isolates of three independent experiments. Determined by Lea and Coulsons method of the median (Lea & Coulson, 1949) using clinafloxacin as the selective antibiotic at 2 x MIC (section 2.3.6).

6.4.5 Sequence Analysis of the QRDR of *gyrA*

```
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDTAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 1
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDAAVYGTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 2
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDSAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 3
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDSAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 4
Codon 54     ------NRAYKKSARIVGDVIGKYHPHGDSAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 5
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDTAVYGTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 6
Codon 54     ------NRAYKKSARIVGDVIGKYHPHGDTAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 7
Codon 54     KLNNDWNRAYKKSARIVGDVIGKYHPHGDTAVYDTIVRMAQDFSLRYMLIDGQGNFGSID      BCH 8
                   ********************* *** ** ************************

Codon 114    GDNAAAMRYTEIRMAKIGHELLADID----------                              BCH 1
Codon 114    GDNAAAMRYTEIRMAKIGHELLADID----------                              BCH 2
Codon 114    GDNAAAMRYTEIRMAKIGHELLADIDKET---------                            BCH 3
Codon 114    GDNAAAMRYTEIRMAKIGHELLADID----------                              BCH 4
Codon 114    GDNAAAMRYTEIRMAKIGHELLADID----------                              BCH 5
Codon 114    GDNAAAMRYTEIRMAKIGHELLADIDKETVDFEPNYDG                            BCH 6
Codon 114    GDNAAAMRYTEIRMAKIGHELLADID----------                              BCH 7
Codon 114    GDNAAAMRYTEIRMAKIGHELLADIDKETVDFEPNYDG                            BCH 8
             *************************
```

Figure 6.4 Amino acid sequences of QRDRs of *gyrA* of clinical isolates. Polymorphisms found at codon 83 (shown in red) and 87 (shown in blue). At codon 83 a serine residue would be expected in a susceptible isolate. Isolates BCH 4 and BCH 5 contain a serine residue at codon 83. Isolate BCH 2 contains a mutation to alanine residue at codon 83, while BCH 1, BCH 3, BCH 6, BCH 7, and BCH 8 contain mutations to threonine. At codon 87 an

BCH 4, BCH 5, BCH 7 and BCH 8 contain an aspartic acid at this position while BCH 2 and BCH 6 contain a glycine mutation. No synonymous mutations were found.

Isolate	Ciprofloxacin MIC (mg/L)	Clinafloxacin MIC(mg/L)
BCH 1	6	2
BCH 2	128	16
BCH 3	64	8
BCH 4	1.5	1
BCH 5	3	8
BCH 6	32	8
BCH 7	256	16
BCH 8	32	2

Table 6.4 Comparison of ciprofloxacin and clinafloxacin MIC of clinical Bcc isolates

Ciprofloxacin and ciprofloxacin solutions were prepared (sections 2.3.6 and 2.3.7).
MICs were determined in duplicate using the agar incorporation method (section 2.6.2) according to the CLSI guidelines (CLSI, 2006).

6.5 Discussion

To characterise the pattern of resistance and the potential for resistance to be fixed in the bacterial population a series of clinical isolates were compared for mutation rate, antibiotic susceptibility and fitness as measured by planktonic growth rate, biofilm growth rate and environmental survival.

Isolates did exhibit variation in growth rate. Growth rates of the different isolates were statistically significant, as measured by a modified Youmans and Youmans method (Table 6.2). There appeared to be an intriguing association between generation time and the relative resistance of each isolate (Table 6.2). However isolate numbers were not large enough to determine if there was a relationship between extent of antibiotic resistance and growth rate.

Isolate BCH 6, a *B. cenocepacia* strain, has the longest generation time (120.7 min. +/- 1.13) and is also the most resistant of the eight strains studied and is resistant to all antibiotics tested. Isolates BCH 1, BCH 4 and BCH 5 have the shortest generation times 54.8 min. +/- 0.63, 54.7 +/- 0.61 and 54.6 min. +/- 1.12 respectively but these are also the most susceptible strains. Caraher *et al* demonstrated differences in growth rate and that generation times in *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamensis* and *B. dolosa* ranged from 70 – 186 min. (Caraher *et al.* 2006) and that there was no association between generation time and genomovar status. However this study did not examine the association between antimicrobial susceptibility and growth rate. This thesis lends support to the idea that multidrug resistance does incur fitness costs in the Bcc. The mechanisms of this resistance were not investigated in this thesis.

No significant differences in biofilm formation were found between the majority of the Bcc isolates, as measured by the crystal violet assay (Figure 6.1). However isolate BCH 1 produced extensive biofilm in the crystal violet assay, compared to the other Bcc isolates. The reasons for the propensity of this isolate for forming biofilms are unclear. Conway *et al* also demonstrated differences in ability to form biofilms between isolates of the Bcc and found that there was no correlation between the ability to form biofilms and growth rate. However the correlation between biofilm formation, growth rate and virulence was not explored (Conway, Venu, & Speert 2002). However *recA* type A organisms formed more biofilms than type B organisms (Conway, Venu, & Speert 2002).

However in this study, no significant difference was found in environmental survival, as measured by survival during drying and survival in water, between the clinical Bcc isolates. Drabick *et al* demonstrated Bcc strain to strain differences in survival on environmental

surfaces and differences in survival on different surfaces with the greatest survival occurring on PVC (Drabick *et al.* 1996). Isolates survived longer if suspended in sputum (Drabick *et al.* 1996).

Zhou *et al* reported the *in vitro* activity of antimicrobial agents that were tested in synergy, using checkerboard testing. The most active antibiotics were minocycline, meropenem and ceftazidime, inhibiting 38%, 26%, 23% of strains respectively, while 18% were resistant to all agents tested (Zhou *et al.* 2007). Aaron *et al* performed *in vitro* synergy studies for the Bcc using multiple combination bactericidal tests (MCBT). This measures the activity of peak serum concentrations of antibiotics (Aaron *et al.* 2000). Meropenem combined with minocycline, amikacin or ceftazidime was bactericidal against 76%, 73% and 73% of isolates, respectively. Triple antibiotic combinations such as tobramycin, meropenem and other agents exhibited bactericidal activity against 81-93% of isolates (Aaron *et al.* 2002). In this study all isolates were resistant to colistin. This is unsurprising as colistin is incorporated into *B. cepacia* selective media to isolate Bcc. Additionally all isolates were resistant to trimethoprim-sulfamethoxazole, tetracycline and gentamicin. Variable susceptibility was found for tigecycline, piperacillin-tazobactam, and temocillin. The most active antibiotics against Bcc are meropenem, ciprofloxacin and ceftazidime.

The nucleotide sequences of the *gyrA* QRDRs of the isolates were determined (Figure 6.4). Clinical isolates of the Bcc contain polymorphisms within the *gyrA* QRDR at positions 83 and 87 and there appears to be a correlation between MIC and the identity of amino acids at codon 83 and 87. The direct effect of QRDR polymorphisms in Bcc clinical isolates on growth rate was not assessed in this thesis and therefore. It is not possible to comment on whether differences in observed growth rate were directly related to the identity of amino

acids within the QRDR. At codon 83 a serine residue would be expected in a susceptible isolate, while at codon 87 an aspartic acid residue would be expected in a susceptible isolate. Isolates of *P. aeruginosa* and *C. jejuni* are 10 fold less susceptible to fluoroquinolones than wildtype *E. coli* because they contain a threonine residue rather than serine at codon 83 (Chen & Lo 2003; Okazaki *et al.* 1991). Codons 83 and 87 encode the hydrophilic α helical domain of DNA gyrase. When mutations occur at these positions fluoroquinolones are less able to form a complex with DNA and DNA gyrase, reducing susceptibility to the drug. Isolates BCH 4 and BCH 5 contain Ser83 and Asp87, the most common genotype in susceptible isolates. Isolates BCH 1, BCH 5 and BCH 4 exhibited low level FQ resistance (Table 6.5). However isolate BCH 1 contains Thr83 and Asp87 and has a higher MIC (6 mg/L) than BCH 5 (3 mg/L) and BCH 4 (1.5 mg/L). Mutations in *gyrA* initially result in substitution of Ser83 and subsequently by substitution of Asp87 (Chen & Lo 2003; Hooper 2003). The altered gyrase reduces binding of fluoroquinolones, even if only one resistance mutation is present (Willmott & Maxwell 1993). Although it might be expected that BCH 6 exhibit the highest level of resistance since this strain contains both Thr83 and Gly87, the ciprofloxacin MIC is 32 mg/L. Of the eight clinical strains isolate BCH 7 exhibited the highest level of resistance at 256 mg/L. This isolate contains Thr83 and Asp87 and it is therefore likely that other resistance mechanisms are contributing to the observed resistance. The BCH 2 isolate contained an alanine residue at codon 83 and is highly resistant to ciprofloxacin (128 mg/L). This resistance mutation is not common but has been documented in fluoroquinolone resistant *E. coli* (Tavio *et al.* 1999). The isolate BCH 2 also contains a Gly87 mutation. It is likely that differences in the QRDR sequence of *gyrB*, *parC* and *parE* would have been found in the clinical isolates if sequenced and that these mutations may have contributed to FQ resistance. However the primers used in this thesis to amplify *gyrA* failed to amplify *gyrB*, *parC* and *parE* genes of the clinical isolates,

despite further optimisation of annealing temperatures and $MgCl_2$ concentration. These regions have, therefore, not been sequenced in the clinical Bcc isolates. Differences in nucleotide sequences between the primer and the *parC* region of the clinical isolates may have prevented the primers from annealing. It is likely that other fluoroquinolone resistance mechanisms will be contributing to the observed levels of resistance.

Varied aspects of fitness may be more important depending on growth conditions and mode of growth. An isolate which is more able to survive environmental conditions may have an advantage over other isolates during transmission from the environment to patients. Similarly isolates that are capable of forming abundant biofilms may have an advantage in the lungs of CF patients. However it is possible that an isolate fittest one assay may not be fittest in all assays that have been selected.

Large SEM values were observed for the environmental survival experiments. Therefore these assays would only be able to detect reliably large differences in fitness.

Clinafloxacin is a broad spectrum FQ, contains a chlorine atom at C8, and is more active against Gram-positive bacteria than ciprofloxacin. Activity of clinafloxacin against 354 Gram-positive and Gram-negative organisms was compared to ciprofloxacin, levofloxacin, sparfloxacin, trovafloxacin, piperacillin, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, ceftazidime and imipenem (Brisse *et al.* 1999). MICs of clinafloxacin were lowest of the FQ drugs tested and had the lowest overall MICs of compounds tested. Clinafloxacin was also the most active against *B. cepacia* (Ednie, Jacobs, & Appelbaum 1998). Resistance to clinafloxacin was used to determine mutation rate in the clinical isolates. In this study mutants selected at low selective concentrations (2 x MIC) of

clinafloxacin did not contain mutations in the QRDR of *gyrA*. MIC values were reduced in the presence of reserpine, therefore it is likely that this low level resistance was due to an increase in efflux activity. No mutants were selected at 4 x and 6 x MIC of clinafloxacin. It was not possible to select clinafloxacin resistant mutants that contained confirmed topoisomerase mutations.

The pattern of clinafloxacin resistance is different to that of other FQ antibiotics. A combination of *gyrA* and *parC* mutations is required for clinically significant FQ resistance to emerge (Nagai *et al.* 2000; Pan & Fisher 1998). This is because single step mutations in *gyrA* or *parC* confer a two fold increase in clinafloxacin MIC. This does not raise the MIC above the susceptibility breakpoint. Mutation frequencies of this initial mutation were very low and have been reported to be in the region of 10^{-10} to 10^{-11}. Pan *et al* also found that it was difficult to select clinafloxacin resistant mutants and proposed that clinafloxacin had no target preference and targeted both gyrase and topoisomerase IV equally (Pan & Fisher 1998). As spontaneous acquisition of two mutations is required for significant resistance, the mutation rate would therefore be very low. If an average mutation rate is 10^{-8} mutations/division the mutation rate of two spontaneous mutations occurring would be 10^{-16}, which is unlikely to occur. Nagai *et al* selected FQ resistant mutants by sequential subculture in sub inhibitory concentrations of clinafloxacin, ciprofloxacin and trovafloxacin (Nagai *et al.* 2000). Observed clinafloxacin mutation frequencies for each mutation step were lower than ciprofloxacin and trovafloxacin. This suggests that clinafloxacin would be less likely than trovafloxacin or ciprofloxacin to result in the development of resistance. Most clinafloxacin mutations did not raise the MIC above the susceptibility breakpoint. The mutation frequency of initial resistance mutation to low level resistance in clinical isolates

of *S. pneumoniae*, which were fully susceptible (MIC 0.06 mg/L), ranged from 8.8×10^{-10}-1.0×10^{-11} (Nagai *et al.* 2000).

Alternative selective tools that could have been used to detect point mutations are heavy metals e.g. copper, mercury, lead, boron, cadmium and tungsten. Bacteria have a number of resistance mechanisms that allow survival in the presence of heavy metals. These include sequestration of heavy metals, reduction of the metal to a less toxic species and efflux (Nies 1999; Nies & Silver 1995). Heavy metal resistance has been described in *Acinetobacter* spp, Staphylococci, *P. aeruginosa* and *S. typhi* (Dhakephalkar & Chopade 1994; Du Bois, Davison, & Pinney 1995; Harnett *et al.* 1998; Nucifora *et al.* 1989; Teitzel & Parsek 2003).

Hypermutability was not observed in this limited panel of clinical Bcc isolates investigated by E-test or estimation of mutation rate but this does not preclude the possibility of observing hypermutability in a wider screen. In this chapter, the necessary methods to determine mutation rate in clinical Bcc isolates have been optimised and used to screen for hypermutability in a small number of isolates that could be applied to screen larger numbers of isolates.

Between the isolates, differences in growth rates were observed. However the isolate numbers were not large enough to determine if there was a relationship between species status or extent of antibiotic resistance in growth rate.

7.0 Final Discussion

The extensive use of antimicrobial compounds has led to the emergence and dissemination of multidrug resistant pathogens (Rogues *et al* 2007; Mutnick *et al* 2004). Additionally the lack of new antimicrobials being developed and introduced into clinical use (Norrby *et al* 2005) has limited treatment options. Approval of new antimicrobials by the U.S. Food and Drug administration decreased by 56% from 1983-1987 to 1998-2002. Since 1998 only 10 new antibiotics have been developed. Of these only linezolid, daptomycin and tigecycline have novel mechanisms of action (Spellberg *et al* 2004). Antibiotic resistant Gram-negative pathogens that are resistant to treatment by almost all available drugs are becoming a particular problem, especially due to their involvement in nosocomial outbreaks (Canton, Coque, & Baquero 2003). Members of the *B. cepacia* complex are highly antibiotic resistant opportunistic pathogens causing lung infection in cystic fibrosis patients. Resistance to fluoroquinolones in the Bcc emerges via mutation in topoisomerase genes and by upregulation of efflux pathways which extrude antimicrobial compounds from the cell.

Most resistance acquisitions, occuring by chromosomal point mutations, are associated with a fitness cost (Andersson & Levin 1999). When this is the case a resistant strain will be outcompeted by a susceptible strain in the absence of the antibiotic selective pressure. Mutants with a low or non existent fitness cost, as described in chapter 5, are therefore more likely to become fixed in the population than those with large fitness deficits. Mutations that confer no cost *in vitro*, with moderate levels of resistance, commonly occur in *B. cepacia* while high level resistant mutants (less common) incur a significant fitness deficit, as measured by growth rate. Programmes to restrict use of antibiotics in order to reduce rates of resistance are based on the assumption that antibiotic resistance has an

associated cost. Where there are no or low cost mutations these strains may remain in the population following the cessation or judicious control of fluoroquinolone use in the community, hospital (Shlaes *et al.* 1997) and within animal husbandry (Nawaz *et al.* 2003; Wallmann 2006). These pre-existing mutants may then re-emerge rapidly in the bacterial population as a consequence of resumed prescribing of fluoroquinolone, as illustrated by Cohen and Murray in their studies of multidrug-resistant tuberculosis (Cohen & Murray 2004). Exposure of a bacterial population already containing resistance conferring mutations to the antibiotic will select subsequent mutations that will further increase MIC and will select in favour of the pre-existing resistant mutants rather than the wildtype. The extent to which the resistant subpopulation will take over the population will depend on three factors; the level of resistance conferred by the genetic change, the fitness deficit associated with the mutation and the level of antibiotic exposure. The probability of resistance mutations occurring and the extent of the associated fitness deficit are important as these factors affect the likelihood of resistance emerging and being maintained in the population. This thesis addresses these factors.

In this thesis novel methods for measuring fitness in the Bcc have been developed. Measures of fitness are context specific and appropriate models of fitness should be relevant to the organism, simple to perform and able to detect small fitness deficits. Growth rate is an accepted method for measuring fitness deficits and has been used previously both as monocultures, or in paired competition assays (Billington, McHugh, & and Gillespie 1999; Gillespie, Voelker, & Dickens 2002), to measure fitness deficits. However no single measure of fitness is sufficient. As Bcc bacteria are likely to form biofilms within the CF lung a biofilm assay was included as a fitness assay, described in chapter 4. Models have been selected for this study, described in chapter 5, that represent

growth and survival conditions for *B. cepacia* (Conway, Venu, & Speert 2002; O'Toole & Kolter 1998; Sanchez *et al.* 2002). These were planktonic growth rate, pair wise competitive culture (Gillespie, Voelker, & Dickens 2002), biofilm growth (Conway, Venu, & Speert 2002; O'Toole & Kolter 1998), survival in water and resistance to drying (Sanchez *et al.* 2002). To measure planktonic growth rate a liquid based semi automated growth rate determination method, modified from the method of Youmans and Youmans (Youmans & Youmans 1949) has been used. This method avoids the use of laborious growth curve techniques and has been shown to be reproducible in this study (Pope *et al.* 2007).

No measurable fitness cost was associated with single mutations in *gyrA*. As these models can not detect fitness costs of <1% it is not possible to conclude that *gyrA* mutations were 'no cost' and are likely to be 'no cost or low cost'. It was notable that mutants containing mutations in both *gyrA* and *parC* were significantly impaired in fitness compared to the susceptible parent (chapter 4). Cystic fibrosis patients are frequently colonised by Bcc bacteria that exhibit high level FQ resistance (Zhou *et al*) even when there is not an FQ selective pressure. As described in chapter 6, clinical Bcc isolates often exhibit moderate to high level fluoroquinolone resistance and exhibit polymorphisms with in the QRDR regions of *gyrA* As multiple mutations are required for moderate and high level resistance it is likely that the fitness costs measured in this thesis do not impair the ability of Bcc to cause clinical disease. Additionally the isolates studied in this thesis were obtained from CF patients with chronic Bcc infection. The size of fitness cost that would be required to have an effect clinically is not known and may be hard to investigate as these are likely to be the isolates that do not survive.

Mutants selected *in vitro* or mutations that occur during treatment may have an undefined genetic background. In order to interpret the effect of resistance on fitness the nucleotide sequence of the susceptible parent and resistant mutants should be determined. Consecutively isolated isogenic clinical strains, containing successive resistance mutations, may be available. It is not possible to establish with confidence the contribution to resistance of topoisomerase mutations selected *in vitro* because it is not possible to exclude the presence of other mutations that may have occurred and contributed to the observed resistance. In this study only the QRDR regions of the topoisomerase genes of mutants selected *in vitro* were sequenced. The possibility of mutations affecting fitness outside these regions cannot be excluded. Use of directed mutagenesis could overcome this limitation. Bagel *et al.* examined the effects of point mutations, created by directed mutagenesis, on fluoroquinolone resistance and found that extent of the fitness cost varied depending on the *gyrA* and *parC* mutations present (Bagel *et al.* 1999). Different *gyrA* mutations had different effects on the degree of supercoiling. It is therefore likely that supercoiling alterations affect growth rate (Bagel *et al.* 1999). Compensatory mutations may occur that restore supercoiling activity of gyrase and may restore growth rates to levels comparable to the susceptible parent therefore restoring the fitness deficit. Restoration of growth rate by compensatory mutation has been demonstrated in *P. aeruginosa* (Kugelberg *et al.* 2005).

During drug development the likelihood of resistance arising is affected by mutation rate. The cost of these mutations may be relevant as even if the mutation rate is high resistant mutants will not proliferate in the population if the fitness cost is high. Novel antimicrobials to which only resistance mechanisms that incur large fitness costs are likely would be promising agents to develop. Previous data show that use of low dose

fluoroquinolone as therapy may increase the rate of resistance mutations occurring in other pathogens that may have colonised the cystic fibrosis lung due to ability of fluoroquinolones to increase mutation rate at sub inhibitory concentrations (Gillespie *et al.* 2005).

Previous treatment with fluoroquinolones may allow amplification of a mutant subpopulation to over 70% resistance if fluoroquinolone treatment is re-initiated (Peloquin *et al.* 1989). It is accepted that fitter more susceptible bacteria can out compete resistant bacteria when the antibiotic selective pressure is removed. However it is apparent that resistance may persist in the population for longer than previously thought. Resistant *S. epidermidis* were found on human skin 4 years after single course of clarithromycin (Sjolund *et al.* 2005). Resistant bacteria are unlikely to disappear even if antibiotic use is reduced and can persist due to no cost mutations, compensatory mutations and co-selection of resistance markers. In the UK a 97% reduction in sulphonamide use was observed during the 1990s as a consequence of a national prescribing restriction prompting a switch from trimethoprim-sulfamethoxazole to trimethoprim. The prevalence of sulphonamide resistance in *E. coli* remained at 40-45% (Enne *et al.* 2001). Antibiotic resistance determinants responsible for resistance to a drug which is no longer in use can be linked to genes conferring resistance to antibiotics still in use. The *sul2* plasmid, containing genes conferring sulphonamide resistance, did not disappear during decreased sulphonamide use, even though these plasmids reduce fitness (Enne *et al.* 2004).

The risk of resistance arising depends on the mutagenicity of the fluoroquinlone, the dose and length of treatment. Use of antimicrobial agents in the hospital and the community can be rationalised. However we can not control the remaining factors. Rational use of

antibiotics will not alone reduce the rate of infections caused by resistant bacteria as resistant bacteria may already have become fixed in the population. This could include prescribing an antibiotic dose that does not select resistant mutants and effective methods for reducing transmission.

A method for measurement of mutation rate has been standardised in this thesis. In bacterial populations some clones may have a higher mutation rate than the rest of the population due to defects in proof reading and repair mechanisms. Hypermutability in populations of pathogenic bacteria has been described in *E. coli* (LeClerc, Li, & Payne 1996; Matic *et al.* 1997), *Salmonella* spp. and *P. aeruginosa* isolated from the lungs of CF patients (Oliver *et al.* 2000; Oliver *et al.* 2004). Although Bcc inhabit a similar niche to *P. aeruginosa* within the CF lung hypermutability has not been reported in the Bcc. In this study no evidence for hypermutability in the Bcc has been found (chapter 6).

Although the rate of mutation in topoisomerase genes conferring FQ resistance in *B. cepacia* is low (chapter 3) the mutants containing a single mutation in *gyrA* which arise, are not associated with a fitness cost (chapter 5). Mutants are likely therefore to persist in the population. The mutation rate of the second step mutation is higher than the mutation rate of the first step mutation (chapter 3). Our group have previously found that the mutation rate of second step mutations in *S. pneumoniae* isolates already containing a *gyrA* or *parC* alteration was higher than the first step mutation rates, using ciprofloxacin and gemifloxacin as the selective agents (Gillespie *et al.* 2003). This is evidence that single mutations in gyrase or topoisomerase IV may predispose the genome to further mutation.

Differences in antibiotic susceptibility were observed between the clinical Bcc isolates. Polymorphisms at codons 83 and 87 of the *gyrA* QRDR were found and the identity of the amino acids affects level of FQ resistance (chapter 6). Variation in generation times was observed for the Bcc clinical isolates. The determinants responsible for the reduced growth are unknown. One isolate was an abundant biofilm producer, compared to the other isolates (chapter 6). However no statistically significant differences in environmental survival were found between clinical isolates.

In this thesis appropriate methods were developed to measure fitness and mutation rate in the Bcc. The path to fluoroquinolone resistance in *B. cepacia* is initially by an efflux mechanism at low selective concentrations, the genetic basis of which was not elucidated. At higher selective concentrations mutations in *gyrA* occur, conferring moderate level resistance, that incur no fitness cost. Second step mutants contain mutation in *gyrA* and *parC*, conferring step wise increases in resistance with significant fitness deficits. No evidence for hypermutability in the Bcc was found.

Future Work

In this thesis appropriate methods have been developed to measure fitness and mutation rate in the Bcc. The isolates investigated in this thesis represented a small number of *B. cenocepacia* and *B. multivorans* CF strains. To build on the experience gained in this study the tools standardised in this thesis can now be used to investigate mechanisms of fluoroquinolone resistance and fitness and to screen for hypermutability in a larger panel of isolates to include isolates from other genomovars.

Mutation rate in planktonic culture in Bcc was determined in this thesis. It would be interesting to determine the mutation rate of Bcc cells growing as a biofilm and to compare this to the planktonic rate as the biofilm mutation rate may be higher and there may be a link between increased mutation rate and antibiotic resistance in biofilms. The maximum final inoculum of bacterial cells growing as a biofilm in the CDFF that could be obtained was too low to enable estimation of mutation rate. Use of the microtitre plate would not have been suitable for measurement of biofilm mutation rate because this model may not adequately represent an *in vivo* biofilm. Therefore this was not pursued in this thesis.

Creation of genetically defined topoisomerase and efflux mutations in isogenic strains would have allowed the fitness costs of each mutation to be measured without the possibility of other mutations occurring elsewhere in the genome. Additionally this would allow the contribution of each mutation on MIC to be elucidated.

Efflux mechanisms were not a primary focus of this thesis. However FQ efflux has emerged as an important mechanism of FQ resistance in Bcc. Fitness of the FQ resistant *in vitro* mutants where resistance was presumptively conferred by increase in efflux activity was not measured and therefore the fitness cost of increased efflux is not known. Additionally the mechanism of FQ efflux in these isolates was not characterised.

Characterisation of the quinolone resistance determining regions of topoisomerase genes from a larger number of clinical Bcc isolates from all genomovars could be performed and correlated with the level of fluoroquinolone MIC. This would enable clarification of the

role of topoisomerase point mutations in FQ resistance and would serve to enhance our understanding of the evolution of antibiotic resistance in Bcc.

REFERENCES

Aaron, S. D., Ferris, W., Henry, D. A., Speert, D. P., & Macdonald, N. E. 2000, "Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with *Burkholderia cepacia*", *Am.J.Respir.Crit Care Med.*, vol. 161, no. 4 Pt 1, pp. 1206-1212.

Aaron, S. D., Ferris, W., Ramotar, K., Vandemheen, K., Chan, F., & Saginur, R. 2002, "Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis", *J.Clin.Microbiol.*, vol. 40, pp. 4172-4179

Abraham, E. P. & Chain. E. 1940, "An enzyme from bacteria able to destroy penicillin", *Nature*, vol. 146, p. 837.

Agodi, A., Mahenthiralingam, E., Barchitta, M., Giannino, V., Sciacca, A., & Stefani, S. 2001, "*Burkholderia cepacia* complex infection in Italian patients with cystic fibrosis: prevalence, epidemiology, and genomovar status", *J.Clin.Microbiol.*, vol. 39, no. 8, pp. 2891-2896.

Aguiar, J. M., Chacon, J., Canton, R., & Baquero, F. 1992, "The emergence of highly fluoroquinolone-resistant *Escherichia coli* in community-acquired urinary tract infections", *J.Antimicrob.Chemother.*, vol. 29, no. 3, pp. 349-350.

Aguilar, C., Bertani, I., & Venturi, V. 2003, "Quorum-sensing system and stationary-phase sigma factor (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416", *Appl.Environ.Microbiol.*, vol. 69, no. 3, pp. 1739-1747.

Aguilar, C., Friscina, A., Devescovi, G., Kojic, M., & Venturi, V. 2003, "Identification of quorum-sensing-regulated genes of *Burkholderia cepacia*", *J.Bacteriol.*, vol. 185, no. 21, pp. 6456-6462.

Akasaka, T., Tanaka, M., Yamaguchi, A., & Sato, K. 2001, "Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance", *Antimicrob.Agents Chemother.*, vol. 45, no. 8, pp. 2263-2268.

Al Bakri, A. G., Gilbert, P., & Allison, D. G. 2004, "Immigration and emigration of *Burkholderia cepacia* and *Pseudomonas aeruginosa* between and within mixed biofilm communities", *J.Appl.Microbiol.*, vol. 96, no. 3, pp. 455-463.

Albrecht, M. T., Wang, W., Shamova, O., Lehrer, R. I., & Schiller, N. L. 2002, "Binding of protegrin-1 to *Pseudomonas aeruginosa* and *Burkholderia cepacia*", *Respir.Res.*, vol. 3, p. 18.

Allison, D. G., McBain, A. J., & Gilbert, P. 2000, "Biofilms: problems of control" in *Community structure and cooperation in Biofilms*, D. G. Allison et al., eds., University Press, Cambridge, pp. 309-327.

Anderl, J. N., Franklin, M. J., & Stewart, P. S. 2000, "Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin", *Antimicrob.Agents Chemother.*, vol. 44, no. 7, pp. 1818-1824.

Andersen, S. R. & Sandaa, R. A. 1994, "Distribution of tetracycline resistance determinants among Gram-negative bacteria isolated from polluted and unpolluted marine sediments", *Appl.Environ.Microbiol.*, vol. 60, no. 3, pp. 908-912.

Andersson, D. I., Bjorkman, J., & Hughes, D. 1998, "[Antibiotic resistance here to stay? Compensatory mutations restore virulence of resistant bacteria]", *Lakartidningen*, vol. 95, no. 37, pp. 3940, 3943-3940, 3944.

Andersson, D. I. & Hughes, D. 1996, "Muller's ratchet decreases fitness of a DNA-based microbe", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 2, pp. 906-907.

Andersson, D. I. & Levin, B. R. 1999, "The biological cost of antibiotic resistance", *Curr.Opin.Microbiol*, vol. 2, no. 5, pp. 489-493.

Anwar, H., van Biesen, T., Dasgupta, M., Lam, K., & Costerton, J. W. 1989, "Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system", *Antimicrob.Agents Chemother.*, vol. 33, no. 10, pp. 1824-1826.

Appelbaum, P. C. & Hunter, P. A. 2000, "The fluoroquinolone antibacterials: past, present and future perspectives", *Int.J.Antimicrob.Agents*, vol. 16, no. 1, pp. 5-15.

Arason, V. A., Gunnlaugsson, A., Sigurdsson, J. A., Erlendsdottir, H., Gudmundsson, S., & Kristinsson, K. G. 2002, "Clonal spread of resistant pneumococci despite diminished antimicrobial use", *Microb.Drug Resist.*, vol. 8, no. 3, pp. 187-192.

Arason, V. A., Sigurdsson, J. A., Erlendsdottir, H., Gudmundsson, S., & Kristinsson, K. G. 2006, "The role of antimicrobial use in the epidemiology of resistant pneumococci: A 10-year follow up", *Microb.Drug Resist.*, vol. 12, no. 3, pp. 169-176.

Aris, R. M., Routh, J. C., LiPuma, J. J., Heath, D. G., & Gilligan, P. H. 2001, "Lung transplantation for cystic fibrosis patients with *Burkholderia cepacia* complex. Survival linked to genomovar type", *Am.J.Respir.Crit Care Med.*, vol. 164, no. 11, pp. 2102-2106.

Ariza, R. R., Cohen, S. P., Bachhawat, N., Levy, S. B., & Demple, B. 1994, "Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*", *J.Bacteriol.*, vol. 176, no. 1, pp. 143-148.

Armitage, P. 1952, "The statistical theory of bacterial populations subject to mutation", *J.R.Statist.Soc.*, vol. 14, pp. 1-40.

Armitage, P. 1953, "Statistical concepts in the theory of bacterial mutation", *J.Hyg.*, vol. 51, pp. 162-184.

Asako, H., Nakajima, H., Kobayashi, K., Kobayashi, M., & Aono, R. 1997, "Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*", *Appl.Environ.Microbiol*, vol. 63, no. 4, pp. 1428-1433.

Asteris, G. & Sarkar, S. 1996, "Bayesian procedures for the estimation of mutation rates from fluctuation experiments", *Genetics*, vol. 142, no. 1, pp. 313-326.

Austin, D. J., Kristinsson, K. G., & Anderson, R. M. 1999, "The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 3, pp. 1152-1156.

Avery, O. T., MacLeod, C. M., & McCarty, M. 1944, "Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III", *Journal of Experimental Medicine*, vol. 79, no. 2, pp. 137-158.

Bachoual, R., Ouabdesselam, S., Mory, F., Lascols, C., Soussy, C. J., & Tankovic, J. 2001, "Single or double mutational alterations of *gyrA* associated with fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*", *Microb.Drug Resist.*, vol. 7, no. 3, pp. 257-261.

Bagel, S., Hullen, V., Wiedemann, B., & Heisig, P. 1999, "Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*", *Antimicrob.Agents Chemother.*, vol. 43, no. 4, pp. 868-875.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P., & Hoiby, N. 2004, "*Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production", *Antimicrob.Agents Chemother.*, vol. 48, no. 4, pp. 1175-1187.

Baird, R. M., Brown, H., Smith, A. W., & Watson, M. L. 1999, "*Burkholderia cepacia* is resistant to the antimicrobial activity of airway epithelial cells", *Immunopharmacology*, vol. 44, no. 3, pp. 267-272.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., & Leibler, S. 2004, "Bacterial persistence as a phenotypic switch", *Science*, vol. 305, no. 5690, pp. 1622-1625.

Baldwin, A., Mahenthiralingam, E., Drevinek, P, Pope, C., Waine, D.J., Henry, D.A., Speert, D. P., Carter, P., Vandamme P., LiPuma, J. J. & Dowson, C.G. 2008. "Elucidating global epidemiology of Burkholderia multivorans in cases of cystic fibrosis by multilocus sequence typing". *J. Clin.Micro*, vol. 46, pp. 290-295.

Baldwin, A., Sokol, P. A., Parkhill, J., & Mahenthiralingam, E. 2004, "The Burkholderia cepacia epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*", *Infect.Immun.*, vol. 72, no. 3, pp. 1537-1547.

Ballard R.W., Palleroni, N. J., Doudoroff, M., & Stanier, R. Y. 1970, "Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia*, *P.marginata*, *P.alliicola* and *P.caryophlli*", *Journal of General Microbiology*, vol. 60, pp. 199-214.

Baranova, N. N. & Neyfakh, A. A. 1997, "Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*", *Antimicrob.Agents Chemother.*, vol. 41, no. 6, pp. 1396-1398.

Barlow, C. W., Robbins, R. C., Moon, M. R., Akindipe, O., Theodore, J., & Reitz, B. A. 2000, "Heart-lung versus double-lung transplantation for suppurative lung disease", *J.Thorac.Cardiovasc.Surg.*, vol. 119, no. 3, pp. 466-476.

Barnard, F. M. & Maxwell, A. 2001, "Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87)", *Antimicrob.Agents Chemother.*, vol. 45, no. 7, pp. 1994-2000.

Barnett, M., Busby, S. R., & Mitchison, D. A. 1953, "Tubercle bacilli res to isoniazid: virulence and response to treatment with isoniazid in guinea pigs", *Br.J.Exp.Pathol.*, vol. 34(5), pp. 568-581.

Barth, A. L. & Pitt, T. L. 1995, "Auxotrophic variants of *Pseudomonas aeruginosa* are selected from prototrophic wild-type strains in respiratory infections in patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 33, no. 1, pp. 37-40.

Barth, A. L. & Pitt, T. L. 1995, "Auxotrophy of *Burkholderia* (*Pseudomonas*) *cepacia* from cystic fibrosis patients", *J.Clin.Microbiol.*, vol. 33, no. 8, pp. 2192-2194.

Barthelemy, P., Autissier, D., Gerbaud, G., & Courvalin, P. 1984, "Enzymic hydrolysis of erythromycin by a strain of *Escherichia coli*. A new mechanism of resistance", *J.Antibiot.(Tokyo)*, vol. 37, no. 12, pp. 1692-1696.

Bast, D. J., Low, D. E., Duncan, C. L., Kilburn, L., Mandell, L. A., Davidson, R. J., & de Azavedo, J. C. 2000, "Fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*: contributions of type II topoisomerase mutations and efflux to levels of resistance", *Antimicrob.Agents Chemother.*, vol. 44, no. 11, pp. 3049-3054.

Beaber, J. W., Hochhut, B., & Waldor, M. K. 2004, "SOS response promotes horizontal dissemination of antibiotic resistance genes", *Nature*, vol. 427, no. 6969, pp. 72-74.

Beckman, W. & Lessie, T. G. 1979, "Response of *Pseudomonas cepacia* to beta-Lactam antibiotics: utilization of penicillin G as the carbon source", *J.Bacteriol.*, vol. 140, no. 3, pp. 1126-1128.

Bedenic, B. & Zagar, Z. 1998, "Extended-spectrum beta-lactamases in clinical isolates of *Klebsiella pneumoniae* from Zagreb, Croatia", *J.Chemother.*, vol. 10, no. 6, pp. 449-459.

Bennett, A. F., Dao, K. M., & Lenski, R. E. 1990, "Rapid evolution in response to high-temperature selection", *Nature*, vol. 346, no. 6279, pp. 79-81.

Bernier, S. P., Silo-Suh, L., Woods, D. E., Ohman, D. E., & Sokol, P. A. 2003, "Comparative analysis of plant and animal models for characterization of *Burkholderia cepacia* virulence", *Infect.Immun.*, vol. 71, no. 9, pp. 5306-5313.

Beyer, R., Pestova, E., Millichap, J. J., Stosor, V., Noskin, G. A., & Peterson, L. R. 2000, "A convenient assay for estimating the possible involvement of efflux of fluoroquinolones by *Streptococcus pneumoniae* and *Staphylococcus aureus*: evidence for diminished moxifloxacin, sparfloxacin, and trovafloxacin efflux", *Antimicrob.Agents Chemother.*, vol. 44, no. 3, pp. 798-801.

Biddick, R., Spilker, T., Martin, A., & LiPuma, J. J. 2003, "Evidence of transmission of *Burkholderia cepacia*, *Burkholderia multivorans* and *Burkholderia dolosa* among persons with cystic fibrosis", *FEMS Microbiol.Lett.*, vol. 228, no. 1, pp. 57-62.

Bigger, J. W. 1944, "Treatment of staphylococcal infections", *Lancet* pp. 497-500.

Billington, O. J., McHugh, T. D., & Gillespie, S. H. 1999, "Physiological cost of rifampin resistance induced *in vitro* in *Mycobacterium tuberculosis*", *J.Antimicrob.Chemother.*, vol. 43, pp. 1866-1869.
Bjorkman, J., Hughes, D., & Andersson, D. I. 1998, "Virulence of antibiotic-resistant *Salmonella typhimurium*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 95, no. 7, pp. 3949-3953.

Bjorkman, J., Nagaev, I., Berg, O. G., Hughes, D., & Andersson, D. I. 2000, "Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance", *Science*, vol. 287, no. 5457, pp. 1479-1482.

Bjorkholm, B., Sjolund, M., Falk, P. G., Berg, O. G., Engstrand, L., & Andersson, D. I. 2001, "Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 25, pp. 14607-14612.

Blahova, J., Kralikova, K & Krcmery, V. 1992 "Imipenem and cefotaxime resistance: transduction by wild-type phages in hospital strains of *Pseudomonas aeruginosa*", *J.Chem.*, vol. 4, no. 6, pp. 335-7.

Boles, B. R., Thoendel, M., & Singh, P. K. 2004, "From the Cover: Self-generated diversity produces "insurance effects" in biofilm communities", *Proceedings of the National Academy of Sciences*, vol. 101, no. 47, pp. 16630-16635.

Bouma, J. E. & Lenski, R. E. 1988, "Evolution of a bacteria/plasmid association", *Nature*, vol. 335, no. 6188, pp. 351-352.

Breines, D. M., Ouabdesselam, S., Ng, E. Y., Tankovic, J., Shah, S., Soussy, C. J., & Hooper, D. C. 1997, "Quinolone resistance locus *nfxD* of *Escherichia coli* is a mutant allele of the *parE* gene encoding a subunit of topoisomerase IV", *Antimicrob.Agents Chemother.*, vol. 41, no. 1, pp. 175-179.

Brenwald, N. P., Gill, M. J., & Wise, R. 1998, "Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*", *Antimicrob.Agents Chemother.*, vol. 42, no. 8, pp. 2032-2035.

Brenwald, N. P., Gill, M. J., & Wise, R. 1997, "The effect of reserpine, an inhibitor of multi-drug efflux pumps, on the in-vitro susceptibilities of fluoroquinolone-resistant strains of *Streptococcus pneumoniae* to norfloxacin", *J.Antimicrob.Chemother.*, vol. 40, no. 3, pp. 458-460.

Bressler, A.M, Keith, Kaye, K.S., LiPuma, M.D., Alexander, M.D., Moore, C.M., Reller, B.R. & Woods, C.W. 2007. "Risk factors for *Burkholderia cepacia* complex bacteremia among intensive care unit patients without cystic fibrosis: a case control study" Infection. Con. and Hosp.Epidem., vol 28, no. 8, pp. 951-958.

Brisse, S., Milatovic, D., Fluit, A. C., Verhoef, J., Martin, N., Scheuring, S., Kohrer, K., & Schmitz, F. J. 1999, "Comparative *in vitro* activities of ciprofloxacin, clinafloxacin,

gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes* clinical isolates with alterations in GyrA and ParC proteins", *Antimicrob.Agents Chemother.*, vol. 43, no. 8, pp. 2051-2055.

Brooun, A., Liu, S., & Lewis, K. 2000, "A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms", *Antimicrob.Agents Chemother.*, vol. 44, no. 3, pp. 640-646.

Broschat, S. L., Loge, F. J., Peppin, J. D., White, D., Call, D. R., & Kuhn, E. 2005, "Optical reflectance assay for the detection of biofilm formation", *J.Biomed.Opt.*, vol. 10, no. 4, p. 44027.

Brown, E. W., LeClerc, J. E., Li, B., Payne, W. L., & Cebula, T. A. 2001, "Phylogenetic evidence for horizontal transfer of *mutS* alleles among naturally occurring *Escherichia coli* strains", *J.Bacteriol.*, vol. 183, no. 5, pp. 1631-1644.

Brown, M. H., Paulsen, I. T., & Skurray, R. A. 1999, "The multidrug efflux protein NorM is a prototype of a new family of transporters", *Mol.Microbiol.*, vol. 31, no. 1, pp. 394-395.

Burch, C. L. & Chao, L. 2000, "Evolvability of an RNA virus is determined by its mutational neighbourhood", *Nature*, vol. 406, no. 6796, pp. 625-628.

Burgos, M., DeRiemer, K., Small, P. M., Hopewell, P. C., & Daley, C. L. 2003, "Effect of drug resistance on the generation of secondary cases of tuberculosis", *J.Infect.Dis.*, vol. 188, no. 12, pp. 1878-1884.

Burkholder, W. H. 1950, "Sour skin, a bacterial rot of onion bulbs.", *Phytopathology*, vol. 40, pp. 115-117.

Burns, J. L., Jonas, M., Chi, E. Y., Clark, D. K., Berger, A., & Griffith, A. 1996, "Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*", *Infect.Immun.*, vol. 64, no. 10, pp. 4054-4059.

Burns, J. L., Hedin. L. A. & Lien., D. M. 1989, "Chloramphenicol resistance in *Pseudomonas aeruginosa* because of decreased permeability.", *Antimicrob.Agents Chemother.*, vol. 33, pp. 136-141.

Butler, S. L., Doherty, C. J., Hughes, J. E., Nelson, J. W., & Govan, J. R. 1995, "*Burkholderia cepacia* and cystic fibrosis: do natural environments present a potential hazard?", *J.Clin.Microbiol.*, vol. 33, no. 4, pp. 1001-1004.

Cairns, J., Overbaugh, J., & Miller, S. 1988, "The origin of mutants", *Nature*, vol. 335, no. 6186, pp. 142-145.

Campbell, A. 1962, "Episomes", *Adv.Genetics*, vol.. 11, pp. 101-146.

Campbell, P. W., III, Phillips, J. A., III, Heidecker, G. J., Krishnamani, M. R., Zahorchak, R., & Stull, T. L. 1995, "Detection of *Pseudomonas* (*Burkholderia*) *cepacia* using PCR", *Pediatr.Pulmonol.*, vol. 20, no. 1, pp. 44-49.

Cambau, E., Perani, E., Dib, C., Petinon, C., Trias, J., & Jarlier, V. 1995, "Role of mutations in DNA gyrase genes in ciprofloxacin resistance of *Pseudomonas aeruginosa* susceptible or resistant to imipenem", *Antimicrob.Agents Chemother.*, vol. 39, no. 10, pp. 2248-2252.

Canton, R., Coque, T. M., & Baquero, F. 2003, "Multi-resistant Gram-negative bacilli: from epidemics to endemics", *Curr.Opin.Infect.Dis.*, vol. 16, no. 4, pp. 315-325.

Caraher, E., Duff, C., Mullen, T., Mc, K. S., Murphy, P., Callaghan, M., & McClean, S. 2006, "Invasion and biofilm formation of *Burkholderia dolosa* is comparable with *Burkholderia cenocepacia* and *Burkholderia multivorans*", *J.Cyst.Fibros.*

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., & Buret, A. 1999, "The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms", *J.Clin.Microbiol.*, vol. 37, no. 6, pp. 1771-1776.

Chao, L. & Cox. E. C. 1983, "Competition between high and low mutating strains of *E. coli*", *Evolution*, vol. 37, pp. 125-134.

Chaowagul, W., White, N. J., Dance, D. A., Wattanagoon, Y., Naigowit, P., Davis, T. M., Looareesuwan, S., & Pitakwatchara, N. 1989, "Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand", *J.Infect.Dis.*, vol. 159, no. 5, pp. 890-899.

Chaparro, C., Maurer, J., Gutierrez, C., Krajden, M., Chan, C., Winton, T., Keshavjee, S., Scavuzzo, M., Tullis, E., Hutcheon, M., & Kesten, S. 2001, "Infection with *Burkholderia cepacia* in cystic fibrosis: outcome following lung transplantation", *Am.J.Respir.Crit Care Med.*, vol. 163, no. 1, pp. 43-48.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M., & Bernardi, T. 2007, "A new device for rapid evaluation of biofilm formation potential by bacteria", *J.Microbiol.Methods*, vol. 68, no. 3, pp. 605-612.

Chen, D.K., McGeer, A, de Azavedo, J.C. & Low, P.E, 1999. Decreased susceptibility of *S. pneumoniae* to fluoroquinolones in Canada. *N. Engl.J.Med.*, vol. 341, pp. 233-235.

Chen, F. J. & Lo, H. J. 2003, "Molecular mechanisms of fluoroquinolone resistance", *J.Microbiol.Immunol.Infect.*, vol. 36, no. 1, pp. 1-9.

Chen, J. S., Witzmann, K. A., Spilker, T., Fink, R. J., & LiPuma, J. J. 2001, "Endemicity and inter-city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis", *J.Pediatr.*, vol. 139, no. 5, pp. 643-649.

Chin, N. X., Figueredo, V. M., Novelli, A., & Neu, H. C. 1988, "*In vitro* activity of temafloxacin, a new difluoro quinolone antimicrobial agent", *Eur.J.Clin.Microbiol.Infect.Dis.*, vol. 7, no. 1, pp. 58-63.

Chin, N. X., Novelli, A., & Neu, H. C. 1988, "*In* vitro activity of lomefloxacin (SC-47111; NY-198), a difluoroquinolone 3-carboxylic acid, compared with those of other quinolones", *J.Antimicrob.Chemother.*, vol. 32, no. 5, pp. 656-662.

Chiu, C. H., Wu, T. L., Su, L. H., Chu, C., Chia, J. H., Kuo, A. J., Chien, M. S., & Lin, T. Y. 2002, "The emergence in Taiwan of fluoroquinolone resistance in *Salmonella enterica* serotype choleraesuis", *N.Engl.J.Med.*, vol. 346, no. 6, pp. 413-419.

Chu, E. H. & Malling, H. V. 1968, "Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells *in vitro*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 61, no. 4, pp. 1306-1312.

Chu, K. K., Davidson, D. J., Halsey, T. K., Chung, J. W., & Speert, D. P. 2002, "Differential persistence among genomovars of the *Burkholderia cepacia* complex in a murine model of pulmonary infection", *Infect.Immun.*, vol. 70, no. 5, pp. 2715-2720.

Cieri, M. V., Mayer-Hamblett, N., Griffith, A., & Burns, J. L. 2002, "Correlation between an *in vitro* invasion assay and a murine model of *Burkholderia cepacia* lung infection", *Infect.Immun.*, vol. 70, no. 3, pp. 1081-1086.

Cirillo, D. M., Valdivia, R. H., Monack, D. M., & Falkow, S. 1998, "Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival", *Mol.Microbiol.*, vol. 30, no. 1, pp. 175-188.

Clinical and Laboratory Standards Institute. 2006. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. Seventh edition M7-A6.* Villanove, PA, USA. NCCLS.

Clode, F. E., Kaufmann, M. E., Malnick, H., & Pitt, T. L. 2000, "Distribution of genes encoding putative transmissibility factors among epidemic and nonepidemic strains of *Burkholderia cepacia* from cystic fibrosis patients in the United Kingdom", *J.Clin.Microbiol*, vol. 38, no. 5, pp. 1763-1766.

Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J. J., Laevens, S., Gillis, M., Speert, D. P., & Vandamme, P. 2001, "*Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates", *Int.J.Syst.Evol.Microbiol.*, vol. 51, no. Pt 4, pp. 1481-1490.

Coenye, T., Schouls, L. M., Govan, J. R., Kersters, K., & Vandamme, P. 1999, "Identification of *Burkholderia* species and genomovars from cystic fibrosis patients by AFLP fingerprinting", *Int.J.Syst.Bacteriol.*, vol. 49 Pt 4, pp. 1657-1666.

Coenye, T. & Vandamme, P. 2005, "Overrepresentation of immunostimulatory CpG motifs in *Burkholderia* genomes", *J.Cyst.Fibros.*, vol. 4, no. 3, pp. 193-196.

Coenye, T., Vandamme, P., Govan, J. R., & LiPuma, J. J. 2001, "Taxonomy and identification of the *Burkholderia cepacia* complex", *J.Clin.Microbiol.*, vol. 39, no. 10, pp. 3427-3436.

Cohen, S. P., McMurry, L. M., & Levy, S. B. 1988, "*marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*", *J.Bacteriol.*, vol. 170, no. 12, pp. 5416-5422.

Cohen, S. P., Yan, W., & Levy, S. B. 1993, "A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria", *J.Infect.Dis.*, vol. 168, no. 2, pp. 484-488.

Cohen, T. & Murray, M. 2004, "Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness", *Nat.Med.*, vol. 10, no. 10, pp. 1117-1121.

Coldham, N. G., Randall, L. P., Piddock, L. J., & Woodward, M. J. 2006, "Effect of fluoroquinolone exposure on the proteome of *Salmonella enterica* serovar Typhimurium", *J.Antimicrob.Chemother.*, vol. 58, no. 6, pp. 1145-1153.

Collazo, C. M. & Galan, J. E. 1996, "Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella typhimurium*", *Infect.Immun.*, vol. 64, no. 9, pp. 3524-3531.

Colten, H. R. 1990, "Screening for cystic fibrosis; public policy and personal choices", *N.Engl.J.Med.*, vol. 322, no. 5, pp. 328-329.

Conway, B. A., Venu, V., & Speert, D. P. 2002, "Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex", *J.Bacteriol.*, vol. 184, no. 20, pp. 5678-5685.

Corbett, C. R., Burtnick, M. N., Kooi, C., Woods, D. E., & Sokol, P. A. 2003, "An extracellular zinc metalloprotease gene of *Burkholderia cepacia*", *Microbiology*, vol. 149, no. Pt 8, pp. 2263-2271.

Cornelis, G. R. & Wolf-Watz, H. 1997, "The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells", *Mol.Microbiol.*, vol. 23, no. 5, pp. 861-867.

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. 1987, "Bacterial biofilms in nature and disease", *Annu.Rev.Microbiol.*, vol. 41, pp. 435-464.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. 1999, "Bacterial biofilms: a common cause of persistent infections", *Science*, vol. 284, no. 5418, pp. 1318-1322.

Courvalin, P. 2006, "Vancomycin resistance in Gram-positive cocci", *Clin.Infect.Dis.*, vol. 42 Suppl 1, p. S25-S34.

Cox, A. D. & Wilkinson, S. G. 1991, "Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance", *Mol Microbiol*, vol. 5, no. 3, pp. 641-646.

Crane, G. J., Thomas, S. M., & Jones, M. E. 1996, "A modified Luria-Delbruck fluctuation assay for estimating and comparing mutation rates", *Mutat.Res.*, vol. 354, no. 2, pp. 171-182.

Criswell, D., Tobiason, V. L., Lodmell, J. S., & Samuels, D. S. 2006, "Mutations conferring aminoglycoside and spectinomycin resistance in *Borrelia burgdorferi*", *Antimicrob.Agents Chemother.*, vol. 50, no. 2, pp. 445-452.

Cunha, M. V., Sousa, S. A., Leitao, J. H., Moreira, L. M., Videira, P. A., & Sa-Correia, I. 2004, "Studies on the involvement of the exopolysaccharide produced by cystic fibrosis-associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections", *J.Clin.Microbiol*, vol. 42, no. 7, pp. 3052-3058.

D'Argenio, D. A., Gallagher, L. A., Berg, C. A., & Manoil, C. 2001, "Drosophila as a model host for *Pseudomonas aeruginosa* infection", *J.Bacteriol.*, vol. 183, no. 4, pp. 1466-1471.

Darling, P., Chan, M., Cox, A. D., & Sokol, P. A. 1998, "Siderophore production by cystic fibrosis isolates of *Burkholderia cepacia*", *Infect.Immun.*, vol. 66, no. 2, pp. 874-877.

Darveau, R. P., Tanner, A., & Page, R. C. 1997, "The microbial challenge in periodontitis", *Periodontol.*., vol. 14, pp. 12-32.

Davies, A. P., Billington, O. J., Bannister, B. A., Weir, W. R., McHugh, T. D., & Gillespie, S. H. 2000, "Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment", *J.Infect.*, vol. 41, no. 2, pp. 184-187.

Davies, D. G., Chakrabarty, A. M., & Geesey, G. G. 1993, "Exopolysaccharide production in Biofilms: substratum activation of alginate gene expression in *Pseudomonas aerugiosa*", *Appl. Environ. Microbiol.*, vol. 59, pp. 1181-1186.

Davies, J. 1994, "Inactivation of antibiotics and the dissemination of resistance genes", *Science*, vol. 264, no. 5157, pp. 375-382.

Davison, J. 1999. "Genetic exchange between bacteria and environment", *Plasmid*, vol. 42, pp. 73-91.

De Soyza, A., Ellis, C.D., Khan, C.M., Corns, P.A. & Dermarco de Hormaeche, R, 2004, "*B. cenocepacia* lipopolysaccharide, lipid A and proinflammatory ability". Am. J. Respir. Crit. Care Med. vol. pp. 170, 70-77.

Dessus-Babus, S., Bebear, C. M., Charron, A., Bebear, C., & de Barbeyrac, B. 1998, "Sequencing of gyrase and topoisomerase IV quinolone-resistance-determining regions of *Chlamydia trachomatis* and characterization of quinolone-resistant mutants obtained *in vitro*", *Antimicrob.Agents Chemother.*, vol. 42, no. 10, pp. 2474-2481.

Devaud, M., Kayser, F.H & Bächi, B. 1982. "Transposon mediated antibiotic resistance in *Acinetobacter* strains", *Antimicrob. Agents. Chem.*, vol. 22, no. 2, pp. 323-329.

Dever, L.A. & Dermody, T.S. 1991. "Mechanisms of bacterial resistance to antibiotics", *Arch.Intern.Med.*, vol. 151, no.6, pp. 886-95.

Devine, M., Moore, J.E., Xu, J., Millar, B.C., Dunbar, K., Stanley, T., Murphy, P.G., Redmond, A.O. & Elborn, J.S. 2004. "Detection of mycobacterial DNA from sputum of patients with cystic fibrosis", *Ir.J.Med.Sci.*, vol. 173, no.2, pp. 96-98.

de Visser, J.AG.M. 2002. "The fate of microbial mutators", *Microbiology*, vol.148, pp. 1247-1252.

Dhakephalkar, P. K. & Chopade, B. A. 1994, "High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*", *Biometals*, vol. 7, no. 1, pp. 67-74.

Djordjevic, D., Wiedmann, M., & McLandsborough, L. A. 2002, "Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation", *Appl.Environ.Microbiol.*, vol. 68, no. 6, pp. 2950-2958.

Dodge, J. A., Lewis, P. A., Stanton, M., & Wilsher, J. 2007, "Cystic fibrosis mortality and survival in the UK: 1947-2003", *European Respiratory Journal*, vol. 29, no. 3, pp. 522-526.

Donlan, R. M. & Costerton, J. W. 2002, "Biofilms: survival mechanisms of clinically relevant microorganisms", *Clin.Microbiol Rev.*, vol. 15, no. 2, pp. 167-193.

Drabick, J. A., Gracely, E. J., Heidecker, G. J., & LiPuma, J. J. 1996, "Survival of *Burkholderia cepacia* on environmental surfaces", *J.Hosp.Infect.*, vol. 32, no. 4, pp. 267-276.

Drake, J. W. 1991, "Constant rate of spontaneous muations in DNA based microbes", *Proc.Natl.Acad.Sci.USA* no. 88, pp. 7160-7164.

Drlica, K. & Hooper, D.C. 2003, "Mechanisms of Quinolone Action," in *Quinolone Antimicrobial Agents*, 3 edn, Hooper, D. C. & Rubenstein, E., ASM Press, Washington D.C., pp. 19-40.

Drlica, K. 2003, "The mutant selection window and antimicrobial resistance", *J.Antimicrob.Chemother.*, vol. 52, no. 1, pp. 11-17.

Drlica, K. & Malik M. 2003 "Fluoroquinolones:Action and Resistance". *Current Topics in Med. Chem.*, vol. 3, pp. 249-282.

Drlica, K. & Zhou, X 1997, "DNA gyrase, topoisomerase IV, and the 4-quinolones", *Microbiol. Mol. Biol. Rev.*, vol. 61, no. 3, pp. 377-392.

Du Bois, S. K., Davison, A. L., & Pinney, R. J. 1995, "Epidemiology and susceptibilities to mercury preservatives of staphylococci isolated from used eye-drops preserved with thiomersal", *J.Pharm.Pharmacol.*, vol. 47, no. 3, pp. 193-196.

Dunne, W. M., Jr. 2002, "Bacterial adhesion: seen any good biofilms lately?", *Clin.Microbiol.Rev.*, vol. 15, no. 2, pp. 155-166.

Durso, L. M., Smith, D., & Hutkins, R. W. 2004, "Measurements of fitness and competition in commensal *Escherichia coli* and *E. coli* O157:H7 strains", *Appl.Environ.Microbiol.*, vol. 70, no. 11, pp. 6466-6472.

Eaves, D. J., Randall, L., Gray, D. T., Buckley, A., Woodward, M. J., White, A. P., & Piddock, L. J. 2004, "Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*", *Antimicrob.Agents Chemother.*, vol. 48, no. 10, pp. 4012-4015.

Ednie, L. M., Jacobs, M. R., & Appelbaum, P. C. 1998, "Comparative activities of clinafloxacin against Gram-positive and negative bacteria", *Antimicrob.Agents Chemother.*, vol. 42, no. 5, pp. 1269-1273.

Egan, J. J., McNeil, K., Bookless, B., Gould, K., Corris, P., Higenbottam, T., Webb, A. K., & Woodcock, A. A. 1994, "Post-transplantation survival of cystic fibrosis patients infected with *Pseudomonas cepacia*", *Lancet*, vol. 344, no. 8921, pp. 552-553.

Elborn, J. S., Shale, D. J., & Britton, J. R. 1991, "Cystic fibrosis: current survival and population estimates to the year 2000", *Thorax*, vol. 46, no. 12, pp. 881-885.

Ender, M., McCallum, N., Adhikari, R., & Berger-Bachi, B. 2004, "Fitness cost of SCCmec and methicillin resistance levels in *Staphylococcus aureus*", *Antimicrob.Agents Chemother.*, vol. 48, no. 6, pp. 2295-2297.

Enne, V. I., Bennett, P. M., Livermore, D. M., & Hall, L. M. 2004, "Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure", *J.Antimicrob.Chemother.*, vol. 53, no. 6, pp. 958-963.

Enne, V. I., Livermore, D. M., Stephens, P., & Hall, L. M. 2001, "Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction", *Lancet*, vol. 357, no. 9265, pp. 1325-1328.

Ensor, E., Humphreys, H., Peckham, D., Webster, C., & Knox, A. J. 1996, "Is *Burkholderia* (*Pseudomonas*) *cepacia* disseminated from cystic fibrosis patients during physiotherapy?", *J. Hos. Inf.*, vol. 32, no. 1, pp. 9-15.

Espinoza, A. M., Chin, N. X., Novelli, A., & Neu, H. C. 1988, "Comparative in vitro activity of a new fluorinated 4-quinolone, T-3262 (A-60969)", *Antimicrob.Agents Chemother.*, vol. 32, no. 5, pp. 663-670.

Everett, M. J., Jin, Y. F., Ricci, V., & Piddock, L. J. 1996, "Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals", *Antimicrob.Agents Chemother.*, vol. 40, no. 10, pp. 2380-2386.

Fergie, N., Bayston, R., Pearson, J. P., & Birchall, J. P. 2004, "Is otitis media with effusion a biofilm infection?", *Clin.Otolaryngol.Allied Sci.*, vol. 29, no. 1, pp. 38-46.

Ferrero, L., Cameron, B., & Crouzet, J. 1995, "Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*", *Antimicrob.Agents Chemother.*, vol. 39, no. 7, pp. 1554-1558.

Ferrero, L., Cameron, B., Manse, B., Lagneaux, D., Crouzet, J., Famechon, A., & Blanche, F. 1994, "Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones", *Mol.Microbiol.*, vol. 13, no. 4, pp. 641-653.

Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L., & Frank, D. W. 1997, "ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury", *Mol.Microbiol.*, vol. 25, no. 3, pp. 547-557.

Fluit, A. C., Wielders, C. L., Verhoef, J., & Schmitz, F. J. 2001, "Epidemiology and susceptibility of 3,051 *Staphylococcus aureus* isolates from 25 university hospitals participating in the European SENTRY study", *J.Clin.Microbiol*, vol. 39, no. 10, pp. 3727-3732.

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M. D., Fujii, C., Garland, S., Hatch, B., Horst, K., Roberts, K., Sandusky, M., Weidman, J., Smith, H. O., & Venter, J. C. 1998, "Complete genome sequence of *Treponema pallidum*, the syphilis spirochete", *Science*, vol. 281, no. 5375, pp. 375-388.

Furet, Y. X., Deshusses, J., & Pechere, J. C. 1992, "Transport of pefloxacin across the bacterial cytoplasmic membrane in quinolone-susceptible *Staphylococcus aureus*", *Antimicrob.Agents Chemother.*, vol. 36, no. 11, pp. 2506-2511.

Gagneux, S., Long, C. D., Small, P. M., Van, T., Schoolnik, G. K., & Bohannan, B. J. 2006, "The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*", *Science*, vol. 312, no. 5782, pp. 1944-1946.

Gangoue-Pieboji, J., Bedenic, B., Koulla-Shiro, S., Randegger, C., Adiogo, D., Ngassam, P., Ndumbe, P., & Hachler, H. 2005, "Extended-spectrum-beta-lactamase-producing Enterobacteriaceae in Yaounde, Cameroon", *J.Clin.Microbiol*, vol. 43, no. 7, pp. 3273-3277.

Garcia-Rey, C., Martin-Herrero, J. E., & Baquero, F. 2006, "Antibiotic consumption and generation of resistance in *Streptococcus pneumoniae*: the paradoxical impact of quinolones in a complex selective landscape", *Clin.Microbiol.Infect.*, vol. 12 Suppl 3, pp. 55-66.

Garrido, M. C., Herrero, M., Kolter, R., & Moreno, F. 1988, "The export of the DNA replication inhibitor Microcin B17 provides immunity for the host cell", *EMBO J.*, vol. 7, no. 6, pp. 1853-1862.

Gee, J. E., Sacchi, C. T., Glass, M. B., De, B. K., Weyant, R. S., Levett, P. N., Whitney, A. M., Hoffmaster, A. R., & Popovic, T. 2003, "Use of 16S rRNA gene sequencing for rapid identification and differentiation of *Burkholderia pseudomallei* and *B. mallei*", *J.Clin.Microbiol.*, vol. 41, no. 10, pp. 4647-4654.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., & Tomizawa, J. I. 1977, "Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity", *Proc.Natl.Acad.Sci.U.S.A*, vol. 74, no. 11, pp. 4772-4776.

Gellert, M., Mizuuchi, K., O'Dea, M. H., & Nash, H. A. 1976, "DNA gyrase: an enzyme that introduces superhelical turns into DNA", *Proc.Natl.Acad.Sci.U.S.A*, vol. 73, no. 11, pp. 3872-3876.

George, A. M. & Levy, S. B. 1983, "Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline", *J.Bacteriol.*, vol. 155, no. 2, pp. 531-540.

Gerding, D. N., Larson, T. A., Hughes, R. A., Weiler, M., Shanholtzer, C., & Peterson, L. R. 1991, "Aminoglycoside resistance and aminoglycoside usage: ten years of experience in one hospital", *Antimicrob.Agents Chemother.*, vol. 35, no. 7, pp. 1284-1290.

Gessner, A. R. & Mortensen, J. E. 1990, "Pathogenic factors of *Pseudomonas cepacia* isolates from patients with cystic fibrosis", *J.Med.Microbiol.*, vol. 33, no. 2, pp. 115-120.

Geesey, G. G. 2001, "Bacterial behavior at surfaces", *Curr.Opin.Microbiol.*, vol. 4, no. 3, pp. 296-300.

Gibson, R. L., Burns, J. L., & Ramsey, B. W. 2003, "Pathophysiology and management of pulmonary infections in cystic fibrosis", *Am.J.Respir.Crit Care Med.*, vol. 168, no. 8, pp. 918-951.

Gillespie, S. H. 2001, "Antibiotic resistance in the absence of selective pressure", *Int.J.Antimicrob.Agents*, vol. 17, no. 3, pp. 171-176.

Gillespie, S. H., Basu, S., Dickens, A. L., O'Sullivan, D. M., & McHugh, T. D. 2005, "Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates", *J.Antimicrob.Chemother.*, vol. 56, no. 2, pp. 344-348.

Gillespie, S. H., Billington, O. J., Breathnach, A., & McHugh, T. D. 2002, "Multiple drug-resistant *Mycobacterium tuberculosis*: evidence for changing fitness following passage through human hosts", *Microb.Drug Resist.*, vol. 8, no. 4, pp. 273-279.

Gillespie, S. H. & McHugh, T. D. 1997, "The biological cost of antimicrobial resistance", *Trends Microbiol*, vol. 5, no. 9, pp. 337-339.

Gillespie, S. H., Voelker, L. L., Ambler, J. E., Traini, C., & Dickens, A. 2003, "Fluoroquinolone resistance in *Streptococcus pneumoniae*:evidence that *gyrA* mutations arise at a lower rate and that mutation in *gyrA* or *parC* predisposes to further mutation", *Microbial Drug Resistance*, vol. 9, pp. 17-24.

Gillespie, S. H., Voelker, L. L., & Dickens, A. 2002, "Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae*", *Microb.Drug Resist.*, vol. 8, no. 2, pp. 79-84.

Gilligan, P. H. 1991, "Microbiology of airway disease in patients with cystic fibrosis", *Clin.Microbiol.Rev.*, vol. 4, no. 1, pp. 35-51.

Gilligan, P. H., Gage, P. A., Welch, D. F., Muszynski, M. J., & Wait, K. R. 1987, "Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 25, no. 7, pp. 1258-1261.

Gillis, M., Van Van, T., Bardin, R., Goor, M., Hebbar, P., Williams, A., Segers, P., & Kersters, K. 1995, "Polyphasic taxonomy in the genus *Burkholderia* leading to an amended description of the genus and proposition of *Burkholderia vietnamensis* sp. nov for nitrogen fixing isolates from rice in Vietnam", *International Journal of Systemic and Evolutionary Microbiology*, vol. 45, pp. 274-289.

Giraud, E., Cloeckaert, A., Baucheron, S., Mouline, C., & Chaslus-Dancla, E. 2003, "Fitness cost of fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium", *J.Med.Microbiol*, vol. 52, no. Pt 8, pp. 697-703.

Giraud, A., Matic, I., Tenaillon, O., Clara, A., Radman, M., Fons, M., & Taddei, F. 2001, "Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut", *Science*, vol. 291, no. 5513, pp. 2606-2608.

Glendinning, K. J., Parsons, Y. N., Duangsonk, K., Hales, B. A., Humphreys, D., Hart, C. A., & Winstanley, C. 2004, "Sequence divergence in type III secretion gene clusters of the *Burkholderia cepacia* complex", *FEMS Microbiol Lett.*, vol. 235, no. 2, pp. 229-235.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M., & Wilson, J. M. 1997, "Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis", *Cell*, vol. 88, no. 4, pp. 553-560.

Gonzalez, C. F. & Vidaver, A. K. 1979, "Bacteriocin, plasmid and pectolytic diversity in *Pseudomonas cepacia* of clinical and plant origin", *J.Gen.Microbiol*, vol. 110, no. 1, pp. 161-170.

Gootz, T. D. & Martin, B. A. 1991, "Characterization of high-level quinolone resistance in *Campylobacter jejuni*", *Antimicrob.Agents Chemother.*, vol. 35, no. 5, pp. 840-845.

Goss, W. A., Deitz, W. H., & Cook, T. M. 1964, "Mechanism of action of nalidixic acid on *Escherchia coli*", *J.Bacteriol.*, vol. 88, pp. 1112-1118.

Gotschlich, A., Huber, B., Geisenberger, O., Togl, A., Steidle, A., Riedel, K., Hill, P., Tummler, B., Vandamme, P., Middleton, B., Camara, M., Williams, P., Hardman, A., & Eberl, L. 2001, "Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex", *Syst.Appl.Microbiol.*, vol. 24, no. 1, pp. 1-14.

Gotz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschape, H., van Elsas, J. D., & Smalla, K. 1996, "Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR", *Appl.Environ.Microbiol.*, vol. 62, no. 7, pp. 2621-2628.

Govan, J. R., Brown, P. H., Maddison, J., Doherty, C. J., Nelson, J. W., Dodd, M., Greening, A. P., & Webb, A. K. 1993, "Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis", *Lancet*, vol. 342, no. 8862, pp. 15-19.

Govan, J. R. & Deretic, V. 1996, "Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*", *Microbiol.Rev.*, vol. 60, no. 3, pp. 539-574.

Govan, J. R. & Nelson, J. W. 1993, "Microbiology of cystic fibrosis lung infections: themes and issues", *J.R.Soc.Med.*, vol. 86 Suppl 20, pp. 11-18.

Govan, J. R. & Vandamme, P. 1998, "Agricultural and medical microbiology: a time for bridging gaps", *Microbiology*, vol. 144, pp. 2373-2375.

Griffiths, A. J. F., Gelbert, W. M., Lewontin, R. C., Wessler, S. R., Suzuki, D. T. and Miller J. H. 2000. "An Introduction to Genetic Analysis", 7th ed, W.H. Freeman and Company.

Griffiths, F. 1928, "The significance of pneumococcal types", *Journal of Hygiene (Lond)*, vol. 27, pp. 113-159.

Guiney, D. G., Jr. 1984, "Promiscuous transfer of drug resistance in Gram-negative bacteria", *J.Infect.Dis.*, vol. 149, no. 3, pp. 320-329.

Gustafsson, I., Cars, O., & Andersson, D. I. 2003, "Fitness of antibiotic resistant *Staphylococcus epidermidis* assessed by competition on the skin of human volunteers", *J.Antimicrob.Chemother.*, vol. 52, no. 2, pp. 258-263.

Gustafsson, I., Sjolund, M., Torell, E., Johannesson, M., Engstrand, L., Cars, O., & Andersson, D. I. 2003, "Bacteria with increased mutation frequency and antibiotic resistance are enriched in the commensal flora of patients with high antibiotic usage", *J.Antimicrob.Chemother.*, vol. 52, no. 4, pp. 645-650.

Hachler, H., Cohen, S. P., & Levy, S. B. 1991, "*marA*, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *Escherichia coli*", *J.Bacteriol.*, vol. 173, no. 17, pp. 5532-5538.

Hales, B. A., Morgan, J. A., Hart, C. A., & Winstanley, C. 1998, "Variation in flagellin genes and proteins of *Burkholderia cepacia*", *J.Bacteriol.*, vol. 180, no. 5, pp. 1110-1118.

Hall-Stoodley, L., Hu, F. Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J., Forbes, M., Greenberg, D. P., Dice, B., Burrows, A., Wackym, P. A., Stoodley, P., Post, J. C., Ehrlich, G. D., & Kerschner, J. E. 2006, "Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media", *JAMA*, vol. 296, no. 2, pp. 202-211.

Hallett, P. & Maxwell, A. 1991, "Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins", *Antimicrob.Agents Chemother.*, vol. 35, no. 2, pp. 335-340.

Hakanen, A., Siitonen, A., Kotilainen, P. & Huonnen, P. 1999. Increasing fluoroquinolone resistance in *Salmonella* serotypes in Finland during 1995-1997. *J.Antimicrob. Agents.*, vol. 43, pp. 145-148.

Hancock, R. E. 1998, "Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria", *Clin.Infect.Dis.*, vol. 27 Suppl 1, p. S93-S99.

Hanzelka, B. L. & Greenberg, E. P. 1995, "Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain", *J.Bacteriol.*, vol. 177, no. 3, pp. 815-817.

Harnett, N., McLeod, S., AuYong, Y., Wan, J., Alexander, S., Khakhria, R., & Krishnan, C. 1998, "Molecular characterization of multiresistant strains of *Salmonella typhi* from South Asia isolated in Ontario, Canada", *Can.J.Microbiol.*, vol. 44, no. 4, pp. 356-363.

Harris, M. 1971, "Mutation rates in cells at different ploidy levels", *J.Cell Physiol*, vol. 78, no. 2, pp. 177-184.

Hart, C. A. & Winstanley, C. 2002, "Persistent and aggressive bacteria in the lungs of cystic fibrosis children", *Br.Med.Bull.*, vol. 61, pp. 81-96.

Harwig, S. S., Waring, A., Yang, H. J., Cho, Y., Tan, L., & Lehrer, R. I. 1996, "Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations", *Eur.J.Biochem.*, vol. 240, no. 2, pp. 352-357.

Hata, M., Suzuki, M., Matsumoto, M., Takahashi, M., Sato, K., Ibe, S., & Sakae, K. 2005, "Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri*", *Antimicrob.Agents Chemother.*, vol. 49, no. 2, pp. 801-803.

Henderson-Begg, S. K., Livermore, D. M., & Hall, L. M. 2006, "Effect of subinhibitory concentrations of antibiotics on mutation frequency in *Streptococcus pneumoniae*", *J.Antimicrob.Chemother.*, vol. 57, no. 5, pp. 849-854.

Hendrickson, H., Slechta, E. S., Bergthorsson, U., Andersson, D. I., & Roth, J. R. 2002, "Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification", *Proc.Natl.Acad.Sci.U.S.A*, vol. 99, no. 4, pp. 2164-2169.

Hendry, J., Elborn, J.S., Nixon, L., Shale, D.J. & Webb, A.K. 1999. "Cystic fibrosis: inflammatory response to infection with *Burkholderia cepacia* and *Pseudomonas aeruginosa". Eur. Respir. J.*, vol. 14, pp. 435-438.

Hengtrakool, C., Pearson, G. J., & Wilson, M. 2006, "Interaction between GIC and *S. sanguis* biofilms: antibacterial properties and changes of surface hardness", *J.Dent.*, vol. 34, no. 8, pp. 588-597.

Henry, D. A., Campbell, M. E., LiPuma, J. J., & Speert, D. P. 1997, "Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium", *J.Clin.Microbiol.*, vol. 35, no. 3, pp. 614-619.

Hogberg, L., Ekdahl, K., Sjostrom, K., Olsson-Liljequist, B., Walder, M., Melander, E., Ringberg, H., & Normark, B. H. 2006, "Penicillin-resistant pneumococci in Sweden 1997-2003: increased multiresistance despite stable prevalence and decreased antibiotic use", *Microb.Drug Resist.*, vol. 12, no. 1, pp. 16-22.

Hodgson, A. E., Nelson, S. M., Brown, M. R., & Gilbert, P. 1995, "A simple *in vitro* model for growth control of bacterial biofilms", *J.Appl.Bacteriol.*, vol. 79, no. 1, pp. 87-93.

Holden, M. T., Titball, R. W., Peacock, S. J., Cerdeno-Tarraga, A. M., Atkins, T., Crossman, L. C., Pitt, T., Churcher, C., Mungall, K., Bentley, S. D., Sebaihia, M., Thomson, N. R., Bason, N., Beacham, I. R., Brooks, K., Brown, K. A., Brown, N. F., Challis, G. L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, B., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K. E., Maddison, M., Moule, S., Price, C., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B. G., Oyston, P. C., & Parkhill, J. 2004, "Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 39, pp. 14240-14245.

Holmes, A., Nolan, R., Taylor, R., Finley R., Riley, M., Jiang, R.Z., Steinbach, S. & Goldstein, R. 1999. "An epidemic of *Burkholderia cepacia* transmitted between patients with and without cystic fibrosis". *J. Infect. Dis.*, vol 179, pp. 1197-1205.

Hooper, D. C. 1998, "Clinical applications of quinolones", *Biochim.Biophys.Acta*, vol. 1400, no. 1-3, pp. 45-61.

Honraet, K. & Nelis, H. J. 2006, "Use of the modified robbins device and fluorescent staining to screen plant extracts for the inhibition of *S. mutans* biofilm formation", *J.Microbiol.Methods*, vol. 64, no. 2, pp. 217-224.

Hope, C. K. & Wilson, M. 2006, "Biofilm structure and cell vitality in a laboratory model of subgingival plaque", *J.Microbiol.Methods*, vol. 66, no. 3, pp. 390-398.

Hope, C. K. & Wilson, M. 2003, "Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping", *J.Microbiol.Methods*, vol. 54, no. 3, pp. 403-410.

Hooper, D. C. 2003, "Resistance to Quinolones," in *Quinolone Antimicrobial Agents*, 3 edn, E. Hooper D.C.& Rubenstein, ed., ASM Press, Washington, D.C.

Hoyle, B. D., Williams, L. J. and Costerton, J.W. 1993, "Production of mucoid exopolysaccharide during development of *Pseudomonas aeruginosa* biofilms", *Infect.Immun.* vol. 61, no. 2, pp. 777-780.

Huang, C. T., Xu, K. D., McFeters, G. A., & Stewart, P. S. 1998, "Spatial Patterns of Alkaline Phosphatase Expression within Bacterial Colonies and Biofilms in Response to Phosphate Starvation", *Applied and Environmental Microbiology*, vol. 64, no. 4, pp. 1526-1531.

Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S., & Eberl, L. 2001, "The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility", *Microbiology*, vol. 147, no. Pt 9, pp. 2517-2528.

Hughes, D. & Anderssen D.I. 1997. "Carbon starvation of *Salmonella typhimurium* does not cause a general increase of mutation rates", *J. Bacteriol.*, vol. 11, pp. 6688-6691.

Hurdle, J. G., O'Neill, A. J., & Chopra, I. 2004, "The isoleucyl-tRNA synthetase mutation V588F conferring mupirocin resistance in glycopeptide-intermediate *Staphylococcus aureus* is not associated with a significant fitness burden", *J.Antimicrob.Chemother.*, vol. 53, no. 1, pp. 102-104.

Hurdle, J. G., O'Neill, A. J., Ingham, E., Fishwick, C., & Chopra, I. 2004, "Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques", *Antimicrob.Agents Chemother.*, vol. 48, no. 11, pp. 4366-4376.

Hutchinson, G. R., Parker, S., Pryor, J. A., Duncan-Skingle, F., Hoffman, P. N., Hodson, M. E., Kaufmann, M. E., & Pitt, T. L. 1996, "Home-use nebulizers: a potential primary source of *Burkholderia cepacia* and other colistin-resistant, gram-negative bacteria in patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 34, no. 3, pp. 584-587.

Hutchison, M. L., Poxton, I. R., & Govan, J. R. 1998, "*Burkholderia cepacia* produces a hemolysin that is capable of inducing apoptosis and degranulation of mammalian phagocytes", *Infect.Immun.*, vol. 66, no. 5, pp. 2033-2039.

Hyde, J. A., Darouiche, R. O., & Costerton, J. W. 1998, "Strategies for prophylaxis against prosthetic valve endocarditis: a review article", *J.Heart Valve Dis.*, vol. 7, no. 3, pp. 316-326.

Ince, D. & Hooper, D. C. 2003, "Quinolone resistance due to reduced target enzyme expression", *J.Bacteriol.*, vol. 185, no. 23, pp. 6883-6892.

Ishida, H., Ishida, Y., Kurosaka, Y., Otani, T., Sato, K., & Kobayashi, H. 1998, "In vitro and *in vivo* activities of levofloxacin against biofilm-producing *Pseudomonas aeruginosa*", *Antimicrob.Agents Chemother.*, vol. 42, no. 7, pp. 1641-1645.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., & Levison, H. 1984, "*Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem", *J.Pediatr.*, vol. 104, no. 2, pp. 206-210.

Jacoby, G. A., Chow, N., & Waites, K. B. 2003, "Prevalence of plasmid-mediated quinolone resistance", *Antimicrob.Agents Chemother.*, vol. 47, no. 2, pp. 559-562.

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A., & Hooper, D. C. 2006, "*qnrB*, another plasmid-mediated gene for quinolone resistance", *Antimicrob.Agents Chemother.*, vol. 50, no. 4, pp. 1178-1182.

Jander, G., Rahme, L. G., & Ausubel, F. M. 2000, "Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects", *J.Bacteriol.*, vol. 182, no. 13, pp. 3843-3845.

Jarvis, K. G., Giron, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S., & Kaper, J. B. 1995, "Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation", *Proc.Natl.Acad.Sci.U.S.A*, vol. 92, no. 17, pp. 7996-8000.

Johnsborg, O., Eldholm, V & Havarstien, L.S. 2007 "Natural genetic transformation: prevalence, mechanisms and function", *Res.Micobiol.*, In press.

Johnson, C. N., Briles, D. E., Benjamin, W. H., Jr., Hollingshead, S. K., & Waites, K. B. 2005, "Relative fitness of fluoroquinolone-resistant *Streptococcus pneumoniae*", *Emerg.Infect.Dis.*, vol. 11, no. 6, pp. 814-820.

Johnston, R. B., Jr. 2001, "Clinical aspects of chronic granulomatous disease", *Curr.Opin.Hematol.*, vol. 8, no. 1, pp. 17-22.

Jones, A., Dodd, M., Govan, J., Barcus, V., Doherty, C., Morris, J., & Webb, A. 2004. "*Burkholderia cenocepacia* and *Burkholderia multivorans*: influence on survival in cystic fibrosis", *Thorax,* vol 59, no 11, pp 948-951.

Jones, M. E., Sahm, D. F., Martin, N., Scheuring, S., Heisig, P., Thornsberry, C., Kohrer, K., & Schmitz, F. J. 2000, "Prevalence of *gyrA*, *gyrB*, *parC*, and *parE* mutations in clinical isolates of *Streptococcus pneumoniae* with decreased susceptibilities to different fluoroquinolones and originating from Worldwide Surveillance Studies during the 1997-1998 respiratory season", *Antimicrob.Agents Chemother.*, vol. 44, no. 2, pp. 462-466.

Jones, M. E., Thomas, S. M. & Rogers, A. 1994, "Luria-Delbrück fluctuation experiments: design and analysis", *Genetics*, vol. 136, no. 3, pp. 1209-1216.

Jonsson, V. 1970, "Proposal of a new species of *Pseudomonas kingii*", *Int.J.Syst.Bacteriol.*, vol. 20, pp. 255-257.

Jordon, P.W, Stanley, T., Donnelly, F.M., Elborn, J.S., McClurg, R.B., Millar, B.C., C.E. Goldsmith & Moore, J.E. 2007. "Atypical mycobacterial infection in patients with cystic fibrosis: update on clinical microbiology methods", *Letters in Applied Microbiology*, vol 44, pp. 459-466.

Kadurugamuwa, J. L., Sin, L., Albert, E., Yu, J., Francis, K., DeBoer, M., Rubin, M., Bellinger-Kawahara, C., Parr, J. T., Jr., & Contag, P. R. 2003, "Direct continuous method for monitoring biofilm infection in a mouse model", *Infect.Immun.*, vol. 71, no. 2, pp. 882-890.

Kadurugamuwa, J. L., Sin, L. V., Yu, J., Francis, K. P., Kimura, R., Purchio, T., & Contag, P. R. 2003, "Rapid direct method for monitoring antibiotics in a mouse model of bacterial biofilm infection", *Antimicrob.Agents Chemother.*, vol. 47, no. 10, pp. 3130-3137.

Kaitwatcharachai, C., Silpapojakul, K., Jitsurong, S., & Kalnauwakul, S. 2000, "An outbreak of *Burkholderia cepacia* bacteremia in hemodialysis patients: an epidemiologic and molecular study", *Am.J.Kidney Dis.*, vol. 36, no. 1, pp. 199-204.

Karpati, F. & Jonasson, J. 1996, "Polymerase chain reaction for the detection of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* in sputum of patients with cystic fibrosis", *Mol.Cell Probes*, vol. 10, no. 6, pp. 397-403.

Kato, J., Nishimura, Y., Imamura, R., Niki, H., Hiraga, S., & Suzuki, H. 1990, "New topoisomerase essential for chromosome segregation in *E. coli*", *Cell*, vol. 63, no. 2, pp. 393-404.

Kendal, W. S. & Frost, P. 1988, "Pitfalls and practice of Luria-Delbruck fluctuation analysis: a review", *Cancer Res.*, vol. 48, no. 5, pp. 1060-1065.

Kepler, T. B. & Oprea, M. 2001, "Improved inference of mutation rates: I. An integral representation for the Luria-Delbruck distribution", *Theor.Popul.Biol.*, vol. 59, no. 1, pp. 41-48.

Kern, W. V., Oethinger, M., Jellen-Ritter, A. S., & Levy, S. B. 2000, "Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*", *Antimicrob.Agents Chemother.*, vol. 44, no. 4, pp. 814-820.

Khan, S.A., Everest, P, Servcs, S, Foxwell, N, Zahrige, U., Brade, H., Rietschel, E.T., Dougan, G., Charles, I.G. Maskell, D.J. "A lethal role for lipid A in *Salmonella* infections". Mol. Microbiol. 1998; 571-529.

Kingston, C. W. 1971, "Chronic or latent melioidosis", *Med.J.Aust.*, vol. 2, no. 12, pp. 618-621.

Kishony, R. & Leibler, S. 2003, "Environmental stresses can alleviate the average deleterious effect of mutations", *J.Biol.*, vol. 2, no. 2, pp. 14.

Kiska, D. L., Kerr, A., Jones, M. C., Caracciolo, J. A., Eskridge, B., Jordan, M., Miller, S., Hughes, D., King, N., & Gilligan, P. H. 1996, "Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 34, no. 4, pp. 886-891.

Knothe, H., Shah, P., Krcmery, V., Antal, M., & Mitsuhashi, S. 1983, "Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*", *Infection*, vol. 11, pp. 315-317.

Koch, A. L. 1982, "Mutation and growth rates from Luria-Delbruck fluctuation tests 28", *Mutat.Res.*, vol. 95, pp. 129-143.

Koch, C. & Hoiby, N. 1993, "Pathogenesis of cystic fibrosis", *Lancet*, vol. 341, no. 8852, pp. 1065-1069.

Kohler, T., M. Michea-Hamzehpour, P. Plesiat, A. L. Kahr, & Pechere, J.C. 1997. "Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*". *Antimicrob. Agents Chemother.*, vol. 141, pp. 2540-2543

Kolar, M., Urbanek, K., Vagnerova, I., & Koukalova, D. 2006, "The influence of antibiotic use on the occurrence of vancomycin-resistant enterococci", *J.Clin.Pharm.Ther.*, vol. 31, no. 1, pp. 67-72.

Komp, L. P., Marcusson, L. L., Sandvang, D., Frimodt-Moller, N., & Hughes, D. 2005, "Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections", *Antimicrob.Agents Chemother.*, vol. 49, no. 6, pp. 2343-2351.

Kooi, C., Subsin, B., Chen, R., Pohorelic, B., & Sokol, P. A. 2006, "*Burkholderia cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in virulence", *Infect.Immun.*, vol. 74, no. 7, pp. 4083-4093.

Kothe, M., Antl, M., Huber, B., Stoecker, K., Ebrecht, D., Steinmetz, I., & Eberl, L. 2003, "Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the cep quorum-sensing system", *Cell Microbiol.*, vol. 5, no. 5, pp. 343-351.

Kresken, M. & Wiedemann, B. 1988, "Development of resistance to nalidixic acid and the fluoroquinolones after the introduction of norfloxacin and ofloxacin", *Antimicrob.Agents Chemother.*, vol. 32, no. 8, pp. 1285-1288.

Kugelberg, E., Lofmark, S., Wretlind, B., & Andersson, D. I. 2005, "Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*", *J.Antimicrob.Chemother.*, vol. 55, no. 1, pp. 22-30.

Kumon, H., Tomochika, K., Matunaga, T., Ogawa, M., & Ohmori, H. 1994, "A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides", *Microbiol Immunol.*, vol. 38, no. 8, pp. 615-619.

Lacey, R. W. 1984, "Evolution of microorganisms and antibiotic resistance", *Lancet*, vol. 2, no. 8410, pp. 1022-1025.

Lafleur, M., Kumamoto, C & Lewis, K. 2006. "Candida albicans biofilms produce antifungal-tolerant persister cells", *Antimicrob. Agents Chemo.*, vol. 50., pp. 3839-3846.

Lam, J., Chan, R., Lam, K., & Costeron, J. W. 1980, "Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis", *Infect.Immun.*, vol. 28, pp. 546-556.

Lamfon, H., Porter, S. R., McCullough, M., & Pratten, J. 2003, "Formation of *Candida albicans* biofilms on non-shedding oral surfaces", *Eur.J.Oral Sci.*, vol. 111, no. 6, pp. 465-471.

Langley, R., Kenna, D. T., Vandamme, P., Ure, R., & Govan, J. R. 2003, "Lysogeny and bacteriophage host range within the *Burkholderia cepacia* complex", *J.Med.Microbiol.*, vol. 52, no. Pt 6, pp. 483-490.

Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S., Lazdunski, A., & Williams, P. 1995, "Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1", *Mol.Microbiol.*, vol. 17, no. 2, pp. 333-343.

Laurent, F., Lelievre, H., Cornu, M., Vandenesch, F., Carret, G., Etienne, J., & Flandrois, J. P. 2001, "Fitness and competitive growth advantage of new gentamicin-susceptible MRSA clones spreading in French hospitals", *J.Antimicrob.Chemother.*, vol. 47, no. 3, pp. 277-283.

Lawrence, J. G. & Ochman, H. 1997, "Amelioration of bacterial genomes: rates of change and exchange", *J.Mol Evol.*, vol. 44, no. 4, pp. 383-397.

Lea, D. & Coulson, C. 1949, "The distribution of the number of mutants in bacterial populations", *Genetics*, vol. 49, pp. 264-285.

LeClerc, J. E., Li, B., & Payne, W. L. 1996, "High mutation frequencies among *Escherichia coli* and *Salmonella pathogens*", *Science*, vol. 274, pp. 1208-1211.

LeClerc, J. E., Payne, W. L., Kupchella, E., & Cebula, T. A. 1998, "Detection of mutator subpopulations in *Salmonella typhimurium* LT2 by reversion of his alleles", *Mutat.Res.*, vol. 400, no. 1-2, pp. 89-97.

Lederberg, J. & Lederberg, E.M. 1952. "Replica plating and indirect selection of bacterial mutants", *J.Bacteriol.* vol. 63, no 3 pp. 399-406.

Ledson, M. J., Gallagher, M. J., Jackson, M., Hart, C. A., & Walshaw, M. J. 2002, "Outcome of *Burkholderia cepacia* colonisation in an adult cystic fibrosis centre", *Thorax*, vol. 57, no. 2, pp. 142-145.

Lee, J. K., Lee, Y. S., Park, Y. K., & Kim, B. S. 2005, "Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*", *Int.J.Antimicrob.Agents*, vol. 25, no. 4, pp. 290-295.

Lee, S. M., Ender, M., Adhikari, R., Smith, J. M., Berger-Bachi, B., & Cook, G. M. 2007, "Fitness cost of staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus aureus* by way of continuous culture", *Antimicrob.Agents Chemother.*, vol. 51, no. 4, pp. 1497-1499.

Lee, S. W. & Edlin, G. 1985, "Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*", *Gene*, vol. 39, no. 2-3, pp. 173-180.

Lenski, R. E. & Bouma, J. E. 1987, "Effects of segregation and selection on instability of plasmid pACYC184 in *Escherichia coli* ", *J.Bacteriol.*, vol. 169, no. 11, pp. 5314-5316.

Lenski, R. E., Mongold, J. A., Sniegowski, P. D., Travisano, M., Vasi, F., Gerrish, P. J., & Schmidt, T. M. 1998, "Evolution of competitive fitness in experimental populations of *E. coli*: what makes one genotype a better competitor than another?", *Antonie Van Leeuwenhoek*, vol. 73, no. 1, pp. 35-47.

Lenski, R. E., Simpson, S. C., & Nguyen, T. T. 1994, "Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness", *J.Bacteriol.*, vol. 176, no. 11, pp. 3140-3147.

Lenski, R. E., Slatkin, M., & Ayala, F. J. 1989, "Mutation and selection in bacterial populations: alternatives to the hypothesis of directed mutation", *Proc.Natl.Acad.Sci.U.S.A*, vol. 86, no. 8, pp. 2775-2778.

Leung, J. W., Sung, J. Y., & Costerton, J. W. 1989, "Bacteriological and electron microscopy examination of brown pigment stones", *J.Clin.Microbiol.*, vol. 27, no. 5, pp. 915-921.

Leverstein-van Hall, M. A., Block ,M., Paauw, A., Fluit, A. C., & Verhoef, J. 2003, "Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species or isolate origin", *J.Infect.Dis.*, vol. 187, no. 2, pp. 251-259.

Levin, B. R., Perrot, V., & Walker, N. 2000, "Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria", *Genetics*, vol. 154, no. 3, pp. 985-997.

Lewenza, S., Conway, B., Greenberg, E. P., & Sokol, P. A. 1999, "Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI", *J.Bacteriol.*, vol. 181, no. 3, pp. 748-756.

Lewenza, S. & Sokol, P. A. 2001, "Regulation of ornibactin biosynthesis and N-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*", *J.Bacteriol.*, vol. 183, no. 7, pp. 2212-2218.

Lewin, C., Doherty, C., & Govan, J. 1993, "*In vitro* activities of meropenem, PD 127391, PD 131628, ceftazidime, chloramphenicol, co-trimoxazole, and ciprofloxacin against *Pseudomonas cepacia*", *Antimicrob.Agents Chemother.*, vol. 37, no. 1, pp. 123-125.

Li, X. Z. & Nikaido, H. 2004. Efflux mediated drug resistance in bacteria. *Drugs* 64, pp.159-204.

Li, X. Z., Nikaido, H., & Poole, K. 1995, "Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*", *Antimicrob.Agents Chemother.*, vol. 39, no. 9, pp. 1948-1953.

Linares, J, de la Campa, A.G. and Palla. 1999. Fluoroquinolone resistance in *Streptococcus pneumoniae*. *N. Engl. J.Med.* vol. 11., pp. 1546-7.

Lin, J., Michel, L. O., & Zhang, Q. 2002, "CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*", *Antimicrob.Agents Chemother.*, vol. 46, no. 7, pp. 2124-2131.

Lindsay, D. & von Holy, A. 1997, "Evaluation of dislodging methods for laboratory-grown bacterial biofilms", *Food Microbiology*, vol. 14, no. 4, pp. 383-390.

LiPuma, J. J. 1998, "*Burkholderia cepacia*. Management issues and new insights", *Clin.Chest Med.*, vol. 19, no. 3, pp. 473-86, vi.

LiPuma, J. J., Dasen, S. E., Nielson, D. W., Stern, R. C., & Stull, T. L. 1990, "Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis", *Lancet*, vol. 336, no. 8723, pp. 1094-1096.

LiPuma, J. J., Dulaney, B. J., McMenamin, J. D., Whitby, P. W., Stull, T. L., Coenye, T., & Vandamme, P. 1999, "Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patients", *J.Clin.Microbiol.*, vol. 37, no. 10, pp. 3167-3170.

LiPuma, J. J., Mortensen, J. E., Dasen, S. E., Edlind, T. D., Schidlow, D. V., Burns, J. L., & Stull, T. L. 1988, "Ribotype analysis of *Pseudomonas cepacia* from cystic fibrosis treatment centers", *J.Pediatr.*, vol. 113, no. 5, pp. 859-862.

LiPuma, J. J., Spilker, T., Coenye, T., & Gonzalez, C. F. 2002, "An epidemic *Burkholderia cepacia* complex strain identified in soil", *Lancet*, vol. 359, no. 9322, pp. 2002-2003.

LiPuma, J. J., Spilker, T., Gill, L. H., Campbell, P. W., III, Liu, L., & Mahenthiralingam, E. 2001, "Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis", *Am.J.Respir.Crit Care Med.*, vol. 164, no. 1, pp. 92-96.

Little, J. W. & Mount, D. W. 1982, "The SOS regulatory system of *Escherichia coli*", *Cell*, vol. 29, no. 1, pp. 11-22.

Lomovskaya, O., Kawai, F., & Matin, A. 1996, "Differential regulation of the *mcb* and *emr* operons of *Escherichia coli*: role of *mcb* in multidrug resistance", *Antimicrob.Agents Chemother.*, vol. 40, no. 4, pp. 1050-1052.

Loo, C. Y., Corliss, D. A., & Ganeshkumar, N. 2000, "*Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes", *J.Bacteriol.*, vol. 182, no. 5, pp. 1374-1382.

Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L., & Zhang, Q. 2005, "Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the

absence of antibiotic selection pressure", *Proc.Natl.Acad.Sci.U.S.A*, vol. 102, no. 3, pp. 541-546.

Luria, S. & Delbrück, M. 1943, "Mutations of Bacteria from Virus Sensitivity to Virus Resistance", *Genetics*, vol. 28, pp. 491-511.

Lutter, E., Lewenza, S., Dennis, J. J., Visser, M. B., & Sokol, P. A. 2001, "Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex", *Infect.Immun.*, vol. 69, no. 7, pp. 4661-4666.

Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., & Hearst, J. E. 1995, "Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*", *Mol.Microbiol.*, vol. 16, no. 1, pp. 45-55.

Ma, W. T., Sandri, G. V. H. & Sarkar. S. 1992, "Analysis of the Luria Delbrück distribution using discrete convolution powers", *J.Appl.Probab.*, vol. 29, pp. 255-267.

MacDougall, C., Harpe, S. E., Powell, J. P., Johnson, C. K., Edmond, M. B., & Polk, R. E. 2005, "*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and fluoroquinolone use", *Emerg.Infect.Dis.*, vol. 11, no. 8, pp. 1197-1204.

Macia, M. D., Borrell, N., Perez, J. L., & Oliver, A. 2004, "Detection and susceptibility testing of hypermutable *Pseudomonas aeruginosa* strains with the E-test and disk diffusion", *Antimicrob.Agents Chemother.*, vol. 48, no. 7, pp. 2665-2672.

Maeda, Y., Kiba, A., Ohnishi, K., & Hikichi, Y. 2004, "Implications of amino acid substitutions in GyrA at position 83 in terms of oxolinic acid resistance in field isolates of *Burkholderia glumae*, a causal agent of bacterial seedling rot and grain rot of rice", *Appl.Environ.Microbiol.*, vol. 70, no. 9, pp. 5613-5620.

Mah, T. F. & O'Toole, G. A. 2001, "Mechanisms of biofilm resistance to antimicrobial agents", *Trends Microbiol.*, vol. 9, no. 1, pp. 34-39.

Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., & O'Toole, G. A. 2003, "A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance", *Nature*, vol. 426, no. 6964, pp. 306-310.

Mahenthiralingam, E., Baldwin, A., & Vandamme, P. 2002, "*Burkholderia cepacia* complex infection in patients with cystic fibrosis", *J.Med.Microbiol*, vol. 51, no. 7, pp. 533-538.

Mahenthiralingam, E., Bischof, J., Byrne, S. K., Radomski, C., Davies, J. E., Av-Gay, Y., & Vandamme, P. 2000, "DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III", *J.Clin.Microbiol.*, vol. 38, no. 9, pp. 3165-3173.

Mahenthiralingam, E., Coenye, T., Chung, J. W., Speert, D. P., Govan, J. R., Taylor, P., & Vandamme, P. 2000, "Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex", *J.Clin.Microbiol.*, vol. 38, no. 2, pp. 910-913.

Mahenthiralingam, E., Simpson, D. A., & Speert, D. P. 1997, "Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 35, no. 4, pp. 808-816.

Mahenthiralingam, E., Urban, T. A., & Goldberg, J. B. 2005, "The multifarious, multireplicon *Burkholderia cepacia* complex", *Nat.Rev.Microbiol*, vol. 3, no. 2, pp. 144-156.

Mahenthiralingam, E., Vandamme, P., Campbell, M. E., Henry, D. A., Gravelle, A. M., Wong, L. T., Davidson, A. G., Wilcox, P. G., Nakielna, B., & Speert, D. P. 2001, "Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*", *Clin.Infect.Dis.*, vol. 33, no. 9, pp. 1469-1475.

Maiden, M. C. 1998, "Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria", *Clin.Infect.Dis.*, vol. 27 Suppl 1, p. S12-S20.

Maira-Litran, T., Allison, D. G., & Gilbert, P. 2000, "An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump acrAB to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms", *J.Antimicrob.Chemother.*, vol. 45, no. 6, pp. 789-795.

Malott, R. J., Baldwin, A., Mahenthiralingam, E., & Sokol, P. A. 2005, "Characterization of the *cciIR* quorum-sensing system in *Burkholderia cenocepacia*", *Infect.Immun.*, vol. 73, no. 8, pp. 4982-4992.

Mamber, S. W., Kolek, K. W., Brookshire, D. P., Bonner, D. P., & Fung-Tomc, J. 1993, "Activity of quinolones in the Ames Salmonella TA102 mutagenicity test and other bacterial genotoxicity assays", *Antimicrb. Agents Chemother.* pp. 213-217.

Marciano, D.C., Karkouti, O.Y. & Palzkill, T. 2007. "A fitness cost associated with the antibiotic resistance enzyme SME-1 β-lactamase". *Genetics.* vol,176, pp 2381-2392.

Mariam, D. H., Mengistu, Y., Hoffner, S. E., & Andersson, D. I. 2004, "Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*", *Antimicrob.Agents Chemother.*, vol. 48, no. 4, pp. 1289-1294.

Markham, P. N. 1999, "Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine", *Antimicrob.Agents Chemother.*, vol. 43, no. 4, pp. 988-989.

Markowitz, V. M., Korzeniewski, F., Palaniappan, K., Szeto, E., Werner, G., Padki, A., Zhao, X., Dubchak, I., Hugenholtz, P., Anderson, I., Lykidis, A., Mavromatis, K., Ivanova, N., & Kyrpides, N. C. 2006, "The integrated microbial genomes (IMG) system", *Nucleic Acids Res.*, vol. 34, no. Database issue, p. D344-D348.

Martinez-Martinez, L., Pascual, A., & Jacoby, G. A. 1998, "Quinolone resistance from a transferable plasmid", *Lancet*, vol. 351, no. 9105, pp. 797-799.

Marsh, P. D. 1995. "Dental plaque", in H. M. Lappin-Scott and J. W. Costerton (ed.), Microbial biofilms. Cambridge University Press, Cambridge, United Kingdom, pp. 282-300.

Mathee, K., Ciofu, O., Sternberg, C., Lindum, P. W., Campbell, J. I., Jensen, P., Johnsen, A. H., Givskov, M., Ohman, D. E., Molin, S., Hoiby, N., & Kharazmi, A. 1999, "Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung", *Microbiology*, vol. 145 (Pt 6), pp. 1349-1357.

Matic, I., Radman, M., Taddei, F., Picard, B., Doit, C., Bingen, E., Denamur, E., & Elion, J. 1997, "Highly variable mutation rates in commensal and pathogenic *Escherichia coli*", *Science*, vol. 277, no. 5333, pp. 1833-1834.

McDonald, L.C., Chen, F., Lo, H.J., Yin, H.C., Lu, P.L., Huang, C.H., Lauderdale, T.L. & Ho, M. "Emergence of reduced susceptibility and resistance to fluoroquinolones in *Escherichia coli* in Taiwan and contributions of distinct selective pressures", *Antimicrob. Agents. Chemother.*, vol. 45, no.11, pp. 3084-3091.

McGowan, J.E. 2006. " Resistance in non-fermenting Gram-negative bacteria: multidrug resistance to the maximum", *Am. J.Med.*, vol.119, no.6, pp. S29-S36.

McHugh, T.D. 2001. "Application of SSCP to identification of resistance mutations", in . *Antibiotic resistance: Methods and Protocols*. ed. S.H. Gillespie, Humana Press, New Jersey, pp. 31-37.

McKenney, D., Brown, K. E., & Allison, D. G. 1995, "Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication", *J.Bacteriol.*, vol. 177, no. 23, pp. 6989-6992.

McKevitt, A. I., Bajaksouzia, S., Klinger, J. D. & Woods, D. E. 1989, "Purification and characterisation of an extracellular protease from *Pseudomonas cepacia*" *Infect. Immun.* vol. 57, pp 771-778.

McKevitt, A. I & Woods, D. E. 1984, "Characterisation of *Pseudomonas cepacia* isolates from patients with CF", *J. Clin.Micro.*, vol. 19, pp. 291-293.

McManus, T.E., Beattie, D., Graham, C., Moore, J.E. & Elborn, J.S. 2005. "Cystic fibrosis genotype and bacterial infection: a possible connection", *Br.J.Biomed.Sci.*, vol.62, no.2, pp.85-88.

McMurray L.M., Oethinger M., and Levy S.B. 1998. "Triclosan targets lipid synthesis", *Nature,* vol. 394, pp. 531–532.

Meka, V. G., Gold, H. S., Cooke, A., Venkataraman, L., Eliopoulos, G. M., Moellering, R. C., Jr., & Jenkins, S. G. 2004, "Reversion to susceptibility in a linezolid-resistant clinical isolate of *Staphylococcus aureus*", *J.Antimicrob.Chemother.*, vol. 54, no. 4, pp. 818-820.

Meyer, J. M., Hohnadel, D., & Halle, F. 1989, "Cepabactin from *Pseudomonas cepacia*, a new type of siderophore", *J.Gen.Microbiol.*, vol. 135, no. 6, pp. 1479-1487.

Miche, L. & Balandreau, J. 2001, "Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*", *Appl.Environ.Microbiol.*, vol. 67, no. 7, pp. 3046-3052.

Mikuniya, T., Kato, Y., Kariyama, R., Monden, K., Hikida, M., & Kumon, H. 2005, "Synergistic effect of fosfomycin and fluoroquinolones against *Pseudomonas aeruginosa* growing in a biofilm", *Acta Med.Okayama*, vol. 59, no. 5, pp. 209-216.

Miles, A. A. & Misra. S. S. 1938, "The estimation of the bactericidal power of the blood.", *J. Hyg.*, vol. 38, pp. 732-749.

Miller, J. H. 1996, "Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair", *Annu.Rev.Microbiol*, vol. 50, pp. 625-643.

Mingeot-Leclercq, M. P., Glupczynski, Y., & Tulkens, P. M. 1999, "Aminoglycosides: activity and resistance", *Antimicrob.Agents Chemother.*, vol. 43, no. 4, pp. 727-737.

Modi, R. I., Wilke, C. M., Rosenzweig, R. F., & Adams, J. 1991, "Plasmid macro-evolution: selection of deletions during adaptation in a nutrient-limited environment", *Genetica*, vol. 84, no. 3, pp. 195-202.

Moken, M. C., McMurry, L. M., & Levy, S. B. 1997, "Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci", *Antimicrob.Agents Chemother.*, vol. 41, no. 12, pp. 2770-2772.

Moore, J.E., McIhatton, B., Buchanan, J., Gilpin, D. Shaw, A., Hall, V., Murphy, P.G. and Elborn, J.S. 2002. "Occurrence of *Burkholderia cepacia* in the hospital environment", *Ir.J. Med.Sci.*, vol. 171, no. 3, pp.131-133.

Moore, J.E, Shaw, A., Howard, J.L., Dooley, J. S. G. & Elborn, J. S. 2004. "Infection control and the significance of sputum and other respiratory secretions from adult patients with cystic fibrosis". *Annal. Clin. Microbiol. Antim.*, vol. 3, pp. 813.

Moorman, D. R. & Mandell, G. L. 1981, "Characteristics of rifampin-resistant variants obtained from clinical isolates of *Staphylococcus aureus*", *Antimicrob.Agents Chemother.*, vol. 20, no. 6, pp. 709-713.

Morris, N. S., Stickler, D. J., & McLean, R. J. 1999, "The development of bacterial biofilms on indwelling urethral catheters", *World J.Urol.*, vol. 17, no. 6, pp. 345-350.

Mouneimne, H., Robert, J., Jarlier, V., & Cambau, E. 1999, "Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*", *Antimicrob.Agents Chemother.*, vol. 43, no. 1, pp. 62-66.

Moyed, H. S. & Bertrand, K. P. 1983, "*hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis", *J.Bacteriol.*, vol. 155, no. 2, pp. 768-775.

Moyed, H. S. & Broderick, S. H. 1986, "Molecular cloning and expression of *hipA*, a gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis", *J.Bacteriol.*, vol. 166, no. 2, pp. 399-403.

Moxon, E. R., Rainey, P. B., Nowak, M. A., & Lenski, R. E. 1994, "Adaptive evolution of highly mutable loci in pathogenic bacteria", *Curr.Biol.*, vol. 4, no. 1, pp. 24-33.

Mukamolova, G. V., Turapov, O. A., Young, D. I., Kaprelyants, A. S., Kell, D. B., & Young, M. 2002, "A family of autocrine growth factors in *Mycobacterium tuberculosis*", *Mol.Microbiol.*, vol. 46, no. 3, pp. 623-635.

Mukamolova, G. V., Yanopolskaya, N. D., Kell, D. B., & Kaprelyants, A. S. 1998, "On resuscitation from the dormant state of *Micrococcus luteus*", *Antonie Van Leeuwenhoek*, vol. 73, no. 3, pp. 237-243.

Muller, H. J. 1964, "The relation of recombination to mutational advance", *Mutat.Res.*, vol. 1, pp. 2-9.

Murga, R., Miller, J. M., & Donlan, R. M. 2001, "Biofilm formation by Gram-negative bacteria on central venous catheter connectors: effect of conditioning films in a laboratory model", *J.Clin.Microbiol.*, vol. 39, no. 6, pp. 2294-2297.

Mutnick, A. H., Rhomberg, P. R., Sader, H.S. & Jones, R. N. 2004, "Antimicrobial usage and resistance trend relationships from the MYSTIC Programme in North America (1999-2001), *J. Antimicrob. Chemother.* vol. 53, pp 290-6.

Nagai, K., Davies, T. A., Pankuch, G. A., Dewasse, B. E., Jacobs, M. R., & Appelbaum, P. C. 2000, "*In vitro* selection of resistance to clinafloxacin, ciprofloxacin, and trovafloxacin in *Streptococcus pneumoniae*", *Antimicrob.Agents Chemother.*, vol. 44, no. 10, pp. 2740-2746.

Nagano, Y., Millar, B.C., Goldsmith, C.E., Elborn, J.S., Rendall, J. & Moore, J.E. 2007. "Emergence of *Scedosporium apiospermum* in patients with cystic fibrosis", *Arch. Dis. Child.*, vol. 92, pp. 607.

Nakamura, S., Nakamura, M., Kojima, T. & Yoshida, H. 1989."*gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*", *Antimicrob.Agents Chemother*, vol. 33, no.2, pp. 254-255.

Nakazawa, T., Yamada, Y., & Ishibashi, M. 1987, "Characterization of hemolysin in extracellular products of *Pseudomonas cepacia*", *J.Clin.Microbiol.*, vol. 25, no. 2, pp. 195-198.

Nawaz, M. S., Khan, S. A., Khan, A. A., Nayak, R., Steele, R., Paine, D., & Jones, R. 2003, "Molecular characterization of fluoroquinolone-resistant *Campylobacter spp.* isolated from poultry", *Poult.Sci.*, vol. 82, no. 2, pp. 251-258.

Nealson, K. H. & Hastings, J. W. 1979, "Bacterial bioluminescence: its control and ecological significance", *Microbial Reviews*, vol. 43, pp. 496-518.

Nelson, J. M., Chiller, T. M., Powers, J. H., & Angulo, F. J. 2007, "Fluoroquinolone-resistant *Campylobacter* species and the withdrawal of fluoroquinolones from use in poultry: a public health success story", *Clin.Infect.Dis.*, vol. 44, no. 7, pp. 977-980.

Nelson, J. W., Doherty, C. J., Brown, P. H., Greening, A. P., Kaufmann, M. E., & Govan, J. R. 1991, "*Pseudomonas cepacia* in inpatients with cystic fibrosis", *Lancet*, vol. 338, no. 8781, pp. 1525.

Neu, H.C., 1989, "Overview of mechanisms of bacterial resistance", *Diagn. Microbiol. Infect. Dis.*, vol.12, pp.109S-116S.

Neyfakh, A. A., Bidnenko, V. E., & Chen, L. B. 1991, "Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system", *Proc.Natl.Acad.Sci.U.S.A*, vol. 88, no. 11, pp. 4781-4785.

Nguyen, T. N., Phan, Q. G., Duong, L. P., Bertrand, K. P., & Lenski, R. E. 1989, "Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12", *Molecular Biology and Evolution*, vol. 6, no. 3, pp. 213-225.

Nies, D. H. 1999, "Microbial heavy-metal resistance", *Appl.Microbiol.Biotechnol.*, vol. 51, no. 6, pp. 730-750.

Nies, D. H. & Silver, S. 1995, "Ion efflux systems involved in bacterial metal resistances", *J.Ind.Microbiol.*, vol. 14, pp. 186-199.

Nikaido, H. 1996, "Multidrug efflux pumps of gram-negative bacteria", *J.Bacteriol.*, vol. 17, pp. 5853-5898.

Nierman, W. C., DeShazer, D., Kim, H. S., Tettelin, H., Nelson, K. E., Feldblyum, T., Ulrich, R. L., Ronning, C. M., Brinkac, L. M., Daugherty, S. C., Davidsen, T. D., Deboy, R. T., Dimitrov, G., Dodson, R. J., Durkin, A. S., Gwinn, M. L., Haft, D. H., Khouri, H., Kolonay, J. F., Madupu, R., Mohammoud, Y., Nelson, W. C., Radune, D., Romero, C. M., Sarria, S., Selengut, J., Shamblin, C., Sullivan, S. A., White, O., Yu, Y., Zafar, N., Zhou, L., & Fraser, C. M. 2004, "Structural flexibility in the *Burkholderia mallei* genome", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 39, pp. 14246-14251.

Nosanchuk, J. D. & Casadevall, A. 2003, "The contribution of melanin to microbial pathogenesis", *Cell Microbiol.*, vol. 5, no. 4, pp. 203-223.

Nowak, R. 1994, "Hungary sees an improvement in penicillin resistance", *Science*, vol. 264, no. 5157, pp. 364.

Norrby, S.R., Nord, C.E., Finch, R & The European Society of Clinical Microbiology and Infectious Diseases. 2005, "Lack of development of new antimicrobial drugs: a potential serious threat to public health", *Lancet Inf. Dis.*, vol. 5, no. 2, pp. 115- 119.

Nucifora, G., Chu, L., Misra, T. K., & Silver, S. 1989, "Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase", *Proc.Natl.Acad.Sci.U.S.A*, vol. 86, no. 10, pp. 3544-3548.

Nzula, S., Vandamme, P., & Govan, J. R. 2002, "Influence of taxonomic status on the in vitro antimicrobial susceptibility of the *Burkholderia cepacia* complex", *J.Antimicrob.Chemother.*, vol. 50, no. 2, pp. 265-269.

O'Callaghan, E. M., Tanner, M. S., & Boulnois, G. J. 1994, "Development of a PCR probe test for identifying *Pseudomonas aeruginosa* and *Pseudomonas* (*Burkholderia*) *cepacia*", *J.Clin.Pathol.*, vol. 47, no. 3, pp. 222-226.

Ochman, H., Lawrence, J. G., & Groisman, E. A. 2000, "Lateral gene transfer and the nature of bacterial innovation", *Nature*, vol. 405, no. 6784, pp. 299-304.

Oethinger, M., Kern, W. V., Jellen-Ritter, A. S., McMurry, L. M., & Levy, S. B. 2000, "Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump", *Antimicrob.Agents Chemother.*, vol. 44, no. 1, pp. 10-13.

Oh, H., Stenhoff, J., Jalal, S., & Wretlind, B. 2003, "Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains", *Microb.Drug Resist.*, vol. 9, no. 4, pp. 323-328.

Oie, S. & Kamiya, A. 1996, "Microbial contamination of antiseptics and disinfectants", *Am.J.Infect.Control*, vol. 24, no. 5, pp. 389-395.

Okazaki, T., Iyobe, S., Hashimoto, H., & Hirai, K. 1991, "Cloning and characterization of a DNA fragment that complements the *nfxB* mutation in *Pseudomonas aeruginosa* PAO", *FEMS Microbiol.Lett.*, vol. 63, no. 1, pp. 31-35.

Oliver, A., Canton, R., Campo, P., Baquero, F., & Blazquez, J. 2000, "High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection", *Science*, vol. 288, no. 5469, pp. 1251-1254.

Oliver, A., Levin, B. R., Juan, C., Baquero, F., & Blasquez, J. 2004, "Hypermutation and the preexistence of antibiotic resistant *Pseudomonas aeruginosa* mutants:Implications for susceptibility testing and treatment of chronic infections", *Antimicrob.Agents Chemother.*, vol. 48, no. 11, pp. 4226-4233.

O'Neill, A. J., Huovinen, T., Fishwick, C. W., & Chopra, I. 2006, "Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence", *Antimicrob.Agents Chemother.*, vol. 50, no. 1, pp. 298-309.

Ordway, D. J., Sonnenberg, M. G., Donahue, S. A., Belisle, J. T., & Orme, I. M. 1995, "Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice", *Infect.Immun.*, vol. 63, no. 2, pp. 741-743.

O'Sullivan, D. M., McHugh, T. D., & Gillespie, S. H. 2005, "Analysis of *rpoB* and *pncA* mutations in the published literature: an insight into the role of oxidative stress in *Mycobacterium tuberculosis* evolution?", *J.Antimicrob.Chemother.*, vol. 55, no. 5, pp. 674-679.

O'Toole, G. A. 2003, "To build a biofilm", *J.Bacteriol.*, vol. 185, no. 9, pp. 2687-2689.

O'Toole, G. A. & Kolter, R. 1998, "Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis", *Mol Microbiol*, vol. 28, no. 3, pp. 449-461.

Palfreyman, R. W., Watson, M. L., Eden, C., & Smith, A. W. 1997, "Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia (Pseudomonas) cepacia* products", *Infect.Immun.*, vol. 65, no. 2, pp. 617-622.

Pan, X. S. & Fisher, L. M. 1998, "DNA gyrase and topoisomerase IV are dual targets of clinafloxacin action in *Streptococcus pneumoniae*", *Antimicrob.Agents Chemother.*, vol. 42, no. 11, pp. 2810-2816.

Pao, S. S., Paulsen, I. T., & Saier, M. H., Jr. 1998, "Major facilitator superfamily", *Microbiol Mol Biol.Rev.*, vol. 62, no. 1, pp. 1-34.

Parke, J. L. & Gurian-Sherman, D. 2001, "Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains", *Annu.Rev.Phytopathol.*, vol. 39, pp. 225-258.

Parsek, M. R. & Greenberg, E. P. 1999, "Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms", *Methods Enzymol.*, vol. 310, pp. 43-55.

Parsek, M. R. & Greenberg, E. P. 2000, "Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 16, pp. 8789-8793.

Parsons, Y. N., Glendinning, K. J., Thornton, V., Hales, B. A., Hart, C. A., & Winstanley, C. 2001, "A putative type III secretion gene cluster is widely distributed in the *Burkholderia cepacia* complex but absent from genomovar I", *FEMS Microbiol.Lett.*, vol. 203, no. 1, pp. 103-108.

Paschoal, I. A., de, O., V, Bertuzzo, C. S., Cerqueira, E. M., & Pereira, M. C. 2007, "Cystic fibrosis in adults", *Lung*, vol. 185, no. 2, pp. 81-87.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L., & Iglewski, B. H. 1993, "Expression of *Pseudomonas aeruginosa* virulence genes requires cell to cell communication", *Science*, vol. 260, pp. 1127-1130.

Passerini, L., Lam, K., Costerton, J. W., & King, E. G. 1992, "Biofilms on indwelling vascular catheters", *Crit Care Med.*, vol. 20, no. 5, pp. 665-673.

Paulsen, I. T., Brown, M. H., & Skurray, R. A. 1996, "Proton-dependent multidrug efflux systems", *Microbiol Rev.*, vol. 60, no. 4, pp. 575-608.

Paulsen, I. T., Skurray, R. A., Tam, R., Saier, M. H., Jr., Turner, R. J., Weiner, J. H., Goldberg, E. B., & Grinius, L. L. 1996, "The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs", *Mol Microbiol*, vol. 19, no. 6, pp. 1167-1175.

Pearson, J. P., Pesci, E. C., & Iglewski, B. H. 1997, "Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes", *J.Bacteriol.*, vol. 179, no. 18, pp. 5756-5767.

Pegues, D. A., Carson, L. A., Tablan, O. C., FitzSimmons, S. C., Roman, S. B., Miller, J. M., & Jarvis, W. R. 1994, "Acquisition of *Pseudomonas cepacia* at summer camps for

patients with cystic fibrosis. Summer Camp Study Group", *J.Pediatr.*, vol. 124, no. 5 Pt 1, pp. 694-702.

Peloquin, C. A., Cumbo, T. J., Nix, D. E., Sands, M. F., & Schentag, J. J. 1989, "Evaluation of intravenous ciprofloxacin in patients with nosocomial lower respiratory tract infections. Impact of plasma concentrations, organism, minimum inhibitory concentration, and clinical condition on bacterial eradication", *Arch.Intern.Med.*, vol. 149, no. 10, pp. 2269-2273.

Peng, H. & Marians, K. J. 1993, "*Escherichia coli* topoisomerase IV. Purification, characterization, subunit structure, and subunit interactions", *J.Biol.Chem.*, vol. 268, no. 32, pp. 24481-24490.

Peters, A. & Wimpenny, J. 1987, "A constant depth laboratory model film fermenter.", *Biotechnology and Bioengineering*, vol. 32, pp. 263-270.

Piddock, L. J., Walters, R. N., & Diver, J. M. 1990, "Correlation of quinolone MIC and inhibition of DNA, RNA, and protein synthesis and induction of the SOS response in *Escherichia coli*", *Antimicrob.Agents Chemother.*, vol. 34, no. 12, pp. 2331-2336.

Pitt, T. L., Kaufmann, M. E., Patel, P. S., Benge, L. C., Gaskin, S., & Livermore, D. M. 1996, "Type characterisation and antibiotic susceptibility of *Burkholderia* (*Pseudomonas*) *cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland", *J.Med.Microbiol.*, vol. 44, no. 3, pp. 203-210.

Pope, C. F., Gillespie, S. H., Pratten, J. R. & McHugh, T. D. 2007, "Investigation on Fluoroquinolone- Resistant mutants of *Burkholderia cepacia*" *Antimicrob. Agent Chemother.*, vol 52, no. 3, pp. 1203-3.

Poole, K. 2000, "Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria", *Antimicrob.Agents Chemother.*, vol. 44, no. 9, pp. 2233-2241.

Potera, C. 1999, "Forging a link between biofilms and disease", *Science*, vol. 283, no. 5409, pp. 1837, 1839.

Poyart, C. Pierre, C., Quesne, G., Pron, B., Berch, P. & Trien-Cust, P. 1997."Emergence of vancomycin resistance in the genus *Streptococcus*: characterization of a *van*B transferable determinant in *Streptococcus bovis*" *Antimicrob. Agent Chemother.*, vol. 41, pp. 24–29

Pratt, L. A. & Kolter, R. 1998, "Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili", *Mol.Microbiol.*, vol. 30, no. 2, pp. 285-293.

Pratten R, Barnett, P & Wilson, M. 1998. "Composition and Susceptibility to Chlorhexidine of Multispecies Biofilms of Oral Bacteria", *Appli.Environ. Micro.*, vol. 64., no.9, pp 3515-3519.

Purevdorj, B., Costerton, J. W., & Stoodley, P. 2002, "Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms", *Appl.Environ.Microbiol.*, vol. 68, no. 9, pp. 4457-4464.

Rafii, F., Park, M., & Novak, J. S. 2005, "Alterations in DNA gyrase and topoisomerase IV in resistant mutants of *Clostridium perfringens* found after in vitro treatment with fluoroquinolones", *Antimicrob.Agents Chemother.*, vol. 49, no. 2, pp. 488-492.

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., Mahajan-Miklos, S., Plotnikova, J., Tan, M. W., Tsongalis, J., Walendziewicz, C. L., & Tompkins, R. G. 2000, "Plants and animals share functionally common bacterial virulence factors", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 16, pp. 8815-8821.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G., & Ausubel, F. M. 1995, "Common virulence factors for bacterial pathogenicity in plants and animals", *Science*, vol. 268, no. 5219, pp. 1899-1902.

Rahme, L. G., Tan, M. W., Le, L., Wong, S. M., Tompkins, R. G., Calderwood, S. B., & Ausubel, F. M. 1997, "Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors", *Proc.Natl.Acad.Sci.U.S.A*, vol. 94, no. 24, pp. 13245-13250.

Rainbow, L., Hart, C. A., & Winstanley, C. 2002, "Distribution of type III secretion gene clusters in *Burkholderia pseudomallei*, *B. thailandensis* and *B. mallei*", *J.Med.Microbiol*, vol. 51, no. 5, pp. 374-384.

Raj, P. A. & Dentino, A. R. 2002, "Current status of defensins and their role in innate and adaptive immunity", *FEMS Microbiol.Lett.*, vol. 206, no. 1, pp. 9-18.

Ramadhan, A. A. & Hegedus, E. 2005, "Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition", *J.Clin.Pathol.*, vol. 58, no. 7, pp. 744-746.

Randall, L. P. & Woodward, M. J. 2001, "Multiple antibiotic resistance (mar) locus in *Salmonella enterica* serovar typhimurium DT104", *Appl.Environ.Microbiol.*, vol. 67, no. 3, pp. 1190-1197.

Rasmussen, B. 2000, "Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit", *Nature*, vol. 405, no. 6787, pp. 676-679.

Ratjen, F. & Doring, G. 2003, "Cystic fibrosis", *Lancet*, vol. 361, no. 9358, pp. 681-689.

Ratledge, C. & Dover, L. G. 2000, "Iron metabolism in pathogenic bacteria", *Annu.Rev.Microbiol.*, vol. 54, pp. 881-941.

Rayssiguier, C., Thaler, D. S., & Radman, M. 1989, "The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants", *Nature*, vol. 342, no. 6248, pp. 396-401.

Remold, S. K. & Lenski, R. E. 2001, "Contribution of individual random mutations to genotype-by-environment interactions in *Escherichia coli*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 20, pp. 11388-11393.

Richau, J. A., Leitao, J. H., Correia, M., Lito, L., Salgado, M. J., Barreto, C., Cescutti, P., & Sa-Correia, I. 2000, "Molecular typing and exopolysaccharide biosynthesis of *Burkholderia cepacia* isolates from a Portuguese cystic fibrosis center", *J.Clin.Microbiol.*, vol. 38, no. 4, pp. 1651-1655.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S., & Eberl, L. 2001, "N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms", *Microbiology*, vol. 147, no. Pt 12, pp. 3249-3262.

Roberts, M. C., Chung, W. O., & Roe, D. E. 1996, "Characterization of tetracycline and erythromycin resistance determinants in *Treponema denticola*", *Antimicrob.Agents Chemother.*, vol. 40, no. 7, pp. 1690-1694.

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Park, C. H., Bush, K., & Hooper, D. C. 2006, "Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase", *Nat.Med.*, vol. 12, no. 1, pp. 83-88.

Rogues, A. M., Dumartin, C., Amadeo, B., Venier, A. G., Marty, N., Parneix, M.D., Gachie, J. P. 2007, "Relationship between rates of antimicrobial consumption and the incidence of antimicrobial resistance in *Staphylococcus aureus* and *Pseudomonas aeruginosa*", *Infect. Con. Hosp. Epidemiol.*, vol. 28, pp. 1389-1395.

Rosche, W. A. & Foster, P. L. 2000, "Determining mutation rates in bacterial populations", *Methods*, vol. 20, no. 1, pp. 4-17.

Rossman, T. G., Goncharova, E. I., & Nadas, A. 1995, "Modeling and measurement of the spontaneous mutation rate in mammalian cells", *Mutat.Res.*, vol. 328, no. 1, pp. 21-30.

Rotz, L. D., Khan, A. S., Lillibridge, S. R., Ostroff, S. M., & Hughes, J. M. 2002, "Public health assessment of potential biological terrorism agents", *Emerg.Infect.Dis.*, vol. 8, no. 2, pp. 225-230.

Rozen, S. & Skaletsky, H. 2000. Primer3 on the WWW for general use and for biologist programming methods. *Methods. Mol. Biol.* 132; 365-386.

Ruiz, J. 2003, "Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection", *J.Antimicrob.Chemother.*, vol. 51, no. 5, pp. 1109-1117.

Ruiz, J., Goni, P., Marco, F., Gallardo, F., Mirelis, B., Jimenez, d. A., & Vila, J. 1998, "Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates", *Microbiol.Immunol.*, vol. 42, no. 3, pp. 223-226.

Saenz, Y., Zarazaga, M., Brinas, L., Ruiz-Larrea, F., & Torres, C. 2003, "Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals", *J.Antimicrob.Chemother.*, vol. 51, no. 4, pp. 1001-1005.

Sahly, H., Schubert, S., Harder, J., Rautenberg, P., Ullmann, U., Schroder, J., & Podschun, R. 2003, "*Burkholderia* is highly resistant to human Beta-defensin 3", *Antimicrob.Agents Chemother.*, vol. 47, no. 5, pp. 1739-1741.

Saier, M. H., Jr., Tam, R., Reizer, A., & Reizer, J. 1994, "Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport", *Mol Microbiol*, vol. 11, no. 5, pp. 841-847.

Sajjan, U. S. & Forstner, J. F. 1993, "Role of a 22-kilodalton pilin protein in binding of *Pseudomonas cepacia* to buccal epithelial cells", *Infect.Immun.*, vol. 61, no. 8, pp. 3157-3163.

Sajjan, U. S., Sun, L., Goldstein, R., & Forstner, J. F. 1995, "Cable (cbl) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia*: nucleotide sequence of the cblA major subunit pilin gene and novel morphology of the assembled appendage fibers", *J.Bacteriol.*, vol. 177, no. 4, pp. 1030-1038.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M., & Li, L. Y. 1995, "Conjugative transposons: an unusual and diverse set of integrated gene transfer elements", *Microbiol.Rev.*, vol. 59, no. 4, pp. 579-590.

Sanchez, P., Linares, J. F., Ruiz-Diez, B., Campanario, E., Navas, A., Baquero, F., & Martinez, J. L. 2002, "Fitness of *in vitro* selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants", *J.Antimicrob.Chemother.*, vol. 50, no. 5, pp. 657-664.

Sarkar, S. 1991, "Haldane's solution of the Luria-Delbrück distribution", *Genetics*, vol. 127, no. 2, pp. 257-261.

Sarkar, S., Ma, W. T., & Sandri, G. H. 1992, "On fluctuation anlaysis: a new, simple and effiecient method for computing the expected number of mutants", *Genetica*, vol. 85, no. 2, pp. 173-179.

Schmitz, F. J., Jones, M. E., Hofmann, B., Hansen, B., Scheuring, S., Luckefahr, M., Fluit, A., Verhoef, J., Hadding, U., Heinz, H. P., & Kohrer, K. 1998, "Characterization of *grlA*, *grlB*, *gyrA*, and *gyrB* mutations in 116 unrelated isolates of *Staphylococcus aureus* and effects of mutations on ciprofloxacin MIC", *Antimicrob.Agents Chemother.*, vol. 42, no. 5, pp. 1249-1252.

Schrag, S. J., Perrot, V., & Levin, B. R. 1997, "Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*", *Proc.Biol.Sci.*, vol. 264, no. 1386, pp. 1287-1291.

Schroder, J. M. 1999, "Epithelial antimicrobial peptides: innate local host response elements", *Cell Mol.Life Sci.*, vol. 56, no. 1-2, pp. 32-46.

Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Yi, A. K., & Krieg, A. M. 1997, "CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract", *J.Clin.Invest*, vol. 100, no. 1, pp. 68-73.

Segonds, C., Heulin, T., Marty, N., & Chabanon, G. 1999, "Differentiation of *Burkholderia* species by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene and application to cystic fibrosis isolates", *J.Clin.Microbiol.*, vol. 37, no. 7, pp. 2201-2208.

Seppala, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K., & Huovinen, P. 1997, "The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A *Streptococci* in Finland. Finnish Study Group for Antimicrobial Resistance", *N.Engl.J.Med.*, vol. 337, no. 7, pp. 441-446.

Shapiro, J. A. 1997, "Genome organisation, natural genetic engineering and adaptive mutation", *Trends.Genet.*, vol. 13, pp. 98-104.

Shaw, K. J., Miller, N., Liu, X., Lerner, D., Wan, J., Bittner, A., & Morrow, B. J. 2003, "Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents", *J.Mol.Microbiol.Biotechnol.*, vol. 5, no. 2, pp. 105-122.

Shaw, D., Poxton, I.R. & Govan, J.R. 1995, "Biological activity of *B. cepacia* lipopolysaccharide", *FEMs Immunol. Med. Microbiol.* vol. 11, pp. 99-106.

Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E., III, & Stover, C. K. 1996, "Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*", *Science*, vol. 272, no. 5268, pp. 1641-1643.

Shigeta, M., Tanaka, G., Komatsuzawa, H., Sugai, M., Suginaka, H., & Usui, T. 1997, "Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method", *Chemotherapy*, vol. 43, no. 5, pp. 340-345.

Shlaes, D. M., Gerding, D. N., John, J. F., Jr., Craig, W. A., Bornstein, D. L., Duncan, R. A., Eckman, M. R., Farrer, W. E., Greene, W. H., Lorian, V., Levy, S., McGowan, J. E., Jr., Paul, S. M., Ruskin, J., Tenover, F. C., & Watanakunakorn, C. 1997, "Society for Healthcare Epidemiology of America and Infectious Diseases Society of America Joint Committee on the Prevention of Antimicrobial Resistance: guidelines for the prevention of antimicrobial resistance in hospitals", *Clin.Infect.Dis.*, vol. 25, no. 3, pp. 584-599.

Shotton, D. M. 1989, "Confocal scanning optical microscopy and its applications for biological specimens", *J. Cell Sci.*, vol. 94, pp. 175-2.

Sindelar, G., Zhao, X., Liew, A., Dong, Y., Lu, T., Zhou, J., Domagala, J., & Drlica, K. 2000, "Mutant prevention concentration as a measure of fluoroquinolone potency against mycobacteria", *Antimicrob.Agents Chemother.*, vol. 44, no. 12, pp. 3337-3343.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J., & Greenberg, E. P. 2000, "Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms", *Nature*, vol. 407, no. 6805, pp. 762-764.

Sist, P., Cescutti, P., Skerlavaj, S., Urbani, R., Leitao, J. H., Sa-Correia, I., & Rizzo, R. 2003, "Macromolecular and solution properties of Cepacian: the exopolysaccharide produced by a strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient", *Carbohydr.Res.*, vol. 338, no. 18, pp. 1861-1867.

Sjolund, M., Tano, E., Blaser, M. J., Andersson, D. I., & Engstrand, L. 2005, "Persistence of resistant *Staphylococcus epidermidis* after single course of clarithromycin", *Emerg.Infect.Dis.*, vol. 11, no. 9, pp. 1389-1393.

Smith, S. M., Eng, R. H., & Padberg, F. T., Jr. 1996, "Survival of nosocomial pathogenic bacteria at ambient temperature", *J.Med.*, vol. 27, no. 5-6, pp. 293-302.

Snell, G. I., de Hoyos, A., Krajden, M., Winton, T., & Maurer, J. R. 1993, "*Pseudomonas cepacia* in lung transplant recipients with cystic fibrosis", *Chest*, vol. 103, no. 2, pp. 466-471.

Sniegowski, P. D., Gerrish, P. J., Johnson, T., & Shaver, A. 2000, "The evolution of mutation rates: separating causes from consequences", *Bioessays*, vol. 22, no. 12, pp. 1057-1066.

Sokol, P. A. 1986, "Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*", *J.Clin.Microbiol.*, vol. 23, no. 3, pp. 560-562.

Sokol, P. A., Darling, P., Woods, D. E., Mahenthiralingam, E., & Kooi, C. 1999, "Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N(5)-oxygenase", *Infect.Immun.*, vol. 67, no. 9, pp. 4443-4455.

Sokol, P. A., Kooi, C., Hodges, R. S., Cachia, P., & Woods, D. E. 2000, "Immunization with a *Pseudomonas aeruginosa* elastase peptide reduces severity of experimental lung infections due to *P. aeruginosa* or *Burkholderia cepacia*", *J.Infect.Dis.*, vol. 181, no. 5, pp. 1682-1692.

Sokol, P. A., Lewis, C. J., & Dennis, J. J. 1992, "Isolation of a novel siderophore from *Pseudomonas cepacia*", *J.Med.Microbiol.*, vol. 36, no. 3, pp. 184-189.

Sokol, P. A., Sajjan, U., Visser, M. B., Gingues, S., Forstner, J., & Kooi, C. 2003, "The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections", *Microbiology*, vol. 149, no. Pt 12, pp. 3649-3658.

Sokol, P. A. & Woods, D. E. 1988, "Effect of pyochelin on *Pseudomonas cepacia* respiratory infections", *Microb.Pathog.*, vol. 5, no. 3, pp. 197-205.

Speer, B. S. & Salyers, A. A. 1988, "Characterization of a novel tetracycline resistance that functions only in aerobically grown *Escherichia coli*", *J.Bacteriol.*, vol. 170, no. 4, pp. 1423-1429.

Speer, B. S. & Salyers, A. 1989, "Novel aerobic tetracycline resistance gene that chemically modifies tetracycline", *J.Bacteriol.*, vol. 171, no. 1, pp. 148-153.

Speert, D. P. 2002, "Advances in *Burkholderia cepacia* complex", *Paediatr.Respir.Rev.*, vol. 3, no. 3, pp. 230-235.

Speert, D. P., Bond, M., Woodman, R. C., & Curnutte, J. T. 1994, "Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense", *J.Infect.Dis.*, vol. 170, no. 6, pp. 1524-1531.

Speert, D. P., Steen, B., Halsey, K., & Kwan, E. 1999, "A murine model for infection with *Burkholderia cepacia* with sustained persistence in the spleen", *Infect.Immun.*, vol. 67, no. 8, pp. 4027-4032.

Spellberg, B., Powers, J., Brass, E. P., Miller, L.G., Edwards, J. E. 2004, "Trends in antimicrobial drug development: implications for the future", *Clin. Inf. Dis.*, vol. 38, pp. 1279-1286.

Spoering, A. L. & Lewis, K. 2001, "Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials", *J.Bacteriol.*, vol. 183, no. 23, pp. 6746-6751.

Stanier, R. Y. & Doudoroff, M. 1966, "The aerobic pseudomonads: a taxonomic study.", *J. Gen. Microbiol.*, vol. 43, pp. 159-271.

Steck, T. R., Franco, R. J., Wang, J. Y., & Drlica, K. 1993, "Topoisomerase mutations affect the relative abundance of many *Escherichia coli* proteins", *Mol.Microbiol.*, vol. 10, no. 3, pp. 473-481.

Steinberg, D. A., Hurst, M. A., Fujii, C. A., Kung, A. H., Ho, J. F., Cheng, F. C., Loury, D. J., & Fiddes, J. C. 1997, "Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity", *Antimicrob.Agents Chemother.*, vol. 41, no. 8, pp. 1738-1742.

Stephan, H., Freund, S., Beck, W., Jung, G., Meyer, J. M., & Winkelmann, G. 1993, "Ornibactins-a new family of siderophores from *Pseudomonas*", *Biometals*, vol. 6, no. 2, pp. 93-100.

Stepanovic, S., Djukic, N., Djordjevic, V., & Djukic, S. 2003, "Influence of the incubation atmosphere on the production of biofilm by *staphylococci*", *Clin.Microbiol.Infect.*, vol. 9, no. 9, pp. 955-958.

Stevens, M. P., Wood, M. W., Taylor, L. A., Monaghan, P., Hawes, P., Jones, P. W., Wallis, T. S., & Galyov, E. E. 2002, "An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen", *Mol Microbiol*, vol. 46, no. 3, pp. 649-659.

Stewart, P. S. 1996, "Theoretical aspects of antibiotic diffusion into microbial biofilms", *Antimicrob.Agents Chemother.*, vol. 40, no. 11, pp. 2517-2522.

Stewart, P. S. 1998, "A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms", *Biotechnol.Bioeng.*, vol. 59, no. 3, pp. 261-272.

Stewart, P. S. & Costerton, J. W. 2001, "Antibiotic resistance of bacteria in biofilms", *Lancet*, vol. 358, no. 9276, pp. 135-138.

Stokes, H. W., O'Gorman, D. B., Recchia, G. D., Parsekhian, M., & Hall, R. M. 1997, "Structure and function of 59-base element recombination sites associated with mobile gene cassettes", *Mol.Microbiol.*, vol. 26, no. 4, pp. 731-745.

Stoodley, P., Debeer, D., & Lewandowski, Z. 1994, "Liquid Flow in Biofilm Systems", *Appl.Environ.Microbiol.*, vol. 60, no. 8, pp. 2711-2716.

Stoodley, P., Dodds, I., Boyle, J. D., & Lappin-Scott, H. M. 1999, "Influence of hydrodynamics and nutrients on biofilm structure", *J.Appl.Microbiol* pp. 19S-28S.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. 2002, "Biofilms as complex differentiated communities", *Annu.Rev.Microbiol*, vol. 56, pp. 187-209.

Stover, G. B., Drake, D. R., & Montie, T. C. 1983, "Virulence of different *Pseudomonas* species in a burned mouse model: tissue colonization by *Pseudomonas cepacia*", *Infect.Immun.*, vol. 41, no. 3, pp. 1099-1104.

Sun, L., Jiang, R. Z., Steinbach, S., Holmes, A., Campanelli, C., Forstner, J., Sajjan, U., Tan, Y., Riley, M., & Goldstein, R. 1995, "The emergence of a highly transmissible lineage of cbl+ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain", *Nat.Med.*, vol. 1, no. 7, pp. 661-666.

Tablan, O. C., Carson, L. A., Cusick, L. B., Bland, L. A., Martone, W. J., & Jarvis, W. R. 1987, "Laboratory proficiency test results on use of selective media for isolating *Pseudomonas cepacia* from simulated sputum specimens of patients with cystic fibrosis", *J.Clin.Microbiol.*, vol. 25, no. 3, pp. 485-487.

Tablan, O. C., Martone, W. J., Doershuk, C. F., Stern, R. C., Thomassen, M. J., Klinger, J. D., White, J. W., Carson, L. A., & Jarvis, W. R. 1987, "Colonization of the respiratory tract with *Pseudomonas cepacia* in cystic fibrosis. Risk factors and outcomes", *Chest*, vol. 91, no. 4, pp. 527-532.

Takenouchi, T., Sakagawa, E., & Sugawara, M. 1999, "Detection of *gyrA* mutations among 335 *Pseudomonas aeruginosa* strains isolated in Japan and their susceptibilities to fluoroquinolones", *Antimicrob.Agents Chemother.*, vol. 43, no. 2, pp. 406-409.

Tan, M. W. & Ausubel, F. M. 2000, "*Caenorhabditis elegans*: a model genetic host to study *Pseudomonas aeruginosa* pathogenesis", *Curr.Opin.Microbiol*, vol. 3, no. 1, pp. 29-34.

Tan, M. W., Mahajan-Miklos, S., & Ausubel, F. M. 1999, "Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 2, pp. 715-720.

Tanaka, M., Nakayama, H., Haraoka, M., & Saika, T. 2000, "Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998", *J.Clin.Microbiol.*, vol. 38, no. 2, pp. 521-525.

Tanlan, D.A. 2001. "Clinical perspectives on new antimicrobials: Focus on FQs", CID vol. 32 (Suppl), pp. 264-71.

Tavio, M. M., Vila, J., Ruiz, J., Ruiz, J., Martin-Sanchez, A. M., & Jimenez de Anta, M. T. 1999, "Mechanisms involved in the development of resistance to fluoroquinolones in *Escherichia coli* isolates", *J.Antimicrob.Chemother.*, vol. 44, no. 6, pp. 735-742.

Teitzel, G. M. & Parsek, M. R. 2003, "Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*", *Appl.Environ.Microbiol.*, vol. 69, no. 4, pp. 2313-2320.

Then, R.L. 1982. "Mechanisms of resistance to trimethoprim, the suplonamides and trimethoprim-sulfamethoxazole", *Rev. Infect. Dis.*, vol. 4, no.2, pp. 261-269.

Tomich, M., Griffith, A., Herfst, C. A., Burns, J. L., & Mohr, C. D. 2003, "Attenuated virulence of a *Burkholderia cepacia* type III secretion mutant in a murine model of infection", *Infect.Immun.*, vol. 71, no. 3, pp. 1405-1415.

Tomlin, K. L., Clark, S. R., & Ceri, H. 2004, "Green and red fluorescent protein vectors for use in biofilm studies of the intrinsically resistant *Burkholderia cepacia* complex", *J.Microbiol Methods*, vol. 57, no. 1, pp. 95-106.

Tomlin, K. L., Coll, O. P., & Ceri, H. 2001, "Interspecies biofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia*", *Can.J.Microbiol*, vol. 47, no. 10, pp. 949-954.

Tomlin, K. L., Malott, R. J., Ramage, G., Storey, D. G., Sokol, P. A., & Ceri, H. 2005, "Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms", *Appl.Environ.Microbiol*, vol. 71, no. 9, pp. 5208-5218.

Tran, J. H., Jacoby, G. A., & Hooper, D. C. 2005, "Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase", *Anti microb.Agents Chemother.*, vol. 49, no. 1, pp. 118-125.

Truong, Q. C., Nguyen, V. J., Shlaes, D., Gutmann, L., & Moreau, N. J. 1997, "A novel, double mutation in DNA gyrase A of *Escherichia coli* conferring resistance to quinolone antibiotics", *Antimicrob.Agents Chemother.*, vol. 41, no. 1, pp. 85-90.

Tsuchiya, K., Homma, Y., Komoto, Y., & Suzui, T. 1995, "Practical detection of *Pseudomonas cepacia* from rhizosphere antagonistic to plant pathogens with a combination of selective medium and ELISA", *Annals of Phytopathology Society of Japan*, vol. 61, pp. 318-324.

Turner, A. K., Nair, S., & Wain, J. 2006, "The acquisition of full fluoroquinolone resistance in *Salmonella typhi* by accumulation of point mutations in the topoisomerase targets", *J.Antimicrob.Chemother.*, vol. 58, no. 4, pp. 733-740.

Ulrich, R. L. & DeShazer, D. 2004, "Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*", *Infect.Immun.*, vol. 72, no. 2, pp. 1150-1154.

Urban, T. A., Griffith, A., Torok, A. M., Smolkin, M. E., Burns, J. L., & Goldberg, J. B. 2004, "Contribution of *Burkholderia cenocepacia* flagella to infectivity and inflammation", *Infect.Immun.*, vol. 72, no. 9, pp. 5126-5134.

Valdezate, S., Vindel, A., Echeita, A., Baquero, F., & Canto, R. 2002, "Topoisomerase II and IV quinolone resistance-determining regions in *Stenotrophomonas maltophilia* clinical isolates with different levels of quinolone susceptibility", *Antimicrob.Agents Chemother.*, vol. 46, no. 3, pp. 665-671.

Van Bambeke, F., Balzi, E., & Tulkens, P. M. 2000, "Antibiotic efflux pumps", *Biochem.Pharmacol.*, vol. 60, no. 4, pp. 457-470.

van Laer, F., Raes, D., Vandamme, P., Lammens, C., Sion, J. P., Vrints, C., Snoeck, J., & Goossens, H. 1998, "An outbreak of *Burkholderia cepacia* with septicemia on a cardiology ward", *Infect.Control Hosp.Epidemiol.*, vol. 19, no. 2, pp. 112-113.

van Pelt, C., Verduin, C. M., Goessens, W. H., Vos, M. C., Tummler, B., Segonds, C., Reubsaet, F., Verbrugh, H., & van Belkum, A. 1999, "Identification of *Burkholderia spp.* in the clinical microbiology laboratory: comparison of conventional and molecular methods", *J.Clin.Microbiol.*, vol. 37, no. 7, pp. 2158-2164.

van Soolingen, D., Borgdorff, M. W., de Haas, P. E., Sebek, M. M., Veen, J., Dessens, M., Kremer, K., & van Embden, J. D. 1999, "Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997", *J.Infect.Dis.*, vol. 180, no. 3, pp. 726-736.

van Veen, H. W. & Konings, W. N. 1998, "The ABC family of multidrug transporters in microorganisms", *Biochim.Biophys.Acta*, vol. 1365, no. 1-2, pp. 31-36.

Vandamme, P., Govan, J. and LiPuma, J. 2007, "Diversity and role of *Burkholderia* spp" in *Burkholderia. Molecular Microbiology and Genomics*, Coeyne, T. & Vandamme, P. , Horizon Bioscience, Wymondham, Norfolk. Chapter 1. pp 1-28.

Vandamme, P., Henry, D., Coenye, T., Nzula, S., Vancanneyt, M., LiPuma, J. J., Speert, D. P., Govan, J. R., & Mahenthiralingam, E. 2002, "*Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools", *FEMS Immunol.Med.Microbiol.*, vol. 33, no. 2, pp. 143-149.

Vandamme, P., Holmes, B., Coenye, T., Goris, J., Mahenthiralingam, E., LiPuma, J. J., & Govan, J. R. 2003, "*Burkholderia cenocepacia* sp. nov.--a new twist to an old story", *Res.Microbiol*, vol. 154, no. 2, pp. 91-96.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M., Kersters, K., & Govan, J. R. 1997, "Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov", *Int.J.Syst.Bacteriol.*, vol. 47, no. 4, pp. 1188-1200.

Vandamme, P., Mahenthiralingam, E., Holmes, B., Coenye, T., Hoste, B., De Vos, P., Henry, D., & Speert, D. P. 2000, "Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV)", *J.Clin.Microbiol.*, vol. 38, no. 3, pp. 1042-1047.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., & Swings, J. 1996, "Polyphasic taxonomy, a consensus approach to bacterial systematics", *Microbiol.Rev.*, vol. 60, no. 2, pp. 407-438.

Venturi, V., Friscina, A., Bertani, I., Devescovi, G., & Aguilar, C. 2004, "Quorum sensing in the *Burkholderia cepacia* complex", *Res.Microbiol*, vol. 155, no. 4, pp. 238-244.

Vermis, K., Coenye, T., LiPuma, J. J., Mahenthiralingam, E., Nelis, H. J., & Vandamme, P. 2004, "Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov", *Int.J.Syst.Evol.Microbiol.*, vol. 54, no. Pt 3, pp. 689-691.

Vila, J., Ruiz, J., Goni, P., & De Anta, M. T. 1996, "Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*", *Antimicrob.Agents Chemother.*, vol. 40, no. 2, pp. 491-493.

Vila, J., Ruiz, J., Goni, P., Marcos, A., & Jimenez, D. A. 1995, "Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*", *Antimicrob.Agents Chemother.*, vol. 39, no. 5, pp. 1201-1203.

Vila, J., Ruiz, J., Marco, F., Barcelo, A., Goni, P., Giralt, E., & Jimenez, d. A. 1994, "Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs", *Antimicrob.Agents Chemother.*, vol. 38, no. 10, pp. 2477-2479.

Visca, P., Ciervo, A., Sanfilippo, V., & Orsi, N. 1993, "Iron-regulated salicylate synthesis by *Pseudomonas* spp", *J.Gen.Microbiol.*, vol. 139, no. 9, pp. 1995-2001.

Visser, M. B., Majumdar, S., Hani, E., & Sokol, P. A. 2004, "Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections", *Infect.Immun.*, vol. 72, no. 5, pp. 2850-2857.

Vorachit, M., Lam, K., Jayanetra, P., & Costerton, J. W. 1993, "Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole", *Antimicrob.Agents Chemother.*, vol. 37, no. 9, pp. 2000-2002.

Voss, A., Milatovic, D., Wallrauch-Schwarz, C., Rosdahl, V. T., & Braveny, I. 1994, "Methicillin-resistant *Staphylococcus aureus* in Europe", *Eur.J.Clin.Microbiol Infect.Dis.*, vol. 13, no. 1, pp. 50-55.

Vrany, J. D., Stewart, P. S., & Suci, P. A. 1997, "Comparison of recalcitrance to ciprofloxacin and levofloxacin exhibited by *Pseudomonas aeruginosa* bofilms displaying rapid-transport characteristics", *Antimicrob.Agents Chemother.*, vol. 41, no. 6, pp. 1352-1358.

Waldor, M. K. & Mekalanos, J.J. 1996, "Lysogenic conversion by a filamentous phage encoding cholera toxin", *Science*, vol. 272, pp. 1910-1914.

Wallmann, J. 2006, "Monitoring of antimicrobial resistance in pathogenic bacteria from livestock animals", *Int.J.Med.Microbiol.*, vol. 296 Suppl 41, pp. 81-86.

Walsh, C. 2000, "Molecular mechanisms that confer antibacterial drug resistance", *Nature*, vol. 406, no. 6797, pp. 775-781.

Wang, C. X. & Mi, Z. H. 2006, "Imipenem-resistant *Pseudomonas aeruginosa* producing IMP-1 metallo-beta-lactamases and lacking the outer-membrane protein OprD", *J.Med.Microbiol.*, vol. 55, no. Pt 3, pp. 353-354.

Wang, H., Dzink-Fox, J. L., Chen, M., & Levy, S. B. 2001, "Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations", *Antimicrob.Agents Chemother.*, vol. 45, no. 5, pp. 1515-1521.

Wang, J. C. 1985, "DNA Topoisomerase", *Annual Review of Biochemistry*, vol. 54, no. 1, pp. 665-697.

Wang, M., Sahm, D. F., Jacoby, G. A., & Hooper, D. C. 2004, "Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States", *Antimicrob.Agents Chemother.*, vol. 48, no. 4, pp. 1295-1299.

Wang, M., Tran, J. H., Jacoby, G. A., Zhang, Y., Wang, F., & Hooper, D. C. 2003, "Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China", *Antimicrob.Agents Chemother.*, vol. 47, no. 7, pp. 2242-2248.

Warnes, A. & Stephenson, J. R. 1986, "The insertion of large pieces of foreign genetic material reduces the stability of bacterial plasmids", *Plasmid*, vol. 16, no. 2, pp. 116-123.

Watnick, P. I. & Kolter, R. 1999, "Steps in the development of a *Vibrio cholerae* El Tor biofilm", *Mol.Microbiol.*, vol. 34, no. 3, pp. 586-595.

Weigel, L. M., Anderson, G. J., & Tenover, F. C. 2002, "DNA gyrase and topoisomerase IV mutations associated with fluoroquinolone resistance in *Proteus mirabilis*", *Antimicrob.Agents Chemother.*, vol. 46, no. 8, pp. 2582-2587.

Wetzstein, H. G., Schmeer, N., & Karl, W. 1997, "Degradation of the fluoroquinolone enrofloxacin by the brown rot fungus *Gloeophyllum striatum*: identification of metabolites", *Appl.Environ.Microbiol.*, vol. 63, no. 11, pp. 4272-4281.

Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., & Greenberg, E. P. 2001, "Gene expression in *Pseudomonas aeruginosa* biofilms", *Nature*, vol. 413, no. 6858, pp. 860-864.

Whiteley, M., Lee, K. M., & Greenberg, E. P. 1999, "Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 24, pp. 13904-13909.

Wichelhaus, T. A., Boddinghaus, B., Besier, S., Schafer, V., Brade, V., & Ludwig, A. 2002, "Biological cost of rifampin resistance from the perspective of *Staphylococcus aureus*", *Antimicrob.Agents Chemother.*, vol. 46, no. 11, pp. 3381-3385.

Wilke, C. O., Wang, J. L., Ofria, C., Lenski, R. E., & Adami, C. 2001, "Evolution of digital organisms at high mutation rates leads to survival of the flattest", *Nature*, vol. 412, no. 6844, pp. 331-333.

Willmott, C. J. & Maxwell, A. 1993, "A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex", *Antimicrob.Agents Chemother.*, vol. 37, no. 1, pp. 126-127.

Wilson, T. M., de Lisle, G. W., & Collins, D. M. 1995, "Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*", *Mol.Microbiol.*, vol. 15, no. 6, pp. 1009-1015.

Witte, W., Klare, I., & Werner, G. 1999, "Selective pressure by antibiotics as feed additives", *Infection*, vol. 27 Suppl 2, p. S35-S38.

Wright, S. 1932, "The roles of mutation, inbreeding, crossbreeding, and selection in evolution", *Proceedings of the Sixth International Congress on Genetics* pp. 355-366.

Wroblewska, M. M., Rudnicka, J., Marchel, H., & Luczak, M. 2006, "Multidrug-resistant bacteria isolated from patients hospitalised in Intensive Care Units", *Int.J.Antimicrob.Agents*, vol. 27, no. 4, pp. 285-289.

Xu, K. D., McFeters, G. A., & Stewart, P. S. 2000, "Biofilm resistance to antimicrobial agents", *Microbiology*, vol. 146 (Pt 3), pp. 547-549.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., & Arakawa, M. 1992, "Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species

Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov", *Microbiol.Immunol.*, vol. 36, no. 12, pp. 1251-1275.

Yabuuchi, E., Kawamura, Y., Ezaki, T., Ikedo, M., Dejsirilert, S., Fujiwara, N., Naka, T., & Kobayashi, K. 2000. *Burkholderia uboniae* sp. nov., L-arabinose assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. Microbiol. Immunol. 44, 307-317.

Yonezawa, M., Takahata, M., Matsubara, N., Watanabe, Y., & Narita, H. 1995, "DNA gyrase *gyrA* mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa*", *Antimicrob.Agents Chemother.*, vol. 39, no. 9, pp. 1970-1972.

Yoshida, H., Bogaki, M., Nakamura, M., & Nakamura, S. 1990a, "Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*", *Antimicrob.Agents Chemother.*, vol. 34, no. 6, pp. 1271-1272.

Yoshida, H., Nakamura, M., Bogaki, M., & Nakamura, S. 1990b, "Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa*", *Antimicrob.Agents Chemother.*, vol. 34, no. 6, pp. 1273-1275.

Yoshida, H., Bogaki, M., Nakamura, M., Yamanaka, C. M. & Nakamura, S.1991, "Quinolone Resistance Determining Region in DNA gyrase *gyrB* gene of *Escherichia coli*". *Antimicrob.Agents Chemother.*, vol. 34, no. 6, pp. 1647-1650.

Youmans, G. P. & Youmans. A. S. 1949, "A method for the determination of the rate of growth of tubercle bacilli by the use of small Inocula", *J.Bacteriol.*, vol. 58, pp. 247-255.

Ysern, P., Clerch, B., Castano, M., Gibert, I., Barbe, J., & Llagostera, M. 1990, "Induction of SOS genes in *Escherichia coli* and mutagenesis in *Salmonella typhimurium* by fluoroquinolones", *Mutagenesis*, vol. 5, no. 1, pp. 63-66.

Zelver, N., Hamilton, M., Pitts, B., Goeves, D., Walker, D., & Sturman, P. H. J. 1999, "Measuring antimicrobial effects on biofilm bacteria: from laboratory to field" in *Biofilms:Methods in Enzymology*, vol. 310 R. Doyle, ed., Academic Press, San Diego, pp. 608-628.

Zhou, J., Chen, Y., Tabibi, S., Alba, L., Garber, E., & Saiman, L. 2007, "Antimicrobial susceptibility and synergy studies of *Burkholderia cepacia* complex isolated from patients with cystic fibrosis", *Antimicrob.Agents Chemother.*, vol. 51, no. 3, pp. 1085-1088.

Zhou, J., Dong, Y., Zhao, X., Lee, S., Amin, A., Ramaswamy, S., Domagala, J., Musser, J. M., & Drlica, K. 2000, "Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations", *J.Infect.Dis.*, vol. 182, no. 2, pp. 517-525.

Publications arising from thesis

Pope, C. F., Gillespie, S. H., Pratten, J. R. & McHugh, T. D. 2008, "Investigation on Fluoroquinolone- Resistant mutants of *Burkholderia cepacia*" *Antimicrob. Agent Chemother.*, vol 52, no. 3, pp. 1203-3.

Pope, C. F., O'Sullivan, D.M., McHugh, T. D. & Gillespie, S. H., 2008, "A practical guide to measuring mutation rates in antibiotic resistance." *Antimicrob. Agent Chemother.*, vol 52, no. 4, pp. 1209-14.

Pope, C. F., McHugh, T. D., Pratten, J. R. & Gillespie, S. H. 2007, "Measuring bacterial fitness" In *Biofilms: coming of age,* Gilbert P., Allison D, Brading M, Pratten J., Spratt D. and Upton M. (Eds.). University of Manchester, Contributions to 8th Meeting of Biofilm Club, 5th-7th Sept, 2007, Powys (UK), pp22-33. ISBN 0-9551030-1-0.

Pope, C. F., McHugh, T. D., & Gillespie, S. H. 2008 "Rapid methods to determine fitness in bacteria using automated culture systems". Commisioned and accepted. *Antibiotic Resistance: Methods and Protocols*, 2nd edition. S. H. Gillespie (Ed). Methods in Molecular Medicine. Humana Press.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2008, p. 1201–1203 Vol. 52, No. 3
0066-4804/08/$08.00+0 doi:10.1128/AAC.00799-07

Fluoroquinolone-Resistant Mutants of *Burkholderia cepacia*▽

C. F. Pope,[1] S. H. Gillespie,[1] J. R. Pratten,[2] and T. D. McHugh[1]*

Centre for Medical Microbiology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2QG,[1] and UCL Eastman Dental Institute, Gray's Inn Road, London WC1X 8LD,[2] United Kingdom

Received 20 June 2007/Returned for modification 6 August 2007/Accepted 16 December 2007

Fluoroquinolone-resistant *Burkholderia cepacia* mutants were selected on ciprofloxacin. The rate of mutation in *gyrA* was estimated to be 9.6×10^{-11} mutations per division. Mutations in *gyrA* conferred 12- to 64-fold increases in MIC, and an additional *parC* mutation conferred a large increase in MIC (>256-fold). Growth rate, biofilm formation, and survival in water and during drying were not impaired in strains containing single *gyrA* mutations. Double mutants were impaired only in growth rate (0.85, relative to the susceptible parent).

Exposure to fluoroquinolones increases mutation rates (9, 12, 20, 26) to various degrees (23). The main mechanism of resistance in gram-negative bacteria develops via stepwise accumulation of mutations in the quinolone resistance-determining region (QRDR) of topoisomerase genes (4, 7, 8, 13).

Opportunistic pathogens of the *Burkholderia cepacia* complex (BCC) consist of genomovars that are important in cystic fibrosis patients (14, 17). Genomovars are species which are phylogenetically distinguishable but phenotypically indistinguishable from each other. Here, BCC refers to the complex, while *B. cepacia* refers to genomovar I. BCC bacteria can survive in respiratory droplets on surfaces (6) and are resistant to many antibacterial agents.

Resistance to drying allows maintenance on environmental surfaces (24) and transmission between hosts. Transmission between colonized patients has been documented (18).

The objective of this work was to investigate the effects of fluoroquinolone resistance mutations on growth rate, biofilm formation, and environmental survival.

B. cepacia 10661 (National Collection of Type Cultures, HPA, London, United Kingdom) and mutants derived from this strain were used. The MICs of parent and mutant strains were determined by the ciprofloxacin Etest (AB Biodisk, Solna, Sweden).

The ciprofloxacin MIC of *B. cepacia* 10661 was 1 μg/ml. Putative resistant mutants were selected at 2×, 4×, and 6× MIC in three separate experiments. Estimation of the mutation rate was performed using ciprofloxacin at 6× MIC. Characterized first-step mutants were used to obtain second-step mutants by selecting first-step mutations on media containing twice the MIC of the first-step mutants. The numbers of viable cells, from three aliquots (approximately 10%), were determined using the method of Miles and Misra in order to determine total cell numbers (3, 11). The plates were incubated at 37°C for 18 h, and the proportion of cultures with mutant colonies were recorded. The mutation rate (μ) was determined using the p_0 method (10, 19, 21).

Approximately 10^2 exponentially growing cells were independently inoculated into 28 tubes, each containing 3 ml of Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom), and incubated at 37°C for 22 h on an orbital shaker (250 rpm; Barloworld Scientific, Rochester, NY). The cells were harvested by centrifugation (2,000 × g, 10 min), the supernatant was removed with a pipette, and the pellet was resuspended in 400 μl of Mueller-Hinton broth and then plated onto Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) containing ciprofloxacin.

The mutants were characterized by sequencing the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* by using the primers listed in Table 1. Standard PCR conditions were employed. Sequencing was performed by the dideoxy method as previously described (15).

No mutations (0/45) were found in the QRDRs of the topoisomerase genes of *B. cepacia* selected at 2× MIC. The MIC of these nontopoisomerase mutants was 4 to 5 μg/ml. At 4× MIC, an Asp87Asn mutation, conferring a 16-fold increase in MIC, was found in colonies from one plate (2/55). All other mutants (53/55) selected at this concentration contained no mutations in the QRDRs (MICs between 4 and 5 μg/ml). All first-step mutants selected at 6× MIC (50/50) contained a Thr83Ile mutation in *gyrA* and had an MIC of 64 μg/ml. Mutations, MIC, and selection step information are shown in Table 2. Mutation rates for second-step mutations were higher than those for the first-step mutations.

To detect efflux activity, the ciprofloxacin MIC of the fluoroquinolone-resistant mutants was determined in the absence and presence of reserpine (25 μg/ml) in Mueller-Hinton agar (2). The MICs of all mutants that did not contain *gyrA* mutations, selected at either 2× MIC (45 mutants tested) or 4× MIC (55 mutants tested), decreased fivefold in the presence of reserpine to the level of the wild type. The MICs of mutants containing topoisomerase mutations did not decrease.

The quantification of biofilm growth was achieved by the spectrophotometric measurement of crystal violet binding by following a previously published method (5). Mutation in *gyrA* and *parC* did not affect biofilm formation in *B. cepacia*. All fitness assays were carried out with one mutation of each type (data not shown).

We modified the method of Youmans and Youmans (25) to determine time to positivity as an indicator of the growth rate.

* Corresponding author. Mailing address: Centre for Medical Microbiology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, United Kingdom. Phone: 44 20 7472 6402. Fax: 44 20 7794 0500, ext. 3540. E-mail: t.mchugh@medsch.ucl.ac.uk.

▽ Published ahead of print on 26 December 2007.

TABLE 1. Primers used to amplify the QRDRs of the topoisomerase genes of *B. cepacia*

Gene	Primer positions[a]	Sequence (5′–3′)	Amplicon size (bp)
gyrA	62–81	5′ ATCTCGATTACGCGATGAGC	449
	493–511	5′ GCCGTTGATCAGCAGGTT	
gyrB	1127–1146	5′ GAGGAAGTTGTGGCGAAGG	400
	1502–1520	5′ AGTCTTCCTTGCCGATGC	
parC	98–118	5′ ATTGGTCAGGGTCGTGAAGA	229
	295–315	5′ GTAGCGCAGCGAGAAATCCT	
parE	1178–1198	5′ CAGGGCAAGGTAGTCGAAAA	380
	1557–1577	5′ GTGAGCAGCAAGGTCTGGAT	

[a] *B. cenocepacia* numbering.

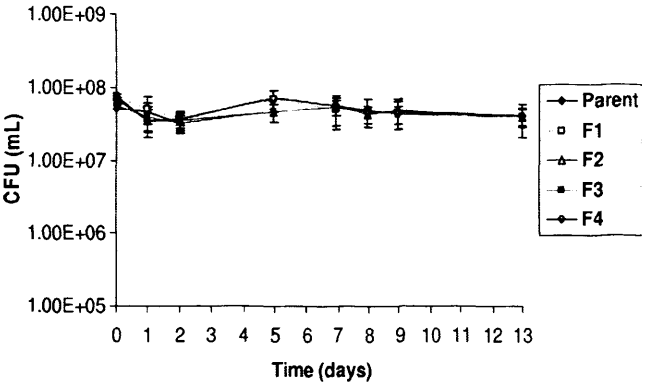


FIG. 1. Effect of topoisomerase mutations on the survival of *B. cepacia* in water. Survival of *B. cepacia* in water was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars indicate the standard errors of the means. Differences in survival are not significant.

The Bactec 9240 continuous blood culture system with standard aerobic medium (Plus Aerobic/F) was used. Aliquots of 100 μl of the diluted exponential culture (1/10 and 1/1,000) were removed using a 0.5-ml syringe and a needle and were aseptically inoculated into duplicate culture vials. The length of time to detection (time to positivity) was measured for all strains. Gram staining and a purity plate assay were performed to confirm the absence of contaminants.

The growth rate constant, k, was determined using equation 1 (where A is the largest inoculum employed, B is the smallest inoculum, and t is the difference in time to positivity in hours). The generation time (G) was determined using equation 2.

$$k = \frac{\log A - \log B}{t} \quad (1)$$

$$G = \frac{\log 2}{k} \quad (2)$$

This experiment was repeated in triplicate. The growth rates of the double mutants relative to those of the parent were 0.88 and 0.83 for mutant strains F3 and F4, respectively, as shown in Table 2.

Competition assays were used to measure the fitness of the fluoroquinolone mutants compared to that of the susceptible parent by the use of a modified version of our previously published method (3, 11). The optical densities of the wild-type and mutant isolates were adjusted to the same value (1.0 optical density unit). Then, 250 μl of each culture was inoculated into 15 ml of LB broth in the absence of antibiotics. This mixed culture was incubated for 10 h (200 rpm). The relative fitness of each strain was calculated from the ratio of the number of generations grown by the resistant strains to the number grown by the susceptible strains. Five independent pairwise cultures were performed for each mutant. The relative growth rates of mutant strains F3 and F4 were 0.80 and 0.78, respectively. The differences in relative growth rates of the strains with the single *gyrA* mutations found during paired competition assays were not significant, as determined by Student's t test. However, these assays cannot measure differences of >1%.

Survival in water and survival during drying were assessed using the method employed by Sánchez et al. (22). No significant differences in environmental survival were found between the mutants and the susceptible parent, as shown in Fig. 1 and Fig. 2.

Selection at lower concentrations of fluoroquinolone resulted in mutants in which resistance was apparently due to an altered expression of an efflux pump. Similarly, Zhou et al. demonstrated that low concentrations of fluoroquinolone selected nongyrase mutants of *Mycobacterium smegmatis* (27).

At higher selection concentrations of ciprofloxacin (4× and 6× MIC), mutations in the topoisomerase genes were found. Lower-level resistance (12- to 64-fold) was caused by single mutations in *gyrA*. Higher-level resistance (MIC of >256 μg/

TABLE 2. Characteristics of fluoroquinolone-resistant *B. cepacia* mutants selected in vitro[a]

Strain	Mutation rate (mutation/division)	MIC (μg/μl)	Selection step	Sequence found in QRDRs of:				Generation time [min (95% confidence interval)]	*P* value	Relative fitness (±SEM)[b]	*P* value
				gyrA	*gyrB*	*parC*	*parE*				
WT		1		WT	WT	WT	WT	38.0 (37.06–38.94)			
F1 mutant	9.6×10^{-11}	12	1st	Asp87Asn	WT	WT	WT	37.0 (36.77–37.23)	0.331	1.01 ± 0.01	0.831
F2 mutant	9.6×10^{-11}	64	1st	Thr83Ile	WT	WT	WT	37.1 (36.9–37.3)	0.377	1.01 ± 0.152	0.868
F3 mutant	1.1×10^{-10}	>256	2nd	Asp87Asn	WT	Ser80Leu	WT	43.0 (41.85–44.15)	0.004	0.80 ± 0.12	0.003
F4 mutant	6.8×10^{-10}	>256	2nd	Thr83Ile	WT	Ser80Leu	WT	45.7 (44.2–47.2)	0.001	0.78 ± 0.18	0.002

[a] Strain F1 was isolated on 2 μg/ml ciprofloxacin (2× MIC); F2 was isolated on 6 μg/ml (6× MIC), F3 was isolated on 24 μg/ml ciprofloxacin by the use of F1 as the starting point; F4 was isolated on 128 μg/ml ciprofloxacin by the use of F2 as the starting point. The statistical significance of generation time differences is shown by a *P* value. WT, wild type.

[b] Competition assays were used to measure the fitness of the fluoroquinolone mutants relative to that of the susceptible parent.

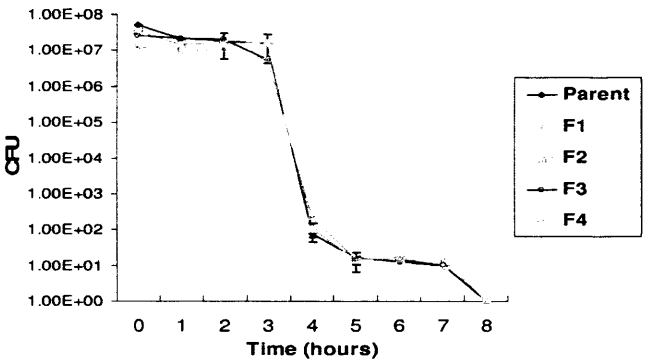


FIG. 2. Effect of topoisomerase mutations on the survival of *B. cepacia* on dry surfaces. Survival of *B. cepacia* in water was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars indicate the standard errors of the means. Error bars are not shown if obscured by the symbol. Differences in survival are not significant.

ml) required mutations in both *gyrA* and *parC*. The same second-step mutation occurred irrespective of the starting point.

Single-step fluoroquinolone resistance in *gyrA* occurs at low or no cost to *B. cepacia*, and this has been observed for other bacteria (11, 1, 16). These mutants may, therefore, remain in the bacterial population in the absence of an antibiotic selective pressure.

We thank Tyrone Pitt (HPA, London, United Kingdom) for advice and for kindly providing reference strains.

REFERENCES

1. **Bagel, S., V. Hullen, B. Wiedemann, and P. Heisig.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob. Agents Chemother. **43**:868–875.
2. **Beyer, R., E. Pestova, J. J. Millichap, V. Stosor, G. A. Noskin, and L. R. Peterson.** 2000. A convenient assay for estimating the possible involvement of efflux of fluoroquinolones by *Streptococcus pneumoniae* and *Staphylococcus aureus*: evidence for diminished moxifloxacin, sparfloxacin, and trovafloxacin efflux. Antimicrob. Agents Chemother. **44**:798–801.
3. **Billington, O. J., T. D. McHugh, and S. H. Gillespie.** 1999. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **43**:1866–1869.
4. **Chen, F. J., and H. J. Lo.** 2003. Molecular mechanisms of fluoroquinolone resistance. J. Microbiol. Immunol. Infect. **36**:1–9.
5. **Conway, B.-A. D., V. Venu, and D. P. Speert.** 2002. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J. Bacteriol. **184**:5678–5685.
6. **Drabick, J. A., E. J. Gracely, G. J. Heidecker, and J. J. LiPuma.** 1996. Survival of *Burkholderia cepacia* on environmental surfaces. J. Hosp. Infect. **32**:267–276.
7. **Drlica, K., and M. Malik.** 2003. Fluoroquinolones: action and resistance. Curr. Top. Med. Chem. **3**:249–282.
8. **Drlica, K., and X. Zhou.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61**:377–392.
9. **Gillespie, S. H., S. Basu, A. L. Dickens, D. M. O'Sullivan, and T. D. McHugh.** 2005. Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. J. Antimicrob. Chemother. **56**:344–348.
10. **Gillespie, S. H., L. L. Voelker, J. E. Ambler, C. Traini, and A. Dickens.** 2003. Fluoroquinolone resistance in *Streptococcus pneumoniae*: evidence that *gyrA* mutations arise at a lower rate and that mutation in *gyrA* or *parC* predisposes to further mutation. Microb. Drug Resist. **9**:17–24.
11. **Gillespie, S. H., L. L. Voelker, and A. Dickens.** 2002. Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae*. Microb. Drug Resist. **8**:79–84.
12. **Gocke, E.** 1991. Mechanism of quinolone mutagenicity in bacteria. Mutat. Res. **248**:135–143.
13. **Hooper, D. C.** 2003. Mechanisms of quinolone resistance, p. 41–67. *In* D. C. Hooper and E. Rubenstein (ed.), Quinolone antimicrobial agents. ASM Press, Washington, DC.
14. **Isles, A., I. Maclusky, M. Corey, R. Gold, C. Prober, P. Fleming, and H. Levison.** 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J. Pediatr. **104**:206–210.
15. **Jenkins, C.** 2005. Rifampicin resistance in tuberculosis outbreak, London, England. Emerg. Infect. Dis. **11**:931–934.
16. **Kugelberg, E., S. Lofmark, B. Wretlind, and D. I. Andersson.** 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **55**:22–30.
17. **LiPuma, J. J.** 1998. *Burkholderia cepacia*. Management issues and new insights. Clin. Chest Med. **19**:473–486.
18. **LiPuma, J. J., S. E. Dasen, D. W. Nielson, R. C. Stern, and T. L. Stull.** 1990. Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. Lancet **336**:1094–1096.
19. **Luria, S., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
20. **Mamber, S. W., B. Kolek, K. W. Brookshire, D. P. Bonner, and J. Fung-Tomc.** 1993. Activity of quinolones in the Ames *Salmonella* TA102 mutagenicity test and other bacterial genotoxicity assays. Antimicrob. Agents Chemother. **37**:213–217.
21. **Rosche, W. A., and P. L. Foster.** 2000. Determining mutation rates in bacterial populations. Methods **20**:4–17.
22. **Sánchez, P., J. F. Linares, B. Ruiz-Diez, E. Campanario, A. Navas, F. Baquero, and J. L. Martinez.** 2002. Fitness of *in vitro* selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants. J. Antimicrob. Chemother. **50**:657–664.
23. **Sierra, J. M., J. G. Cabeza, C. M. Ruiz, T. Montero, J. Hernandez, J. Mensa, M. Llagostera, and J. Vila.** 2005. The selection of resistance to and the mutagenicity of different fluoroquinolones in *Staphylococcus aureus* and *Streptococcus pneumoniae*. Clin. Microbiol. Infect. **11**:750–758.
24. **Smith, S. M., R. H. Eng, and F. T. Padberg, Jr.** 1996. Survival of nosocomial pathogenic bacteria at ambient temperature. J. Med. **27**:293–302.
25. **Youmans, G. P., and A. S. Youmans.** 1949. A method for the determination of the rate of growth of tubercle bacilli by the use of small inocula. J. Bacteriol. **58**:247–255.
26. **Ysern, P., B. Clerch, M. Castano, I. Gibert, J. Barbe, and M. Llagostera.** 1990. Induction of SOS genes in *Escherichia coli* and mutagenesis in *Salmonella typhimurium* by fluoroquinolones. Mutagenesis **5**:63–66.
27. **Zhou, J., Y. Dong, X. Zhao, S. Lee, A. Amin, S. Ramaswamy, J. Domagala, J. M. Musser, and K. Drlica.** 2000. Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. J. Infect. Dis. **182**:517–525.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2008, p. 1209–1214 Vol. 52, No. 4
0066-4804/08/$08.00+0 doi:10.1128/AAC.01152-07

MINIREVIEW

A Practical Guide to Measuring Mutation Rates in Antibiotic Resistance[▽]

Cassie F. Pope, Denise M. O'Sullivan, Timothy D. McHugh, and Stephen H. Gillespie*

Centre for Medical Microbiology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, United Kingdom

Bacteria become resistant to antibacterial agents by three main mechanisms: acquisition of complete resistance genes or gene complexes via plasmids and other transposable elements (12, 16, 21, 26, 30), recombination of DNA from other bacteria into the genome by transformation (6), and spontaneous mutational events in the chromosome and accessory DNA (14). Horizontal gene transfer in bacteria has been reviewed by Thomas and Nielsen (31). This minireview will concentrate on the study of chromosomal mutations that confer resistance. Mutational events are assumed to be stochastic, so that the rate of beneficial mutation does not occur at a higher frequency than those that are neutral or disadvantageous and that mutations are not directed. For bacterial cells, there is a finite probability that a mutation conferring the resistant phenotype will occur, and unless a revertant mutation occurs, all of the progeny of such a cell will be resistant also. An important review by Rosche and Foster which critically analyzes mutation rate determination methods lays the foundation of this minireview (29). The terms and abbreviations used here are defined in Table 1.

MUTATION RATE OR MUTATION FREQUENCY

A mutation rate is an estimation of the probability of a mutation occurring per cell division and corresponds to the probability of a mutation occurring in the lifetime of a bacterial cell. A mutation frequency is simply the proportion of mutant bacteria present in a culture. These terms are often used interchangeably, causing confusion. The relationship between mutation frequency and the rate at which mutations occur is uncertain. If a mutation arises early in the culture period, then a large number of mutant progeny occur and this would be represented by a high frequency. This phenomenon is known as a "jackpot culture" and was first described in 1943 by Luria and Delbrück during their seminal set of experiments investigating the mutation of *Escherichia coli* from bacteriophage T1 sensitivity to resistance (19). Understanding of this phenomenon was the crucial evidence indicating the role of mutation in phage resistance and underpins all of the work on mutation that followed.

FLUCTUATION TEST OF LURIA AND DELBRÜCK

Luria and Delbrück demonstrated that bacteriophage-resistant mutant colonies arise from a sensitive culture of *E. coli* if bacteriophage T1 is present in excess (19). Resistant colonies appeared from sensitive cultures, i.e., in which there was clearing, within 12 to 16 h. These bacteria were resistant to bacteriophage T1 but sensitive to other viruses capable of causing lysis in that strain of *E. coli*. Luria and Delbrück showed that reversion to sensitivity was a rare event and that, in a growing culture, the proportion of resistant bacteria increased with time. They argued that if the presence of the phage was needed to trigger the change to resistance, then the distribution of mutant colonies should demonstrate a Poisson distribution. The high variance in the numbers of mutants in the culture, however, led Luria and Delbrück to conclude that resistant mutants were present in the culture before bacteriophage exposure and that the bacteriophage resistance mutation arose independently. The Luria-Delbrück distribution is different from the Poisson distribution in that its variance is greater than 1.

Luria and Delbrück assumed that for a bacterium there was a small fixed chance that a resistance-conferring mutation could occur per unit of time if the bacteria are "in an identical state." The number of mutated cells in a culture depends on how early the mutation occurred during the growth of the bacterial population. If mutation occurs early in the culture, the number of mutated cells will be higher than if it occurs later. Measurement of the mutation rate, rather than frequency, should be the standard in antibiotic research. Although the protocols and calculation methods are more complex, they are not as inaccessible as it might appear.

DETERMINATION OF MUTATION RATE

Broadly, there are two methods for determination of the mutation rate: mutation accumulation and fluctuation analysis. Mutant accumulation methods have the advantage that they are very accurate, but they are complicated and time-consuming to perform because the culture is sampled at multiple time points. The methodology depends on growing bacteria exponentially until probability dictates that a mutant will be present. If the assumption is made that the growth rates of wild-type and mutant bacteria are the same, then the proportion of mutants will increase linearly with time. Furthermore, if the number of mutants and the total number of bacterial cells are known at each time point, then the mutation rate (μ) can be calculated from the slope of the line describing the rela-

* Corresponding author. Mailing address: Centre for Medical Microbiology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, United Kingdom. Phone: 44 (0)207 794 0500. Fax: 44 (0)207 794 0433. E-mail: s.gillespie@medsch.ucl.ac.uk.

▽ Published ahead of print on 4 February 2008.

TABLE 1. Terms and abbreviations used in this minireview

Term	Definition
m	No. of mutational events/culture
μ	Mutation rate
r	Observed no. of mutants
x	Median no. of mutants
C	No. of cultures
p_0	Proportion of cultures without mutants
N_0	Initial no. of cells
N_t	No. of cells at time t
f	Mutant frequency
n	No. of generations

tionship between the number of mutants against the generation number. The mutation rate can be determined by using the equation $\mu = [(r_2/N_2) - (r_1/N_1)] \times \ln (N_2/N_1) = (f_1 - f_2) \times \ln (N_2/N_1)$, where r_1 is the observed number of mutants at time point 1, r_2 is the observed number of mutants at the next time point, and N_1 and N_2 are the numbers of cells at time points 1 and 2, respectively, while f_1 and f_2 are the mutant frequencies at points 1 and 2.

For this method to be accurate, a very large difference in the total cell number is required between N_1 (the number of cells at the first time point) and N_2 (the number of cells at the second time point). Serial dilutions would make this easier to perform, but this introduces sampling errors. If available, continuous culture would be an alternative but this would allow the selection of waves of bacteria, each better suited than the generation before to take over the culture (25). Moreover, many studies have shown that the acquisition of a mutation providing resistance is associated with a significant fitness deficit, which invalidates one of the basic premises of the mutant accumulation method, as less fit mutants will accumulate at a different rate than the parent (3). For this reason and for greater simplicity, fluctuation methods are more commonly used, and this minireview will concentrate on describing various applications of this approach.

FLUCTUATION ANALYSIS IN ANTIBIOTIC RESEARCH: GENERAL PRINCIPLES

Fluctuation analysis involves estimating the mutation rate from the distribution of mutants in a number of parallel cultures. This method was pioneered by Luria and Delbrück (19). Briefly, an initial inoculum of cells (with a known cell volume) from a growing culture is added to a broth and incubated in the absence of selective pressure. The bacterial cells are concentrated and screened for antibiotic-resistant mutant cells by plating the whole cell population onto solid medium containing a suitable concentration of the test antibiotic, usually at two to four times the MIC. It is assumed that this will inhibit the growth of susceptible cells, leaving only resistant mutants. A plate count is performed on a portion of the culture to determine the number of viable cells in the cell deposit. The method of Miles and Misra can be used to determine viable cell numbers. This method involves the spotting of replicate 20-μl drops of broth onto a plate and counting of the colonies that grow within that spot. This reduces the bacterial cells that are lost by spreading (23). Luria and Delbrück suggested two methods for estimating the overall mutation rate of the population: the p_0 method, which is based on the proportion of cultures in which there are no mutants observed, and the method of the mean, which relies on the determination of the mean number of mutants. Both methods assume a Poisson distribution with a mean and variance equal to the product of the probability of a mutation and the number of bacteria. All of the methods described in this minireview use an estimate of the number of mutational events (not the number of mutants), m, to determine the mutation rate and have a Luria-Delbrück distribution (19). Parameter m will be influenced by the amount of growth and the mutation rate (μ). The estimated value of m can be divided by the total number of cells to give the mutation rate.

DESIGNING A MUTATION RATE EXPERIMENT

Choice of selective antibiotic. Ideally, mutation rates should be calculated by using an antibiotic to which resistance arises via a mutation at a single base pair for the reasons noted above. This situation rarely arises, and consequently, pragmatic compromises must be made. Manipulating the culture volume growth conditions and durations enables these methods to be adapted to answer a wide range of questions in antibiotic research.

The choice of the selecting agent depends on the purpose of the experiment. Antibiotics which are most suitable for mutation rate methods are those to which resistance arises as a result of point mutations in chromosomal genes, including the aminoglycosides, quinolones, rifampin, pyrazinamide, and isoniazid (10). If one wants to measure the rate of resistance to a particular antibiotic, then the nature of the drug-bacterium interaction will dictate how the parameters vary and "ranging" experiments may be required. Not all of the colonies growing on the selective plate will contain the same mutation. Thus, a mutation rate calculated by including confirmed mutations in a single target gene will be lower than a phenotypic mutation rate due to the presence of multiple target genes and nonheritable changes. In antibiotic research, it is usual that lethal selection for preexisting mutations, as in the case of the experiment of Luria and Delbrück, is being tested, and this is different from the nonlethal selection used by Cairns et al. in their "directed-mutation" experiments with Lac, which allowed mutants arising postplating to grow (5).

Parameters. For each mutation rate experiment, there are three main parameters which must be considered, i.e., the expected number of mutational events, the number of cultures to be examined, and the size of the initial inoculum.

If the p_0 method is to be used, m should be between 0.3 and 2.3 mutational events per culture. If m is less than 0.3, then none of the mutation rate methods are reliable. When m is greater than 2.3, the Luria and Delbrück method of the mean can be used to estimate the mutation rate (19). Methods of the mean or median described below have constraints on the number of mutants per culture if the results are to be valid, and these ranges are shown in Table 2.

The number of mutational events present in the culture depends on the mutation rate itself and the amount of growth. Growth conditions will vary between bacterial species. For example, culture aliquots of *Streptococcus pneumoniae* cannot be incubated for extended periods. This is due to the activity of

TABLE 2. Appropriateness of different methods for different values of m

Method	Value of m (no. of mutational events/culture)
p_0 method	$0.3 \leq m \leq 2.3$
Method of the mean	Any
Lea and Coulson method of the median	$1.5 \leq m \leq 15$
Drake formula	$m \geq 30$

the cell wall autolysin, which results in a decrease in the viable cell count following extended incubation (15). The value of m can be manipulated by inoculating different volumes of broth onto solid medium, but this can introduce errors (see below). The choice of methods will vary with different values of m, and therefore the method chosen will depend on the expected value of m.

Number of cultures. The second crucial parameter is the number of parallel cultures (C) chosen to represent the bacterial population. Irrespective of the method used, the precision of m is a function of $1/\sqrt{C}$ and increases as C increases; if more cultures are tested, then precision is increased. Between 20 and 30 cultures are routinely included (2). For the p_0 method, a precision level of 20% is considered necessary to provide a suitable estimate of the number of mutational events per culture (29). Precision is the coefficient of variation, σ_m/m, multiplied by 100% and has been calculated as 0.2 (29) and is a measurement of the reproducibility of results, as opposed to accuracy.

Size of initial inoculum. The final parameter is the size of the initial inoculum (N_0). This inoculum should not contain any preexisting mutants, and thus it should be small. For example, in their *E. coli* experiments, Luria and Delbrück used an initial inoculum of between 50 and 500 bacteria (19). The smaller the initial inoculum, the longer the incubation period. This is especially important when working with slow-growing cultures, e.g., *Mycobacterium tuberculosis*. We have found that between 3,000 and 5,000 cells/ml is sufficient as the initial inoculum for *S. pneumoniae* and *M. tuberculosis*, respectively. There are other complications involved in growing small numbers of organisms. For example, many organisms monitor the density of cells via quorum sensing and only switch on virulence genes after a quorum of bacteria is present (24, 28). A small inoculum may produce a reduction in viability, resulting in greater variation in the final number of cells (N_t). In each parallel culture, the final cell number (N_t) should be the same and the value of N_0 should always be negligible compared to N_t (a ratio of at least <1:1,000 is desirable). Variations in N_t can be eliminated by using a large initial inoculum. Rosche and Foster (29) found that, in their experiments, a pragmatic compromise between the above factors was to use an initial inoculum of total cells of $m\ N_t/10^4$ (3, 9, 10). To reduce variability, the initial inoculum should consist of an even cell suspension. This is especially important when working with organisms such as *M. tuberculosis*, which tend to form cellular aggregates. To overcome this problem, the initial inoculum should be passed through a fine-needle syringe or a filter to form a single-cell suspension. Additionally, Middlebrook 7H9 broths contain Tween 80 to reduce clumping (3).

Additional relevant considerations. (i) Volume. In order to observe a mutation, it is necessary to have a large enough final cell number. The size of this final cell number is a function of the culture volume and the mutation rate. If the mutation rate is high, then a small broth culture can be used, and if the rate is low, then larger cultures must be used.

(ii) Cell cycle. Mutation rates may be influenced by the growth phase of the cell. Determinations of mutation rates are usually performed by using cells growing in exponential phase (3, 10). There are reports, however, that mutation rates in *E. coli* are elevated in stationary phase compared to exponential phase (15, 18). The initial inoculum of cells should contain cells that are in the same phase of the growth cycle in order to compare estimated rates. Therefore, a growth curve should be constructed during method optimization. To reduce the degree of variability in these experiments, all of the above parameters should be kept constant between experiments.

ASSUMPTIONS OF FLUCTUATION ANALYSIS

Each mutation rate method relies on a set of pragmatic assumptions that are made in order to make estimations possible. (i) The probability of the mutation occurring is constant per cell lifetime. (ii) The probability of this mutation occurring does not vary between growth phases. (iii) There is no cell death. (iv) Revertants occur at a negligible rate. (v) Mutation occurs only during cell division and results in only one mutant. (vi) The growth rates of mutants and nonmutants are the same. (vii) Initial cell numbers are negligible compared to the final cell numbers. (viii) All mutants are detected, and no mutants occur after selection is imposed. However, these assumptions may not be true in all situations. Mutation rates of the same organism that are obtained by using the same selection tool and estimated via different methods can be very different.

DEVIATIONS FROM THE ASSUMPTIONS

Fitness of mutants. As noted above, mutation rate calculation methods assume that there is no physiological impairment of mutants with respect to their susceptible parents. If mutants do not grow as efficiently as their parents, they may not be detected and this may affect the calculated mutation rate. There are examples in which mutations responsible for resistance occur at no or low cost. For example, the *rpsL* Lys42Arg mutation, which confers resistance to streptomycin in *Salmonella enterica* serovar Typhimurium, incurs no measurable cost. In contrast, the Lys42Thr and Lys42Asn mutations associated with resistance incur a heavy fitness burden (4). For example, the *parC* and *gyrA* mutations, conferring fluoroquinolone resistance, incur no or low cost in *S. pneumoniae* (11). The extent of a fitness deficit is dependent on the nature of the mutation, as demonstrated by *M. tuberculosis*, where there is a relationship between the rates at which various resistant mutants are found in clinical practice and the initial fitness deficit of the mutant strain (3, 8, 22).

Completeness of detection. It is possible that not all mutations are detected. For example, mutations that occur late in the culture may not give rise to colonies and these mutants will not be counted. This phenomenon is known as phenotypic lag. Importantly, it is also possible that mutations may occur after

selection has been imposed; i.e., mutants may arise on antibiotic-containing solid medium. In order to overcome these issues, some preliminary ranging experiments could be preformed which would ensure that the correct initial inoculum and the correct final plating volume are used. Colonies should be counted as earlier as possible to minimize the number of postplating mutants that could occur.

Other factors. There are a number of other factors that complicate the calculation of mutation rates. For example, mutation rates are not constant in a population of cells. They can vary depending on the antibiotic concentration (13) and the availability of the carbon source (1).

PLATING A PORTION OF THE CULTURE

It is assumed that all mutants are detected. Plating a portion of the culture can introduce an error in the estimation of m. Some of the methods used to determine the rate of mutation have been derived to take sampling into account. Ma et al. (20) and Jones (14) have altered their fluctuation analysis method to show that it is possible to plate an aliquot of the culture volume when there is a large final inoculum. It is also possible to plate a portion of a large culture rather than using multiple small cultures, and Crane et al. have proposed a modified fluctuation assay for the estimation of mutation rates where small increases in the mutation rate are expected (6).

VARATIONS ON THE LURIA-DELBRÜCK METHOD FOR MUTATION RATE ESTIMATION

No satisfactory solution of the Luria and Delbrück distribution has been found that effectively describes the distribution numerically. Therefore, extensive attempts have been made to improve the accuracy of the estimates (2, 15). The practical effect of this is that mutation rates estimated via different methods cannot be compared.

CALCULATION METHODS

The p_0 method. The p_0 method is the simplest method to calculate and is the one originally described by Luria and Delbrück in their seminal paper (19). It is most suitable when the number of mutational events in a culture is low. This method has successfully been used to estimate mutation rates in *M. tuberculosis* (3) and *S. pneumoniae* (10).

The proportion of cultures without mutants (p_0) is the zero term of the Poisson distribution given by the equation $p_0 = e^{-m}$. This method should only be used if the proportion of cultures without mutants is between 0.1 and 0.7, i.e., the number of mutational events per culture is between 0.3 and 2.3. The formula can be rearranged to give the number of mutational events as follows: $m = -\ln p_0$.

Multiple parallel cultures are performed and scored as positive if they yield a resistant mutant, i.e., show growth. When the proportion of mutants detected is known, then the actual value of m can be calculated. There is no need to enumerate the colonies, and this simplifies the process. It should be noted that the precision of m varies depending on the value of p_0. Compared with other methods, the p_0 method requires more cultures for the same level of precision when $m \geq 1.2$. As cultures are scored as either positive or negative for growth, mutations that affect the growth rate of the progeny cells have less effect in the p_0 method than on other methods. A clone that does not give rise to a colony would add to the proportion of cultures without mutants erroneously. Conditions of growth and culture volume need to be chosen so that the proportion of resistant cultures is in the appropriate range. The p_0 estimator method is very sensitive to phenotypic lag, postplating mutations, and decreased plating efficiency, as these will increase the value of p_0. Some of the progeny of each mutant will be lost if the plate efficiency is less than 100%. This will be the normal situation in most culture systems; thus, cultures with few mutants may be counted as cultures with no mutants.

Methods using the mean. The mean estimator methods use the observation that when a population is large enough there will be an extra μN_t mutants after each generation as each of the cells in the final population may undergo mutation. The probability of this occurring is determined by the mutation rate (μ). Therefore, the extra number of mutants will be a product of these two terms. The time period after the point when the bacterial population of all cultures has reached the required size when this may occur is $1/\mu$ and is known as the Luria-Delbrück period. The mean methods should not be used if there is no Luria-Delbrück period, i.e., if $N_t < 1/\mu$. Methods that use the mean are disproportionally inflated by jackpot cultures and are not recommended. They can be made more accurate by removing data points caused by jackpot cultures, but this makes the approach somewhat arbitrary, with data being removed by the investigator. Methods using the median are more accurate and will be discussed in more detail below.

Lea and Coulson method of the median. Lea and Coulson (17) attempted to develop a method with better precision than the method of the mean. The function m is calculated from the equation $(x/m) - \ln m = 1.24$.

The method assumes that if the median number of mutants is large enough, then most mutations occur early enough to be detected. From a practical point of view, a greater number of selective plates (approximately 5 to 10) are needed for this method to give an adequate precision level. An additional drawback to the increased number of plates required is that median methods should not be used if more than half of the plates are devoid of mutants. It is used when all or most of the cultures give rise to mutant colonies, and it has been quoted as the method of choice (excluding maximum-likelihood methods) if m is between 1.5 and 15 and if the median number of mutational events in a culture is between 2.5 and 60. The main drawback of the method is that it is sensitive to any variation in the assumptions, e.g., phenotypic lag and altered growth rate of progeny, described previously, which results in reduced precision.

Drake formula using the median. The Drake formula using the median provides an easy option to make an estimate of the mutation rate from frequency data, given by the equation $\mu = f/\ln(N_t\mu)$, where f is the final mutation frequency (7). By using the median final mutation frequency and not the mean final mutation frequency, the impact of jackpot mutations is reduced. It can be used when the number of mutational events per culture is high, i.e., ≥ 30. This method has been used to estimate rates of mutation of *S. pneumoniae* to fluoroquin-

olone resistance and of *Mycobacterium fortuitum* to fluoroquinolone, macrolide, and aminoglycoside resistance (9).

Jones median estimator. Jones calculated the hypothetical dilutions required so that half of the selective plates in a putative experiment had mutant colonies (14). Under these circumstances, other median methods cannot be used. The Jones method has the advantage that it relies on the observed number of mutant colonies to estimate m by an explicit equation. Jones (14) verified this method against the Lea and Coulson method of the median, by using computer simulations, for values of m between 1.5 and 10 and showed that it is more efficient than the Lea and Coulson method of the median. Crane et al. modified the method so that it can be used to give more precise mutation rate measurements. In this method, a portion of larger-volumes cultures is plated rather than the whole of smaller-volume cultures (6); this allows more mutants to accumulate. We have used this method to estimate the rate of the *rpoB* mutation, which confers rifampin resistance (3).

CHOOSING A METHOD

There may be a number of differences between methods, but they all use similar functions. Since the pivotal experiments by Luria and Delbrück in the last century, novel methods for the calculation of the Luria-Delbrück distribution have made the estimation of mutation rates more accurate and easy to perform. The most useful methods are the p_0 method (2) and the Jones median estimator together with the modification of Crane et al. for partial plating (6).

Mutation rate studies have been performed with a number of organisms related to antibiotic research, including *E. coli*, *S. pneumoniae* (10), *Pseudomonas aeruginosa* (27), and *M. tuberculosis* (3). Oliver et al. used the modification by Crane et al. of the Jones estimator to show that antibiotic-resistant isolates of *P. aeruginosa* were present prior to antibiotic therapy due to the existence of hypermutable bacteria (27). As large broths were used, aliquots from these broths were taken to reduce culture-to-culture variation (27). For example, Billington et al. also used this method for experiments with *M. tuberculosis* for similar reasons (3). Mutation rate experiments with *S. pneumoniae* have been used to show that mutations in the *gyrA* gene occur at a lower rate than *parC* mutations and that mutations in either gene predisposes to further mutation (10). The Drake method was used when ciprofloxacin was the selective antibiotic as the number of mutational events per culture was >30. However, the p_0 method was used with gemifloxacin as the number of mutational events was smaller (between 0.3 and 2.3).

SUMMARY

Whichever method is chosen, the experimental factors should be optimized to improve the precision and accuracy of the estimation. It is usually necessary to perform preliminary experiments to provide estimates of the mutation rate, and as has been stated previously, it is usually helpful to determine growth curves to confirm that the bacteria are in the same growth phase when the mutation rate estimation cultures are inoculated. The growth conditions of the experiment can only be established with the knowledge of the expected mutation rate, which requires preliminary experiments to enable the researcher to develop the necessary protocol. When mutations are likely to be rare, then m is, by definition, small and thus the p_0 method is likely to be the most useful. When m is greater, then median methods are most appropriate. The choice of calculation method will depend on whether all of the cultures were positive, with a median method being chosen for situations in which all are positive and the p_0 when this is not the case.

REFERENCES

1. **Andersson, D. I., J. Björkman, and D. Hughes.** 1998. Antibiotic resistance here to stay? Compensatory mutations restore virulence of resistant bacteria. Lakartidningen **95**:3940, 3943–3944.
2. **Asteris, G., and S. Sarkar.** 1996. Bayesian procedures for the estimation of mutation rates from fluctuation experiments. Genetics **142**:313–326.
3. **Billington, O. J., T. D. McHugh, and S. H. Gillespie.** 1999. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **43**:1866–1869.
4. **Björkman, J., D. Hughes, and D. I. Andersson.** 1998. Virulence of antibiotic-resistant *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **95**: 3949–3953.
5. **Cairns, J., J. Overbaugh, and S. Miller.** 1988. The origin of mutants. Nature **335**:142–145.
6. **Crane, G. J., S. M. Thomas, and M. E. Jones.** 1996. A modified Luria-Delbrück fluctuation assay for estimating and comparing mutation rates. Mutat. Res. **354**:171–182.
7. **Drake, J. W.** 1991. Constant rate of spontaneous mutations in DNA based microbes. Proc. Natl. Acad. Sci. USA 7160–7164.
8. **Gagneux, S., C. D. Long, P. M. Small, T. Van, G. K. Schoolnik, and B. J. Bohannan.** 2006. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science **312**:1944–1946.
9. **Gillespie, S. H., S. Basu, A. L. Dickens, D. M. O'Sullivan, and T. D. McHugh.** 2005. Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. J. Antimicrob. Chemother. **56**:344–348.
10. **Gillespie, S. H., L. L. Voelker, J. E. Ambler, C. Traini, and A. Dickens.** 2003. Fluoroquinolone resistance in *Streptococcus pneumoniae*: evidence that *gyrA* mutations arise at a lower rate and that mutation in *gyrA* or *parC* predisposes to further mutation. Microb. Drug Resist. **9**:17–24.
11. **Gillespie, S. H., L. L. Voelker, and A. Dickens.** 2002. Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae*. Microb. Drug Resist. **8**:79–84.
12. **Guiney, D. G., Jr.** 1984. Promiscuous transfer of drug resistance in gram-negative bacteria. J. Infect. Dis. **149**:320–329.
13. **Hughes, D., and D. I. Anderson.** 1997. Carbon starvation of *Salmonella typhimurium* does not cause a general increase of mutation rates. J. Bacteriol. **179**:6688–6691.
14. **Jones, M. E.** 1993. Accounting for plating efficiency when estimating spontaneous mutation rates. Mutat. Res. **292**:187–189.
15. **Kepler, T. B., and M. Oprea.** 2001. Improved inference of mutation rates. I. An integral representation for the Luria-Delbrück distribution. Theor. Popul. Biol. **59**:41–48.
16. **Kugelberg, E., S. Lofmark, B. Wretlind, and D. Anderssen.** 2005. Reduction of the fitness burden in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **55**:22–30.
17. **Lea, D., and C. Coulson.** 1949. The distribution of the number of mutants in bacterial populations. Genetics **49**:264–285.
18. **Loewe, L., V. Textor, and S. Scherer.** 2003. High deleterious genomic mutation rate in stationary phase of *Escherichia coli*. Science **302**:1558–1560.
19. **Luria, S., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
20. **Ma, W. T., G. V. Sandri, and S. Sarkar.** 1992. Analysis of the Luria-Delbrück distribution using discrete convolution powers. J. Appl. Probability **29**:255–267.
21. **Maiden, M. C.** 1998. Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. Clin. Infect. Dis. **27**(Suppl. 1):S12–S20.
22. **Mariam, D. H., Y. Mengistu, S. E. Hoffner, and D. I. Andersson.** 2004. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **48**:1289–1294.
23. **Miles, A. A., and S. S. Misra.** 1938. The estimation of the bactericidal power of the blood. J. Hyg. **38**:732–749.
24. **Nealson, K. H., and J. W. Hastings.** 1979. Bacterial bioluminescence: its control and ecological significance. Microb. Rev. **43**:496–518.
25. **Novick, A., and L. Szilard.** 1950. Experiments with the chemostat on spontaneous mutations of bacteria. Proc. Natl. Acad. Sci. USA **36**:708–719.
26. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405**:299–304.

27. **Oliver, A., B. R. Levin, C. Juan, F. Baquero, and J. Blasquez.** 2004. Hypermutation and the preexistence of antibiotic resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. Antimicrob. Agents Chemother. **48:**4226–4233.
28. **Parsek, M. R., and E. P. Greenberg.** 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA **97:**8789–8793.
29. **Rosche, W. A., and P. L. Foster.** 2000. Determining mutation rates in bacterial populations. Methods **20:**4–17.
30. **Shapiro, J. A.** 1997. Genome organisation, natural genetic engineering and adaptive mutation. Trends Genet. **13:**98–104.
31. **Thomas, C. M., and K. M. Nielsen.** 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. **3:** 711–721.

Measuring Bacterial Fitness

C.F. Pope[1], T.D. McHugh[1], J.R Pratten[2] and S.H. Gillespie[1]

[1]Centre for Medical Microbiology, Royal Free and University College Medical School, Rowland Hill Street, NW3 2QG and [2]UCL Eastman Dental Institute, Gray's Inn Road, London, WC1X 8LD

Acquisition of antibiotic resistance may or may not be associated with a physiological cost for the bacterium. Measurement of planktonic growth rate, by competitive growth assays between the resistant mutant and the susceptible parent, is a commonly used measure of fitness. However, fitness is a complex characteristic and multiple models are required to measure fitness costs, which may be small and difficult to quantify. Available in vitro models that can be used to quantify this fitness cost when compared to the susceptible parent include quantification of biofilm growth, survival in water, resistance to drying and alternative methods to determine planktonic growth rate. It is an accepted belief that decreased antibiotic use will reduce rates of resistance. However, some mutations conferring resistance result in a small or non existent fitness cost. These isolates may out compete the susceptible isolate and may remain in the bacterial population to form a pool of resistant organisms, which can rapidly proliferate if the selective antibiotic pressure is reapplied.

Introduction

Antibiotic resistance in a bacterial population occurs due to selection of resistant mutants in the presence of antibiotics. The existence of a continuing antibiotic selective pressure is responsible for high levels of antibiotic resistance. When bacteria are exposed to antibiotics a mutation conferring resistance to that antibiotic gives the bacterium an obvious advantage. However it is an accepted dogma that a resistant organism pays a physiological price for resistance, particularly resistance mediated by chromosomal mutations (Andersson & Levin 1999; Levin *et al.* 2000). It is an accepted belief that with rational use of antibiotics, resistant mutants will be out-competed by their susceptible counterparts and will be lost from the population. Although studies have shown that acquisition of antibiotic resistance can incur a biological cost (Andersson & Levin 1999; Gillespie & McHugh 1997) there is evidence that some mutations conferring resistance may result in a small or none existent fitness deficit (Gillespie 2001; Gillespie *et al.* 2002; Kugelberg *et al.* 2005). Furthermore, quantification of fitness costs is important when determining the stability of antibiotic resistance in a population.

MEASURING BACTERIAL FITNESS

Fitness and Antibiotic Resistance

Fitness is a complex characteristic that encompasses the ability of a genotype to reproduce within a host, be transmitted and be cleared. It is also a measure of how well bacteria survive in defined environments. The major factors that influence the frequency of antibiotic resistance in a population of bacteria are the extent of antibiotic use, the cost of resistance and the extent that the bacteria can compensate for this cost. Within a population of bacteria different genotypes must compete with each other to reproduce. Therefore, incidence of resistance can be reduced by the rational use of antibiotics as resistant bacteria can be selected against in the absence of antibiotics due to a fitness cost. However, resistance will not disappear from the population. If mutations conferring resistance have a low fitness cost, or no cost, then these mutants may remain at high levels in the bacterial population if antibiotic use is withdrawn or may return to high frequencies if antibiotic pressure is reintroduced.

Models of Fitness

In vitro models of fitness have been used in order to investigate the evolution of antibiotic resistance and to assess the physiological price associated with acquisition of resistance. The growth rate of a bacteria in culture medium is a commonly used model for evaluating fitness (Bennett *et al.* 1990; Lenski *et al.* 1998; Lenski, Simpson, & Nguyen 1994; Nguyen *et al.* 1989). Relative fitness is often determined by competition assay between isogenic antibiotic susceptible and antibiotic resistant bacteria in culture or in animal models. These models can be adapted for use in many bacterial species. Models should be chosen that reflect growth and environmental survival conditions of the bacterial species of interest. For example, a suitable biofilm model should be included for bacteria which are known to form biofilms within the human body e.g. *Pseudonomas aeruginosa* within the cystic fibrosis lung (Govan & Deretic 1996; Singh *et al.* 2000). Environmental survival e.g. resistance to drying or survival in water is relevant for nosocomial pathogens which can be transmitted via contaminated surfaces.

Fitness Costs Incurred by Antibiotic Resistance

The carriage of plasmids has been shown to reduce the fitness of bacteria (Lee & Edlin 1985; Nguyen *et al.* 1989; Warnes & Stephenson 1986). Insertion of a plasmid reduced fitness of the strain compared to the plasmid free strain. However, this fitness deficit was reduced following passage. Restoration of fitness may be due to loss of plasmid containing bacteria from the population as plasmid free bacteria outgrow them (Lenski & Bouma 1987). This would suggest that following rational antibiotic use the frequency of resistant bacteria may decline, reducing the spread of antibiotic resistance. Subsequently, it has been demonstrated that with time chromosomal changes occur that increase the fitness of the bacteria plasmid carrying bacteria (Lenski *et al.* 1994). Over many generations of association the effects of fitness can be decreased exten-

sively (Bouma & Lenski 1988; Modi *et al.* 1991).

Chromosomal mutations that confer resistance by altering antibiotic targets include DNA gyrase, RNA polymerase, the cell wall or the ribosome and these alterations may cause a reduction in fitness (Andersson & Levin 1999; Gillespie & McHugh 1997). Mutations in *rpsL* confer streptomycin resistance in *Salmonella* Typhimurium due to changes in the ribosomal protein S12. These mutants have been shown to be less fit than the wild type due to a decrease in peptide elongation rate and resulting decrease in slower protein synthesis and growth rate (Bjorkman *et al.* 1998). Chromosomal mutations in RNA polymerase (*rpoB*) that confer resistance to rifampicin are associated with a fitness cost in *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Moorman & Mandell 1981; Wichelhaus *et al.* 2002). The extent of this fitness cost depends on the resistance mutation.

In 1953 Barnett and colleagues showed that resistance to isoniazid in *M. tuberculosis* ameliorated disease a guinea pig model (Barnett, *et al.* 1953). Molecular tools have since shown that point mutations in *katG* confer this isoniazid resistance. Functional *katG*, integrated to the genome, restored virulence to wild type levels (Wilson *et al.* 1995). In the mouse model, resistant strains of *M. tuberculosis* vary in virulence (Ordway *et al.* 1995), however, increased levels of drug resistance were not associated with a reduction in virulence.

Compensation of Fitness Costs

A deleterious mutation may be lost from the population, revert to susceptibility or be compensated for by another mutation. Bacteria which are less fit may acquire compensatory mutations that restore reproductive potential. These are mutations that occur in another site which ameliorate the cost incurred by the initial resistance mutation without the loss of the resistance. These mutations can accumulate to restore fitness and stabilise the population of resistant bacteria.

Most compensatory mutations that restore fitness are not revertants to susceptibility. This may be because the mutation rate for other mutations is higher due to multiple targets. For example, compensation of fluoroquinolone resistance in *S. aureus* occurs by decreased expression of topoisomerase IV (Ince & Hooper 2003). It has also been demonstrated that the fitness cost of mutations in *rpsL*, conferring streptomycin resistance in *E. coli* can be compensated to a restored rate of protein synthesis following adaptation (Schrag *et al.* 1997). Similarly, adaptation experiments in *M. tuberculosis* have demonstrated that rifampicin resistant *rpoB* mutants lose the fitness deficit following serial passage (Billington *et al.* 1999).

Isoniazid resistant *M. tuberculosis* with mutation in *katG*, resulting in loss of a

functional catalase, accumulate compensatory mutations that result in increased expression of the *ahpC* promoter. The *ahpC* gene encodes an alkyl hydroperoxidase reductase and it has been proposed that these mutations increase the expression of this enzyme which protects *M. tuberculosis* from oxidative stress and compensates for the loss of catalase (Sherman *et al.* 1996).

Measuring Fitness

Defining fitness cost can be difficult due to variations in measurements in experimental procedures. No one method is likely to be sufficient in isolation and therefore multiple models are required. The models selected will depend on the organism, its natural lifestyle and its mode of growth. Fitness deficits will vary depending on the resistance mutation, the organism and the model used to quantify the cost. For example, Sanchez *et al.* assessed the fitness costs associated with overproduction of multidrug efflux pumps in *P. aeruginosa* using survival in water, maintenance on dry surfaces, biofilm formation, nematode killing, production of pyocyanin and pyoverdin and quantification of proteases (Sanchez *et al.* 2002). These mutants have been shown to have fitness costs in terms of resistance to desiccation, survival in water, loss of quorum sensing response and loss of virulence in the nematode killing model. However, the *nalB* mutant exhibited greater biofilm formation than the wild type (Sanchez *et al.* 2002). Hence, fitness costs may not be evident in all assays and the models chosen should reflect the how the organism causes disease in the host.

Fitness costs are measured in a number of ways and a variety of *in vitro* and animal models are available. These include comparison of growth rate in monocultures (Kugelberg *et al.* 2005). For example, we have used paired competition assays to assess fitness costs of fluoroquinolone resistance in *Streptococcus pneumoniae* (Gillespie *et al.* 2002) and *Burkholderia cepacia* and rifampicin resistance in *M. tuberculosis* (Billington *et al.* 1999; Davies *et al.* 2000). Relative fitness is defined by the difference in number of generations that have occurred between the susceptible parent and the resistant mutant. We have adapted the method of Youmans and Youmans (1949) to determine generation times in a semi automated liquid culture system for *B. cepacia* and *M. tuberculosis*, using the difference in time to positivity of diluted inoculums. These methods may minimise observed variation and allow fitness costs to be calculated in terms of generations.

Most studies investigating fitness costs use *in vitro* models while few have used *in vivo* models. These *in vivo* studies commonly use competitive colonisation to measure fitness (Johnson *et al.* 2005). For example, fitness of fluoroquinolone resistant *Campylobacter jejuni*, was assessed via colonisation and persistence in chickens in the absence of antibiotic selective pressure (Luo *et al.* 2005). Few studies have used human colonization or infection to measure fitness cost. Andersson *et al.* have assessed the fitness costs conferred by *parC* and *fusR*

mutations, conferring resistance to fluoroquinolones and fusidic acid respectively, in *Staphylococcus epidermidis* using a human competition mode (Gustafsson *et al.* 2003). Susceptible and resistant bacteria were inoculated onto human skin and relative numbers monitored. No loss of fitness associated with *parC* mutation was found. However *fusA* mutations resulted in a considerable loss of fitness as compared to the susceptible isogenic strain during competition. It is unrealistic to assume that *in vitro* assays, using biological rich media, will accurately reflect the fitness costs experienced by the pathogen during infection. Fitness deficits may be affected by growth conditions (Durso *et al.* 2004; Remold & Lenski 2001) and so use of a minimal medium may be more appropriate if *in vivo* models are not possible.

Biofilm Fitness Models

Biofilms have a role in many infectious diseases. For bacteria that grow as biofilms during infection a biofilm quantification assay should be included as a fitness assay. This is because the propensity to form biofilms is likely to affect fitness. There are numerous systems that have been developed and used to model the growth of bacterial biofilms. However, no single model is ideal for all experimental scenarios as each as been designed for a specific purpose. For use as a fitness assay the model of biofilm growth should be simple and reproducible with sufficient replicate biofilms to allow statistical analysis.

Growth within a biofilm can be measured by sacrificing cells from the biofilm by sonication and/or vortexing the biofilm before determining viable cell numbers estimated by plate counting, although using this method biofilm specific characteristics may be lost. Biofilm growth can also be visualised *in situ* with fluorescent probes and reporter genes (Geesey 2001) as well as using imaging software to estimate biofilm coverage of the surface. Bioluminescence is particurarly useful *in vitro* and *in vivo* as it allows biofilms to be monitored in real time (Kadurugamuwa *et al.* 2003a,b). Models of quantifying biofilm formation, in selected models, are shown in Table 1.

The crystal violet microtitre plate assay is a simple and rapid method that quantifies adherence of bacteria to the wells of a microtitre plate. It is especially useful as a fitness assay as replicate biofilms can be grown in large numbers. The use of a robot can increase reproducibility and partly automate the procedure. Bacteria are grown in wells of a microtitre plate containing a suitable medium. Wells are washed to remove planktonic cells and incubated with crystal violet. Unbound crystal violet is removed by repeated washing with water. Ethanol is added to release bound crystal violet and biofilm formation is quantified by determination of the absorbance of the solution at 590 nm. This assay has been used to study biofilm formation in a number of bacteria including *Escherichia coli* (Pratt & Kolter 1998), *B. cepacia* complex (BCC) bacteria (Conway *et al.* 2002), *Pseudomonas fluorescens* (O'Toole & Kolter 1998b), *P. aeruginosa* (O'Toole & Kolter 1998a), *Vibrio cholerae* (Watnick & Kolter 1999)

and *Streptococcus gordonii* (Loo *et al.* 2000) and has been used as a fitness assay to quantify the physiological cost of antibiotic resistance in *P. aeruginosa* (Kugelberg *et al.* 2005) and *B. cepacia.*

Table 1 Models that have been used to quantify bacterial biofilm growth

Model	Organisms	Flow	Substratum	Method of quantifying biofilm	Reference
Constant Depth Film fermenter (CDFF)	*B.cepacia, P.aeruginosa,* oral bacteria	Continuous	Variable	Vortex plug, viable count. Can be observed directly by SEM and CLSM	(Hengtrakool, Pearson, & Wilson 2006; Hope & Wilson 2006; Peters & Wimpenny 1987)
CDC Biofilm Reactor	Gram negative bacteria	Continuous	Plastic connectors	Sonicate, vortex, homogenise, viable count	(Murga *et al.* 2001)
Modified Robbins device	*B. pseudomallei, P. aeruginosa*	Batch	Variable	Viable count	(Honraet & Nelis 2006; Mikuniya *et al.* 2005;Vorachit *et al.* 1993)
Calgary biofilm device	*P. aeruginosa, S. aureus, E. coli*	Batch	Plastic pegs	Sonicate peg, viable count	(Ceri *et al.* 1999)
Sorborads Filter	*S. aureus, P. aeruginosa*	Continuous	Filter paper	Vortex, viable count	(Hodgson *et al.* 1995)

Use of Fitness Models to assess Fitness Costs Associated with Fluoroquinolone Resistance Mutations

Fluoroquinolones (FQs) inhibit two homologous enzymes, DNA gyrase and topoisomerase IV which consist of two subunits, gyrase is encoded by *gyrA* and *gyrB* and topoisomerase IV by *parC* and *pare*, respectively. Target alteration, together with efflux and reduced permeability are the primary mechanisms that confer resistance to FQs in Gram-negative bacteria (Ince & Hooper 2003).. Resistance develops via the stepwise accumulation of mutations in the Quinolone Resistance Determining Regions (QRDR) of topoisomerase genes, increasing the level of resistance with each successive mutation (Everett *et al.* 1996). The cost of mutations conferring fluoroquinolone mutations has been investigated in a number of organisms including *E. coli* (Bagel *et al.* 1999), *S. pneumoniae* (Gillespie *et al.* 2002), *S. typhimurium* (Giraud *et al.* 2003) and *P. aeruginosa* (Kugelberg *et al.* 2005). The fitness cost varies in these organisms and depends on the resistance mutations. Multiple mutations associated with high levels of resistance exhibit reduced fitness.

Conclusion

In a culture, a non fatal deleterious mutation is more frequent than the occurrence of favourable mutations leading to an increase in fitness. Therefore an accumulation of deleterious mutations will occur, a decline in fitness will be observed and the fittest individuals can eventually be lost from the population. This has been referred to as 'Muller's ratchet'(Muller 1964). Andersson and Hughes showed that Muller's ratchet also operates in *Salmonella typhimurium*

(Andersson & Hughes 1996).

A number of fitness models are available that can be used as tools to assess the cost of acquiring antibiotic resistance. The context of a fitness model is important and models should be chosen to represent how the organism survives in the environment and causes disease and therefore multiple models may be required.

Few studies have investigated the effect of reduction in antibiotic use and subsequent levels of resistance in bacterial populations. Austin *et al.* (1999) attempted to quantify the relationship between antibiotic use and frequency of resistance. Their findings suggested that significant reduction in antibiotic use is required to cause a significant decline in resistance. This decline in resistance is likely to occur at a lower rate than the initial emergence of the resistance (Austin *et al.* 1999). Reduction in macrolide use within outpatients in Finland during the 1990s resulted in a decline in erythromycin resistance in Group A streptococci isolated from throat swabs and pus samples (Seppala *et al.* 1997). Isolates containing no cost mutations may not be outcompeted by their susceptible counterparts and may remain in the population to form a pool of resistant organisms. Therefore rational use of antibiotics, in isolation, may not be adequate to reverse the continuing rise in antibiotic resistance.

References

Andersson, D. I. and Hughes, D. (1996) Muller's ratchet decreases fitness of a DNA-based microbe. *Proceedings of the National Academy of Sciences* **93**, 906-907.

Andersson, D. I. and Levin, B. R. (1999) The biological cost of antibiotic resistance. *Current Opinions in Microbiology* **2**, 489-493.

Austin, D. J., Kristinsson, K. G. and Anderson, R. M. (1999) The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proceedings of the National Academy of Sciences* **96**, 1152-1156.

Bagel, S., Hullen, V., Wiedemann, B. and Heisig, P. (1999) Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **43**, 868-875.

Barnett, M., Busby, S. R. and Mitchison, D. A. (1953) Tubercle bacilli res to isoniazid: virulence and response to treatment with isoniazid in guinea pigs. *British Journal of Experimental Pathology* **34**, 568-581.

Bennett, A. F., Dao, K. M. and Lenski, R. E. (1990) Rapid evolution in response to high-temperature selection. *Nature* **346**, 79-81.

Billington, O. J., McHugh, T. D. and Gillespie, S. H. (1999) Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* **43**, 1866-1869.

Bjorkman, J., Hughes, D. and Andersson, D. I. (1998) Virulence of antibiotic-resistant *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences* **95**, 3949-3953.

Bouma, J. E. and Lenski, R. E. (1988) Evolution of a bacteria/plasmid association. *Nature* **335**, 351-352.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. and Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology* **37**, 1771-1776.

Conway, B. A., Venu, V. and Speert, D. P. (2002) Biofilm formation and acyl homoserine lactone

production in the *Burkholderia cepacia* complex. *Journal of Bacteriology* **184**, 5678-5685.
Davies, A. P., Billington, O. J., Bannister, B. A., Weir, W. R., McHugh, T. D. and Gillespie, S. H. (2000) Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment. *The Journal of Infection* **41**, 184-187.
Durso, L. M., Smith, D. and Hutkins, R. W. (2004) Measurements of fitness and competition in commensal *Escherichia coli* and *E. coli* O157:H7 strains. *Applied and Environmental Microbiology* **70**, 6466-6472.
Everett, M. J., Jin, Y. F., Ricci, V. and Piddock, L. J. (1996) Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrobial Agents and Chemotherapy* **40**, 2380-2386.
Geesey, G. G. (2001) Bacterial behavior at surfaces. *Current Opinions in Microbiology* **4**, 296-300.
Gillespie, S. H. (2001) Antibiotic resistance in the absence of selective pressure. *International Journal of Antimicrobial Agents* **17**, 171-176.
Gillespie, S. H. and McHugh, T. D. (1997) The biological cost of antimicrobial resistance. *Trends in Microbiology* **5**, 337-339.
Gillespie, S. H., Voelker, L. L. and Dickens, A. (2002) Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae*. *Microbial Drug Resistance* **8**, 79-84.
Giraud, E., Cloeckaert, A., Baucheron, S., Mouline, C. and Chaslus-Dancla, E. (2003) Fitness cost of fluoroquinolone resistance in Salmonella enterica serovar Typhimurium. *Journal of Medical Microbiology* **52**, 697-703.
Govan, J. R. and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiology Reviews* **60**, 539-574.
Gustafsson, I., Cars, O. and Andersson, D. I. (2003) Fitness of antibiotic resistant *Staphylococcus epidermidis* assessed by competition on the skin of human volunteers. *Journal of Antimicrobial Chemotherapy* **52**, 258-263.
Hengtrakool, C., Pearson, G. J. and Wilson, M. (2006) Interaction between GIC and *S. sanguis* biofilms: antibacterial properties and changes of surface hardness. *Journal of Dentistry* **34**, 588-597.
Hodgson, A. E., Nelson, S. M., Brown, M. R. and Gilbert, P. (1995) A simple in vitro model for growth control of bacterial biofilms. *Journal of Applied Bacteriology* **79**, 87-93.
Honraet, K. and Nelis, H. J. (2006) Use of the modified robbins device and fluorescent staining to screen plant extracts for the inhibition of *S. mutans* biofilm formation. *Journal of Microbiology Methods* **64**, 217-224.
Hope, C. K. and Wilson, M. (2006) Biofilm structure and cell vitality in a laboratory model of subgingival plaque. *Journal of Microbiology Methods* **66**, 390-398.
Ince, D. and Hooper, D. C. (2003) Quinolone resistance due to reduced target enzyme expression. *Journal of Bacteriology* **185**, 6883-6892.
Johnson, C. N., Briles, D. E., Benjamin, W. H., Jr., Hollingshead, S. K. and Waites, K. B. (2005) Relative fitness of fluoroquinolone-resistant *Streptococcus pneumoniae*. *Emerging Infectious Disease* **11**, 814-820.
Kadurugamuwa, J. L., Sin, L., Albert, E., Yu, J., Francis, K., DeBoer, M., Rubin, M., Bellinger-Kawahara, C., Parr, J. T., Jr. and Contag, P. R. (2003a) Direct continuous method for monitoring biofilm infection in a mouse model. *Infection and Immunity* **71**, 882-890.
Kadurugamuwa, J. L., Sin, L. V., Yu, J., Francis, K. P., Kimura, R., Purchio, T. and Contag, P. R. (2003b) Rapid direct method for monitoring antibiotics in a mouse model of bacterial biofilm infection. *Antimicrobial Agents and Chemotherapy* **47**, 3130-3137.
Kugelberg, E., Lofmark, S., Wretlind, B. and Andersson, D. I. (2005) Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **55**, 22-30.
Lee, S. W. and Edlin, G. (1985) Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. *Gene* **39**, 173-180.
Lenski, R. E. and Bouma, J. E. (1987) Effects of segregation and selection on instability of plasmid pACYC184 in *Escherichia coli*. *Journal of Bacteriology* **169**, 5314-5316.
Lenski, R. E., Mongold, J. A., Sniegowski, P. D., Travisano, M., Vasi, F., Gerrish, P. J. and Schmidt, T. M. (1998) Evolution of competitive fitness in experimental populations of *E. coli*: what makes one genotype a better competitor than another? *Antonie Van Leeuwenhoek* **73**, 35-47.

Lenski, R. E., Simpson, S. C. and Nguyen, T. T. (1994) Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *Journal of Bacteriology* **176**, 3140-3147.

Levin, B. R., Perrot, V. and Walker, N. (2000) Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**, 985-997.

Loo, C. Y., Corliss, D. A. and Ganeshkumar, N. (2000) *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *Journal of Bacteriology* **182**, 1374-1382.

Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L. and Zhang, Q. (2005) Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proceedings of the National Academy of Sciences* **102**, 541-546.

Mikuniya, T., Kato, Y., Kariyama, R., Monden, K., Hikida, M. and Kumon, H. (2005) Synergistic effect of fosfomycin and fluoroquinolones against *Pseudomonas aeruginosa* growing in a biofilm. *Acta Medica Okayama* **59**, 209-216.

Modi, R. I., Wilke, C. M., Rosenzweig, R. F. and Adams, J. (1991) Plasmid macro-evolution: selection of deletions during adaptation in a nutrient-limited environment. *Genetica* **84**, 195-202.

Moorman, D. R. and Mandell, G. L. (1981) Characteristics of rifampin-resistant variants obtained from clinical isolates of *Staphylococcus aureus*. *Antimicrobial Agents Chemotherapy* **20**, 709-713.

Muller, H. J. (1964) The relation of recombination to mutational advance. *Mutation Research* **1**, 2-9.

Murga, R., Miller, J. M. and Donlan, R. M. (2001) Biofilm formation by gram-negative bacteria on central venous catheter connectors: effect of conditioning films in a laboratory model. *Journal of Clinical Microbiology* **39**, 2294-2297.

Nguyen, T. N., Phan, Q. G., Duong, L. P., Bertrand, K. P. and Lenski, R. E. (1989) Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Molecular Biology and Evolution* **6**, 213-225.

O'Toole, G. A. and Kolter, R. (1998a) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology* **30**, 295-304.

O'Toole, G. A. and Kolter, R. (1998b) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology* **28**, 449-461.

Ordway, D. J., Sonnenberg, M. G., Donahue, S. A., Belisle, J. T. and Orme, I. M. (1995) Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice. *Infection and Immunity* **63**, 741-743.

Peters, A. and Wimpenny, J. (1987) A constant depth laboratory model film fermenter. *Biotechnology and Bioengineering* **32**, 263-270.

Pratt, L. A. and Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology* **30**, 285-293.

Remold, S. K. and Lenski, R. E. (2001) Contribution of individual random mutations to genotype-by-environment interactions in *Escherichia coli*. *Proceedings of the National Academy of Sciences* **98**, 11388-11393.

Sanchez, P., Linares, J. F., Ruiz-Diez, B., Campanario, E., Navas, A., Baquero, F. and Martinez, J. L. (2002) Fitness of in vitro selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants. *Journal of Antimicrobial Chemotherapy* **50**, 657-664.

Schrag, S. J., Perrot, V. and Levin, B. R. (1997) Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. *Proceedings Biological Sciences* **264**, 1287-1291.

Seppala, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K. and Huovinen, P. (1997) The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A *streptococci* in Finland. Finnish Study Group for Antimicrobial Resistance. *New England Journal of Medicine* **337**, 441-446.

Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E. and Stover, C. K. (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* **272**, 1641-1643.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. and Greenberg, E. P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**, 762-764.

Vorachit, M., Lam, K., Jayanetra, P. and Costerton, J. W. (1993) Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole.

Antimicrobial Agents Chemotherapy **37**, 2000-2002.

Warnes, A. and Stephenson, J. R. (1986) The insertion of large pieces of foreign genetic material reduces the stability of bacterial plasmids. *Plasmid* **16**, 116-123.

Watnick, P. I. and Kolter, R. (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology* **34**, 586-595.

Wichelhaus, T. A., Boddinghaus, B., Besier, S., Schafer, V., Brade, V. and Ludwig, A. (2002) Biological cost of rifampin resistance from the perspective of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **46**, 3381-3385.

Wilson, T. M., de Lisle, G. W. and Collins, D. M. 1995, "Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Molecular Microbiology* **15**, 1009-1015.

Youmans, G. P. and Youmans, A. S. (1949) A Method for the determination of the rate of growth of tubercle bacilli by the use of small inocula. *Journal of Bacteriology* 58, 247-255.

Rapid methods to determine fitness in bacteria using automated culture systems

Cassie Pope, Timothy McHugh and Stephen Gillespie

Abstract

Determination of growth rate and generation time is often used to measure fitness costs associated with antibiotic resistance. However the use of growth curve techniques to determine generation time is laborious, time consuming and can introduce sampling error. We have described the use of a semi automated liquid culture system to estimate generation time in *Burkholderia cepacia* complex bacteria. We have also used the BacT/ALERT system to determine generation time and enumerate bacterial numbers in *Mycobacterium tuberculosis*. These methods, therefore, can be adapted for use with other organisms. This chapter outlines a method to use the Bactec 9000 series of instruments to measure generation time.

Introduction

It is an accepted dogma that a resistant organism pays a physiological price for resistance, particularly resistance mediated by chromosomal mutations ***(1,2)***. Although studies have shown that acquisition of antibiotic resistance can incur a biological cost ***(1,3)*** there is evidence that some mutations conferring resistance may result in a low or no existent fitness deficit ***(4-6)***. *In vitro* models of fitness have been used in order to investigate the evolution of antibiotic resistance and to assess the physiological price associated with acquisition of resistance. The growth rate of bacteria in culture medium is a commonly used model for evaluating fitness as a measure of reproductive potential ***(7-10)***. However fitness is a complex characteristic that encompasses the ability of a genotype to reproduce

within a host, be transmitted and be cleared. Multiple models should therefore be chosen carefully that represent the mode of life of the organism in question. We have used competitive pair wise cultures, growth rate, biofilm formation and environmental survival models to assess the fitness cost of acquisition of fluoroquinolone resistance in *Burkholderia cepacia* ***(11)***. Relative fitness is often determined by competition assay between isogenic antibiotic susceptible and antibiotic resistant bacteria in culture or in animal models. These models can be adapted for use in many bacterial species.

Viable cell count estimation is subjective and dependent on enumeration of colonies that grow under growth conditions provided, introducing sampling error. By using an automated system which is less time consuming and not user dependent, it is possible to determine growth rate for large numbers of strains. Youmans and Youmans used the difference in time to positivity, measured as time to a certain turbidity, of small inoculums of *M. tuberculosis* to determine generation time ***(12)***. We have adapted this method to determine generation times in a semi automated liquid culture system for *B. cepacia* and *M. tuberculosis*, using the difference in time to positivity of diluted inoculums. These methods may minimise observed variation and allow fitness costs to be calculated in terms of generations. Laurent *et al* have also used an automated liquid culture system (MS2 Research System, Abbott Laboratories, Dallas, Tx, USA) and paired competitive cultures to determine growth rate, as a measure of fitness in MRSA ***(13)***. This chapter describes use of a semi automated liquid culture system for measurement of generation time in *Burkholderia cepacia.*

We have used the Bactec 9240 continuous, blood culture system with standard aerobic medium was used to determine growth rate in *B. cepacia.* The Bactec 9000

(9240/9120/9050) series of automated blood culture systems are used to rapidly detect viable microorganisms in clinical specimens and is used in most clinical laboratories with in the UK. Bactec Plus Aerobic vials, contain 25 mL of enriched soybean-caesin digest broth, 0.05% sodium polyanetholesulfonate (SPS), resins, CO_2, O_2 and a sensor. This sensor, within each vial, responds to changes in oxygen and carbon dioxide levels as a result of bacterial metabolism. These changes are measured by an increase in the fluorescence of the sensor which is monitored every ten minutes. A positive fluorescence reading indicates an increase in CO_2 or decrease in oxygen and the presence of microorganisms in that vial.

Materials

1. Test organisms
2. Incubator
3. Muller Hinton Broth
4. Orbital incubator
5. Phosphate buffer saline (PBS)
6. Spectrophotometer
7. 0.5 mL syringe
8. 0.5 mm guage needle
9. Bactec automated blood culture system (Bactec 9000 series)
10. Steret alcohol wipes
11. Aerobic Bactec bottles
12. Blood agar plates
13. Microscope
14. Gram stain reagents
15. Glass slides

Methods

1. Grow *B. cepacia* on Columbia blood agar plates at 37°C for 18 hours
2. Using a sterile loop inoculate one colony of *B. cepacia* in Muller Hinton broth (5mL) and incubate using an orbital incubator (200rpm) for 4 hours to obtain an exponentially growing culture. Dilute culture to standard optical density using PBS.
3. Dilute culture via serial dilution (10^{-1}-10^{-6}) in PBS.
4. Aseptically inoculate triplicate Bactec bottles with 0.5 mL of the 10^{-2} and 10^{-4} dilutions using steret alcohol wipe, needle and syringe.
5. Invert bottles to mix.
6. Load into system.
7. Incubate until bottles flag as positive.

8. Print growth curve and time to positivity.
9. Remove bottles and discard.
10. Confirm absence of contaminants by Gram stain and spreading of one drop of bottle content onto Columbia blood agar.

The growth rate constant and generation time can be determined by the following equations

$$K = \frac{\log a - \log b}{t}$$

$$G = \frac{\log 2}{K}$$

Where K is the growth rate constant, a is the largest inoculum (1:10), b is the smallest inoculum (1:1000), t is the difference in time (h) taken for each of the sets of bottles to signal positive and G is the generation time.

Notes

1. The Bactec 9000 series of instruments are used by most clinical microbiology laboratories and are therefore available for translational research following discussion with the service manager and chief Biomedical Scientists.
2. Bactec bottles should be allowed to equilibrate at room temperature before inoculation.
3. Bottles should be left in the system until time to positivity data can be printed, as the system only stores data temporarily. If with discussion, the Biomedical Scientist wishes to remove bottles immediately after flagging as positive then they should print the growth plot at the same time.
4. Bottles may flag as positive overnight therefore ensure that the on call Biomedical Scientist is aware of the experiment and understands the importance of printing the growth plot or leaving bottles in the system.
5. Print out of growth curve can be integrated into clinical service if required.
6. Bottles must to be booked into the laboratory computer system because otherwise sample data will not be recorded.
7. Bottle bar code stickers must be retained in order to identify bottles.
8. Any bottles that do not become positive must be removed from the system.
9. Cells must be added to bottles while in exponential phase to limit the effect of lag time differences on time to positivity. An initial growth curve experiment should be performed to determine incubation conditions required for bacterial cells to be in exponential phase
10. This method can be adapted for other fast growing bacteria. We have also used the BacT/ALERT system to determine generation time in *M. tuberculosis*.

References

1. Andersson, D. I, and Levin, B. R. (1999) The biological cost of antibiotic resistance. *Curr Opin Microbiol*, **2**, 489-493.

2. Levin, B.R., Perrot, V., and Walker, N. (1999) Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics, **154**, 985-997.

3. Gillespie, S. H., McHugh, T. D. (1997) The biological cost of antimicrobial resistance. *Trends Microbiol*, **5**, 337-339.

4. Kugelberg, E., Lofmark, S., Wretlind, B., Andersson, D. I. (2005) Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa J Antimicrob Chemother*, **55**, 22-30.

5. Gillespie, S. H, Voelker, L. L, Dickens, A.(2002) Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae. Microb Drug Resist,* **8,** 79-84.

6. Gillespie, S. H. (2001) Antibiotic resistance in the absence of selective pressure. *Int J Antimicrob Agents*, **17**, 171-176.

7. Bennett, A. F, Dao, K. M, Lenski, R. E. (1990) Rapid evolution in response to high-temperature selection *Nature*, **346**, 79-81.

8. Lenski, R.E., Simpson, S.C. and Nguyen, T. T. (1994) Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *J Bacteriol* ,**176**, 3140-3147.

9. Lenski, R. E, Mongold, J. A, Sniegowski, P. D, Travisano, M., Vasi, F., Gerrish, P. J. and Schmidt, T. (1998) Evolution of competitive fitness in experimental populations of *E. coli*: what makes one genotype a better competitor than another? *Antonie Van Leeuwenhoek*, **73**, 35-47.

10. Nguyen, T. N., Phan Q. G., Duong L. P., Bertrand K. P. and Lenski R. E. (1989) Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Mol Biol Evol* **6**, 213-225.

11. Pope, C. F., Gillespie, S. H., Pratten, J. R. & McHugh, T. D. 2007, Investigation on Fluoroquinolone- Resistant mutants of *Burkholderia cepacia Antimicrob. Agent Chemother* **52**, 1203-3.

12. Youmans, G. P. and Youmans, A. S. (1949) A method for the determination of the rate of growth of tubercle bacilli by the use of small inocula. *J Bacteriol* **58**, 247-255.

13. Laurent, F., Lelievre, H., Cornu, M., Vandenesch, F., Carret, G., Etienne, J., and Flandrois, J. P. (2001) Fitness and competitive growth advantage of new gentamicin-susceptible MRSA clones spreading in French hospitals. *J.Antimicrob Chemother* **47**, 277-283.