A study of clinical strains of *Pseudomonas aeruginosa* and the investigation of antibiotic resistance mechanisms in the multidrug resistant strain PA13

A thesis submitted to Dublin City University in fulfilment of the requirements for the award of the degree of Doctor of Philosophy

by

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November 2007

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"*Everything that exists in the universe is the result of chance and necessity*"

(Democritus, 460-370 BC)

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Abstract

A study of clinical strains of *Pseudomonas aeruginosa* and the investigation of antibiotic resistance mechanisms in the multidrug resistant strain PA13

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Thirteen clinical strains of bacteria from two Irish hospitals were identified as *Pseudomonas aeruginosa* using classical methods, API 20NE and Biolog GN. Their identification was confirmed by 16S rRNA gene sequencing. The antibiotic resistance profiles of the isolates were determined against forty-one antibiotics belonging to eleven distinct classes. All the isolates were resistant to penicillin G, ampicillin, cephalothin, cloxacillin, oxacillin, amoxicillin, cefotaxime, moxalactam, sulphatriad cotrimoxazole, chloramphenicol and tetracycline. All were sensitive to ceftazidime, piperacillin-tazobactam, cefepime, ceftriaxone, meropenem, aztreonam, amikacin, apramycin, butirosin A, lividomycin and colistin sulphate. One of the isolates, PA13, was resistant to a further fourteen antibiotics and was identified as a multidrug resistant strain. A 2.2 kbp PCR product was amplified from *P. aeruginosa* PA13. When this product was sequenced it was found to contain four open reading frames. BLASTN analysis identified these as being an integrase gene (ORF1), an aminoglycoside acetyltransferase gene, *aac(6')-Ib* (ORF2), an oxacillinase gene (ORF3) and a quaternary ammonium compound resistance gene (ORF4). The presence of the integrase gene and the quaternary ammonium compound gene suggested that the genes were on a Class 1 integron. The acetyltransferase *aac(6')-Ib* gene contained the mutant type of the enzyme with a leucine substitution by serine at position 119. Two expression vectors were chosen to investigate the novel oxacillinase gene. One was a commercially available vector, pET-28a (Novagen) and the other was an in-house vector, pPC. The gene was successfully cloned into both vectors. Following induction the desired protein was not expressed in either the soluble or insoluble fractions.

List of Abbreviations

A = absorbance

AAC = acetyltransferase

AANAT = arylalkylamine *N*-acetyltransferase

ABC = ATP-binding cassette

AcCoA = acetyl coenzyme A

AIDS = acquired immune deficiency syndrome

Ala = alanine

AME = aminoglycoside modifying enzyme

amp = ampicillin

ANT = adenyltransferase

APH = phosphotransferase

API = analytical profile index

Arg = arginine

argU = arginine tRNA gene

argW = arginine tRNA gene

ARP = antibiotic resistance profile

Asp = aspartic acid

Asn = asparagine

ATCC = American Type Culture Collection

ATP = adenosine triphosphate

$BaCl_2$ = barium chloride

$BaSO_4$ = barium sulphate

bla-$_{OXA}$ = β-lactamase (oxacillinase)

be = base element

bp = base pair

BSAC = British Society for Antimicrobial Chemotherapy

Ca^{2+} = calcium

$CaCl_2$ = calcium chloride

CA-SFM = Comite de l'Antibiogramme de la Societe Francaise de Microbiologie

CF = cystic fibrosis

CLSI = Clinical and Laboratory Standards Institute

Cm = chloramphenicol

CO_2 = carbon dioxide

Co-A = coenzyme A

CFU = colony forming units

CRG = Commissie Richtlijenen Gevoeligneids Depalingen

CS = conserved segment

Da = dalton

D-Ala = D-alanine

dATP = deoxyadenosine triphosphate

dcm = DNA cytosine methylase mutation

dCTP = deoxycytidine triphosphate

dGTP = deoxyguanosine triphosphate

dH_2O = distilled water

DHF = dihydrofolic acid

DHFR = dihydrofolate reductase

DIN = German Institute for Standardisation

DNA = deoxyriboucleic acid

dNTP = deoxynucleotide triphosphate

Dsb = disulphide bond forming protein

dTTP = deoxythymidine triphosphate

EDTA = ethylenediaminetetraacetic acid

EIP =energy-independent

EDP = energy-dependent

end = DNA-specific endonuclease 1 mutation

F' = Host contains an F' episome

Fe = iron

g = gram

gal = block catabolism of galactose

Glu = glutamic acid

Gly = glycine

glyT = glycine tRNA gene

GNAT = GCN5-related *N*-acetyltransferases

GN-NENT = Gram-negative non-enteric

GTP = guanosine triphosphate

gyrA = DNA gyrase mutation

H_2O = water

H_2SO_4 = sulphuric acid

HAT = histone *N*-acetyltransferase

HCl = hydrochloric acid

hsdR = host DNA restriction and methylation system mutation

hsdS = mutation of specific determinant for host DNA restriction and methylation system

Ile = isoleucine

ileX = isoleucine tRNA gene

Int = integrase

IPTG = Isopropyl-β-D-thiogalactopyranoside

K_2HPO_4 = potassium phosphate

kan = kanamycin

kbp = kilo base pairs

kDa = kilodaltons

kg = kilogram

KOH = potassium hydroxide

L = litre

$LacI^q$ = overproduction of the lac repressor protein

Leu = leucine

leuW = leucine tRNA gene

LPS = lipospolysaccharide

Lys = lysine

M = molar

mA = milliamp

Mbp = mega base pairs

MCS = multiple cloning site

ml = millilitre

mRNA = messenger ribonucleic acid

Mg^{2+} = magnesium

MFS = major facilitator subfamily

MIC = minimum inhibitory concentration

MOPS = 3-(*N*-morpholino) propanesulfonic acid

MRSA = methicillin resistant *staphylococcus aureus*

NaCl = sodium salt

NADH = nicotinamide adenine dinucleotide

NADPH = nicotinamide adenine dinucleotide phosphate

Na_2-EDTA = disodium ethylenediaminetetraacetic acid

NAG = N-acetylglucosamine

NAM = N-acetylmuramic acid

NaOH = sodium hydroxide

NO_3 = nitrate

NWGA = Norwegian Working Group on Antibiotics

O_2 = oxygen

OD = optical density

omp = mutation of an outer membrane protein

ORF = open reading frame

OXA = oxacillinase

pABA = p-aminobenzoic acid

PAGE = polyacrylamide gel electrophoresis

PBP = penicillin binding protein

PCR = polymerase chain reaction

PEP = phosphoenolpyruvate

Phe = phenylalanine

PIA = *Pseudomonas* isolation agar

Pro = proline

proAB = mutations in proline metabolism

proL = proline tRNA gene

QAC = quaternary ammonium compound

ram = ribosomal ambiguity

RBS = ribosome binding site

recA = mutation in recombination

relA = ppGpp synthase I mutation

RPM = revolutions per minute

RND = resistance-nodulation-cell division

rRNA = ribosomal ribonuleic acid

SDS = sodium dodecyl sulphate

Sec = secretory chaperone

Ser = serine

Skp = generic secretory chaperone

SMR = small multidrug regulator

spp. = species

SRGA = Swedish Reference Group for Antibiotics

SRP = signal recognition pathway

sul = sulphonamide

sup = suppressor mutation

TEMED = N,N,N,N-Tetramethyl-ethylenediamine

Tet = tetracycline

THF = tetrahydrofolic acid

thi1 = mutation in thiamine metabolism

Trp = tryptophan

Tyr = tyrosine

U = uridine

UDP = uridine diphosphate

Und-P = undecaprenyl phosphate

Und-P-P = undecaprenyl pyrophosphate

UV = ultraviolet

tRNA = transfer ribonucleic acid

V = volt

Val = valine

VRE = vancomycin resistant enterococci

WHO = World Health Organisation

Zn = zinc

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1.0 Introduction

1.1 Pseudomonads

The genus *Pseudomonas* belongs to the bacterial family *Pseudomonadaceae* which also contains the genera *Azomonas*, *Azotobacter*, *Cellvibrio*, *Mesophilobacter*, *Rhizobacter* and *Rugamonas*. These bacteria are common inhabitants of soil and water. The term Pseudomonad is used to describe strictly aerobic Gram-negative, non-sporulating bacteria. They are oxidase positive or negative, catalase positive, non-acid fast rods, which are generally straight, but maybe slightly curved, 0.5 – 1 μm in diameter and 1.5 – 5 μm in length. These bacteria are generally motile, with polar flagella and do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic. Most species fail to grow in acidic conditions (pH 4.5 or lower) and do not require organic growth factors. The optimum growth temperature for most strains is 28°C, but many are capable of growth between 4- 45°C (Bergey's Manual of Systematic Bacteriology, 2001).

Members of the genus *Pseudomonas* are free-living organisms and occupy a dominant position in the biosphere in terms of variety of habitats and the number of species in a given habitat (Todar, 2004). One of the most striking properties of the members of this genus is their remarkable nutritional versatility. They play an important role in decomposition, biodegradation and the carbon and nitrogen cycles. Organic compounds such as alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and hydrocarbons are all readily used by *Pseudomonas* species as growth substrates. In fact the only organic compounds that cannot be attacked by the Pseudomonads are teflon, styrofoam and one-carbon organic compounds such as methane, methanol, formaldehyde etc. (Todar, 2004).

The biological identity of the genus *Pseudomonas* has changed dramatically in recent years during the transition between artificial classification based on phenotypic properties and revisionist classification based on genotypic properties (Todar, 2004). In the past, *Pseudomonas* species were subdivided on the basis of rRNA homology into five similarity groups (Palleroni, 1986). There were about forty species. More

recently only members of Group I were held in the genus *Pseudomonas*. Group I is the largest group, including fluorescent strains such as *P. aeruginosa*, *P. fluorescens* and *P. putida* and the plant pathogens *P. syringae* and *P. cichorii*. It also includes many important nonfluorescent species such as *P. stutzeri* and *P. mendocina*. The members of groups II, III, IV, V were moved into new or previously existing genera such as *Burkholderia*, *Xanthomonas* and *Comamonas* based on 16S rRNA gene analysis (Bergey's Manual of Systematic Bacteriology, 2001).

The use of 16S rRNA gene sequene in the classification of bacterial species has now been well established (García-Martínez *et al.*, 2001). It is the part of the DNA now most commonly used for taxonomic purposes for bacteria (Harmsen and Karch, 2004). This gene is present in all bacteria and therefore can be used to measure relationships between them. This gene can be compared not only to other bacteria but also with archaeobacteria and the 18S rRNA gene of eukaryotes function (Clarridge III, 2004). Its degree of conservation is believed to result from the importance of the 16S rRNA as a critical component of cell. Other genes, such as those that make enzymes, can tolerate more frequent mutations because they may affect structures not as unique and essential as rRNA. Therefore, very few genes are as highly conserved as the 16S rRNA gene. Although the absolute rate of change in 16S rRNA is not known, it does mark the evolutionary distance and relatedness of organisms (Clarridge III, 2004).

The ribosome is an organelle in cells that assembles proteins. It is composed of both ribosomal RNA and ribosomal proteins, known as the ribonucleoprotein. Ribosomes can be found floating freely in the cytoplasm or bound to the endoplasmic reticulum or the nuclear envelope and are usually found in large number in cells. Ribosomal RNA (rRNA) is the major proportion of cellular RNA and makes up about 65% of the bacterial ribosome (Rodnina *et al*., 2007).

Ribosomal RNAs are at present the most useful and most used of the molecular chronometers (Clarridge III, 2004). Since ribosomes are an essential component of protein synthesis apparatus and the structures are strictly conserved, the DNA component of the small ribosome subunit has been proven extensively to be an important and useful molecular clock for quantitating evolutionary relationships

between organisms (Ueda *et al*., 1999). They are useful because they occur in all organisms, and different positions in their sequences change at very different rates, allowing phylogenetic relationships, both close and distant, to be measured, which makes their range all encompassing (Clarridge III, 2004). They are large and they consist of many domains. There are about 50 helical stalks in the 16S rRNA structure (Figure 1.1) and almost 100 in the 23S rRNA (Wimberly *et al*., 2000). The number of domains is important because non-random changes affecting one of the units will not appreciably affect the others. This is a major advantage of using the larger rRNAs (16S and 23S) over the smaller 5S rRNA (Woese, 1987).

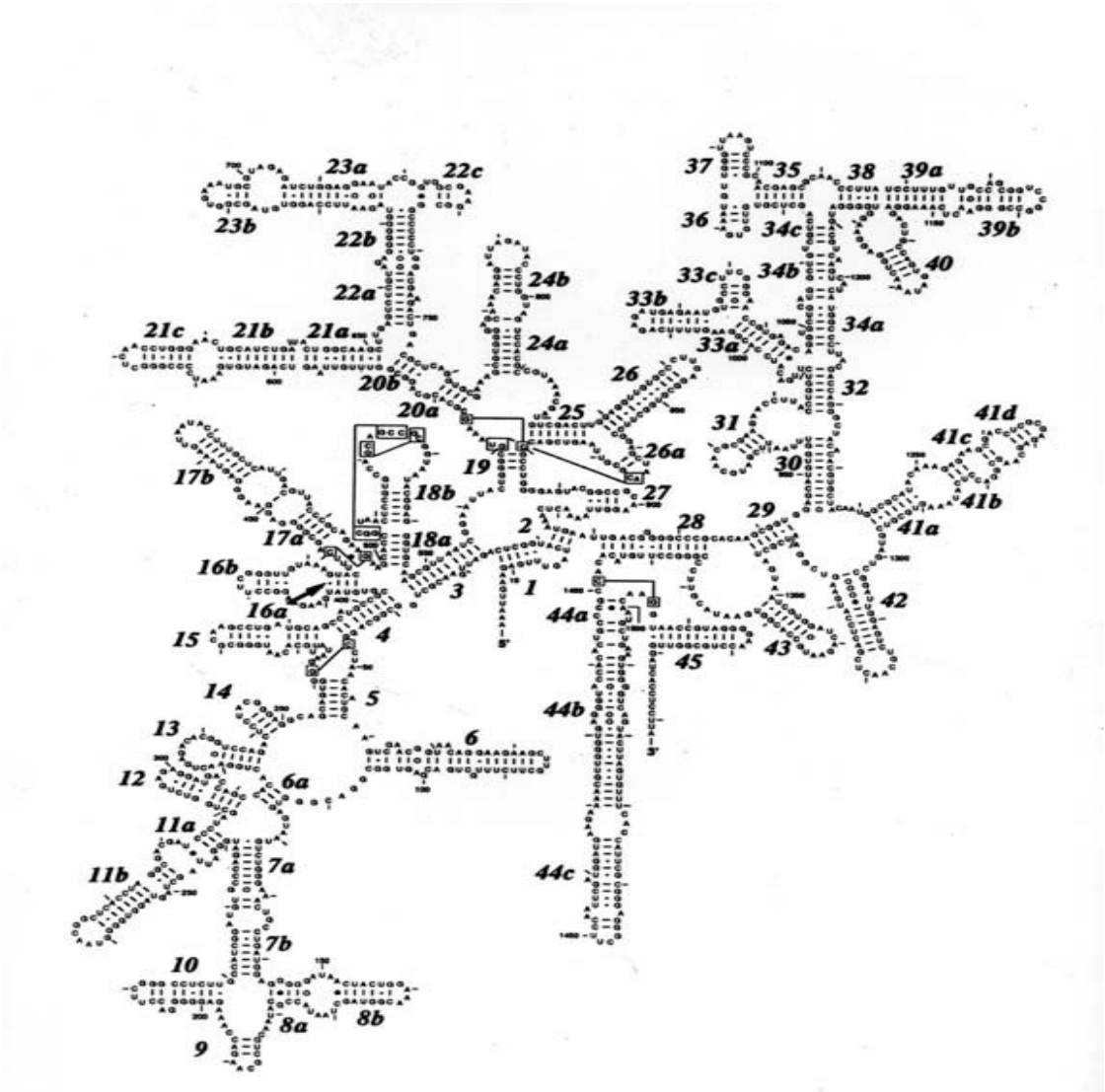


Figure 1.1: The secondary structure of 16S ribosomal RNA (Gutell, 1994). The small numbers indicate nucleotide numbers in *E. coli* and the large numbers indicate the loop number.

Since the development of molecular techniques such as the polymerase chain reaction and DNA sequencing in the 1980s, the phylogenetic structure of bacteria has been studied by comparing sequences of 16S ribosomal RNA. Fox *et al*. (1980) described the process, which changed the way microorganisms were identified and classified. It was identified that the 16S rRNA gene is highly conserved within a species and among a species of the same genus. Nucleotide substitutions occur within ribosomal nucleic acids at steady rate throughout evolutionary history (Woese, 1987). Some regions of rRNA genes evolve at different rates resulting in regions of nucleotide conservation and variability. The conserved regions allow for the selection of universal primers for PCR amplification of almost all prokaryotes.

Bacteria can be identified by amplifying the 16S rRNA gene, sequencing it and comparing it to other bacterial sequences in a database, such as GenBank, the largest database of nucleotide sequences. The reliability of DNA sequences generated in laboratories has been greatly improved by the introduction of automated sequencing systems and DNA alignment software. However, other factors, such as the purity of the DNA template and number of overlapping nucleotide fragments in the alignment, contribute to the reliability of the final sequence (Sacchi *et al.*, 2002).

A phylogenetic tree can be constructed which shows the bacterium's position in the evolutionary order based on base differences between species. This process is fast and very accurate and is aided by the large number of available programmes and databases. Databases are available that have thousands of 16S rRNA sequences from almost all known genera of bacteria (Zhang *et al*., 2002). Advances in sequencing technology have also increased the speed with which sequence information can be obtained. 16S rRNA gene sequencing is now the gold standard of bacterial identification. It enables the identification of non-cultivable microorganisms and elucidates the relationship between unknown species and known ones (Woo *et al*., 2000).

Ribosomal RNA sequences do not always coincide with characterisations based on classic taxonomic methods. Whereas genotypic classifications are based on relatively stable and uniform molecular targets, phenotypic classification is subject to variations in morphology, metabolic status and interpretation. When sequence data are included

with other methods (e.g. API, Biolog identification kits) in a polyphasic approach, a comprehensive taxonomic and phylogenetic assessment can be obtained (Kolbert and Persing, 1999).

1.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was first obtained in pure culture by Gessard in 1882 from wounds that had produced blue-green discoloration (Forkner, 1960). The word 'aeruginosa' comes from the Latin word for verdigris or copper rust. This describes the blue-green bacterial pigment seen in laboratory cultures of *P. aeruginosa*. *Pseudomonas aeruginosa* is a Gram-negative, mesophilic, aerobic rod (measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm) (Bergey's manual of Systematic Bacteriology, 2001). These bacteria are commonly found in soil and water. They occur regularly on the surface of plants and occasionally on the surfaces of animals. The pseudomonads are better known to microbiologists as pathogens of plants rather than animals, but few pseudomonads species are pathogens of humans (Todar, 2004).

Stover *et al*. (2000) accomplished sequencing of the complete 6.3Mbp genome of *P. aeruginosa*. The large genome size and genetic complexity of *P. aeruginosa* reflects evolutionary adaptations permitting it to thrive in diverse ecological niches. *P. aeruginosa* has broad capabilities to transport, metabolize and grow on organic substances, numerous iron siderophore uptake systems and enhanced ability to export compounds, e.g., antibiotics, by a large number of protein secretion and efflux systems.

Pseudomonas aeruginosa is not particularly distinctive as a pseudomonad, but there are a few characteristics that are noteworthy and relate to its pathogenesis (Todar, 2004). *Pseudomonas aeruginosa* possesses the metabolic versatility for which pseudomonads are so renowned. *Pseudomonas aeruginosa* is nonfermentative and derives its energy from oxidation rather than fermentation of carbohydrates. It can utilise at least eighty organic compounds but can grow on minimal media with only acetate for carbon and ammonium sulphate for nitrogen. It does not require any organic growth factors (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas aeruginosa is extremely versatile biochemically and can multiply in an extraordinary assortment of environments including eye drops, soaps, sinks, anaesthesia and resuscitation equipment, fuels, humidifiers and even stored distilled water, which is evidence of its minimal nutritional requirements (Todar, 2004). Although the bacterium is respiratory and never fermentative, it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. Its optimum temperature for growth is 37ºC, and it is able to grow at temperatures as high as 42ºC. Indeed, it is this ability to grow at 42ºC that distinguishes it from many other *Pseudomonas* species. No growth occurs at 5ºC (Bergey's Manual of Systematic Bacteriology, 2001). Its tolerance to a wide variety of physical conditions, including temperature, contributes to its ecological success as an opportunistic pathogen. *Pseudomonas aeruginosa* does, however, show a preference for growth in moist environments, a reflection of its origins in soil and water (Todar, 2004).

Pseudomonas aeruginosa isolates can produce three different colony types. One is large, smooth, with flat edges and an elevated centre ("fried egg" appearance) and the other is small, rough and convex. Clinical materials are, in general, good sources of the large colony type, while the small is commonly obtained from natural sources (Véron and Berche, 1976). A third type (mucous) often can be obtained from respiratory and urinary tract secretions and was first observed by Sonnenshein (1927). The mucus is attributed to the production of alginate slime. The smooth mucoid colonies are presumed to play a role in colonisation and virulence (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas aeruginosa produces many types of soluble pigments of which pyocyanin and pyoverdin are the most common. The latter is produced abundantly in media of low-iron content, and functions in iron metabolism in the bacterium. Pyocyanin refers to "blue pus" which is a characteristic of suppurative infections caused by *Pseudomonas aeruginosa*. (Palleroni, 1986) Other pigments produced are pyorubin (red), pyomelanin (brown) and pyoverdin (yellow/green) (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas aeruginosa is the epitome of an opportunistic pathogen of humans. It rarely causes infections in healthy individuals but is a major cause of hospital acquired nosocomial infections. Even though the bacterium almost never infects uncomprimised tissues, there is hardly any tissue that it cannot infect, if the tissue defences are compromised in some manner (Todar, 2004). It tends to infect people with immunodeficiency or burns and those with indwelling catheters or on respirators. Infection with *P. aeruginosa* can lead to urinary tract infections, sepsis (blood stream infection), pneumonia, endocarditis, pharyngitis, meningitis, and many other medical problems. It colonises the lungs of patients with cystic fibrosis (CF) and contributes to the chronic progressive pulmonary disease and death rate in CF. Although the initial isolation of *P. aeruginosa* from sputum may be intermittent in CF and bronchiectasis, once a chronic infection is established it is almost impossible to eradicate it even with intensive antibiotic treatment (Rayner *et al*., 1994).

Pseudomonas aeruginosa is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen (Seol *et al*., 2002). It has a natural tendency for the development of resistance to antibiotics. This limits future therapeutic uses of antibiotics against this bacterium and increases rates of mortality. The bacterium is naturally resistant to many antibiotics including tetracyclines and benzylpenicillin due to the permeability barrier afforded by its outer membrane lipopolysaccharide (LPS) (Li *et al*., 1994 [a]). It can colonise surfaces in a biofilm form making the cells impervious to antibiotics. *Pseudomonas aeruginosa* has been living in the soil for millions of years in the presence of antibiotic producing bacilli, actinomycetes and moulds. Therefore, it has developed resistance to a variety of their naturally occurring antibiotics. Moreover, *P. aeruginosa* maintains antibiotic resistant plasmids, and is able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including some β-lactams, aminoglycosides and fluoroquinolones, and even these antibiotics are not effective against all strains. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant that it cannot be treated (Todar, 2004).

Pseudomonas aeruginosa produces a variety of virulence factors, which aid it in colonising a host. These include protease enzymes, mucoid exopolysaccharide, pili, exotoxin A, lipopolysaccharide, pigments, lipase, haemolysin, histamine, exoenzyme S, leukocidin and rhamnolipids (Schaber *et al*., 2004) These help the bacteria to adhere and invade to their host by damaging the host's immune responses and forming a barrier to antibiotics. No single virulence factor by itself is potent but the whole array of factors contributes to the pathogenicity of the *P. aeruginosa* (Wilson and Dowling, 1998).

1.2 Antibiotics

Bacteria comprise a large group of unicellular, prokaryotic, microorganisms, which are also able to form spores, i.e., dormant forms produced under adverse conditions, but with the potential to germinate or revert to the cellular, replicating bacterial form in a favourable environment. Some bacterial activities are beneficial to man while others, notably the capacity to cause disease, are detrimental. Undoubtedly, one of the most important scientific achievements of the last century has been the ability to control the detrimental activities of bacteria by the use of antibiotics (Russell and Chopra, 1990).

It is widely accepted that bacteria as living organisms came to existence over 3.5 billion years ago (Schopf and Packer, 1987). As these microorganisms were forced to interact with each other and other living organisms, they became more complex and evolved the biochemical means for influencing the existence of each other. One of these developments was the advent of biochemical pathways for the production of antibiotics. If these antibiotics could inhibit the growth of a competitor, then more resources would be available for the growth of the original organism (Walsh, 2003).

Antibiotics (meaning "against life") are molecules that stop microbes, both bacteria and fungi, from growing or kill them outright. Antibiotics that stop bacteria from growing are bacteriostatic, exemplified by the drug chloramphenicol. Antibiotics that cause bacterial cell death are bactericidal. Penicillins and aminoglycosides are examples of these bactericidal agents (Russell and Chopra, 1990). Antibacterial agents can also destroy spores. These are called sporicidal agents. Some antibiotics

can display bactericidal activity in some circumstances and bacteriostatic activity in others, where sufficient damage to one or more cell pathways or structures occurs that a net bactericidal response is triggered. Some bactericidal agents are also sporicidal and vice versa, but bacteriostatic agents are ineffective against resting spores (Walsh, 2003).

Antibiotic agents can either be natural products or synthetic chemicals, designed to block some crucial process in microbial cells' selectivity. They specifically interfere with the biochemical processes of bacteria and hence they can be safely used in mammalian hosts (Todar, 2002). Many of the antibiotics in human clinical use today are natural products. Both bacteria and fungi produce natural antibiotic products, with the major group of antibiotic-producing bacteria being the actinomycetes. Antimicrobial compounds can be antibacterial or antifungal but there are almost no therapeutically useful agents that are effective as both antibacterial and antifungal agents because of different molecular and cellular targets and microbial cell penetration issues (Walsh, 2003).

The establishment of infections in humans and animals by a pathogenic bacterium usually involves the following steps: (a) attachment to the epithelial surfaces of the respiratory, alimentary or urogenital tracts; (b) penetration of the epithelial surfaces by the pathogen; (c) interference with, or evasion of, host defence mechanisms; (d) multiplication in the environment of the host's tissues; (e) damage of the host tissues. Antibiotics usually interfere at step (d) either by killing the pathogen or by slowing their growth to the point where host defence mechanisms can clear the infection. (Russell and Chopra, 1990).

The worldwide genome sequencing efforts have completed approximately 400 bacterial genomes to date (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The number of genes in most of these organisms varies from between 1000 and 5000 genes. It is estimated that only between 20 and 200 genes are essential for the survival of most bacteria (Fang *et al*., 2005). Therefore the proteins encoded by these genes are potential targets for antibiotics. Other types of antibiotics interfere with assemblies of these gene products or with structural components that result from their actions, such as the cell wall, bacterial envelope or ribosome. Known antibiotics interfere with a

handful of biochemical processes. These are interference in metabolic pathways, disruption of the integrity of the cytoplasmic membrane, inhibition of protein biosynthesis, inhibition of DNA and RNA biosynthesis and disruption of the biosynthesis of cell wall (Golemi-Kotra, 2002) (Figure 1.1). A list of antibiotic classes and their targets are shown in Table 1.1.

Table 1.1: Antibiotics and their targets (Adapted from Todar, 2002)

Antibiotic	Target
β-lactams	Cell wall synthesis
Glycopeptides	Cell wall synthesis
Quinolones	DNA replication and repair
Aminoglycosides	Protein synthesis
Tetracyclines	Protein synthesis
Macrolides	Protein synthesis
Chloramphenicol	Protein synthesis
Sulphonamides	Folic acid pathway
Trimethoprim	Folic acid pathway
Lipopeptides	Cell membrane

1.2.1 Antibiotic classes and their targets

Four major targets of antibiotics are cell wall synthesis, DNA replication and repair, protein synthesis and metabolic pathways. These targets and others are illustrated in Figure 1.2.

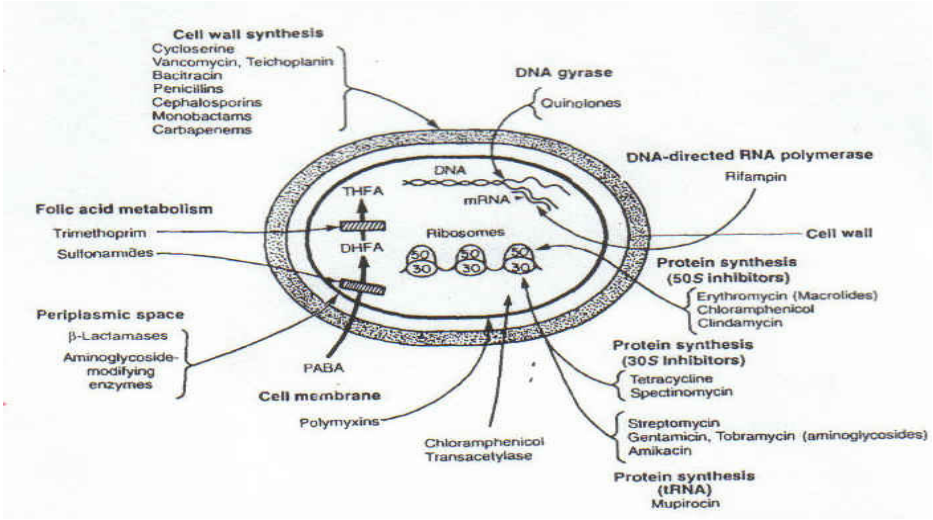


Figure 1.2: Major targets for antibacterial action (Neu, 1992)

1.2.1.1 Antibiotics that act on cell wall biosynthesis

The Gram stain is probably the most widely used staining procedure in microbiology (Forster, 2002). It is a differential stain that differentiates between Gram-positive and Gram-negative bacteria. Gram-positive stain purple and Gram-negative stains pink. Bacteria such as *Pseudomonas aeruginosa* and *E. coli* are Gram-negative whereas streptococci and staphylococci are Gram-positive. Gram-positive and Gram-negative stain differentially because of fundamental differences in the structure of their cell walls (Figure 1.3) (Bergey's Manual for Systematic Bacteriology, 2001).

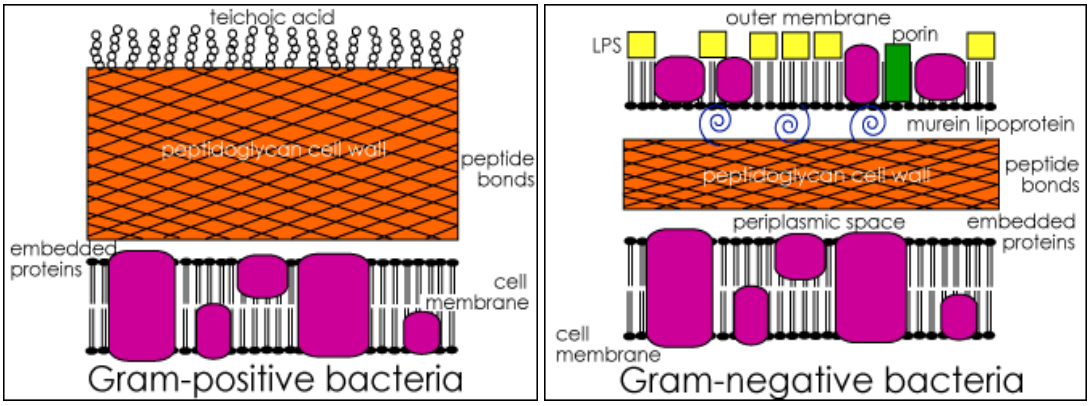


Figure 1.3: Diagram showing the composition of Gram-negative and Gram-positive cell walls (Adapted from http://library.mtandao-afrika.org/TQA01074/english/bio.htm)

The bacterial cell wall serves to give the organism its size and shape and also to prevent osmotic lysis. Peptidoglycan (also called murein) is the component of the cell that confers its rigidity. Both Gram-positive bacteria and Gram-negative bacteria have a peptidoglycan layer as part of their cell wall structure. The peptidoglycan layer is substantially thicker and multilayered in Gram-positive bacteria. It is a vast polymer consisting of interlocking chains of identical peptidoglycan monomers. The monomer consists of two joined sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with a pentapeptide coming off the NAM (Figure 1.4). The monomers are synthesized in the cytoplasm of the bacterium where they attach to a membrane carrier molecule called bactoprenol. Bactoprenols transport the peptidoglycan monomers across the cytoplasmic membrane and work with enzymes to insert the monomers into existing peptidoglycan enabling bacterial growth following binary fission (Russell and Chopra, 1990).

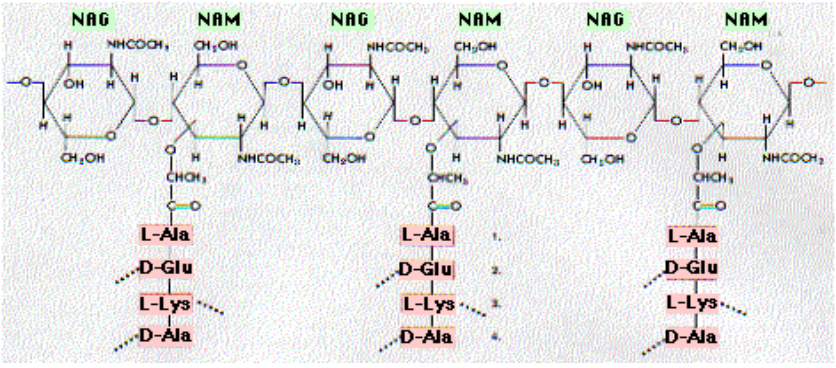


Figure 1.4: Structure of peptidoglycan (Walsh, 2003)

Once the new peptidoglycan monomers are inserted, glycosidic bonds then link these monomers into the growing chains of peptidoglycan. These long sugar chains are then joined to one another by means of peptide cross-links between the peptides coming off the NAMs. The peptide cross-links introduce covalent connectivity to the meshwork, impart mechanical strength and provide the major structural barrier to osmotic pressure forces that could kill the bacterium (Walsh, 2003).

In order for bacteria to increase in size following binary fission, links in the peptidoglycan monomers must be inserted and the peptide cross-links must be resealed. Williams *et al*. (1996) described the synthesis of peptidoglycan.

Peptidoglycan is made in several stages. The initial reactions occur in the cytoplasm. Further transformations are then effected in the cell membrane and the final incorporation of peptidoglycan into the bacterial cell wall occurs at the point of extension of the cell wall.

Cytosolic phase

The basic unit of peptidoglycan made in the cytoplasm consists of NAM attached to the inactivating nucleotide uridine diphosphate (UDP) and bearing a pentapeptide. N-acetylglucosamine-1-phosphate is first converted into UDP-NAG. The lactic acid residue that converts UDP-NAG into UDP-NAM is incorporated as pyruvate from the glycolytic intermediate phosphoenolpyruvate (PEP), followed by the reduction of this pyruvate to lactate. The lactate is joined by an ether link between its hydroxyl and the 3-hydroxyl of the NAG. The peptide is attached to the carbonyl group of the lactic acid residue. The stepwise addition of three amino acids by peptide bonds forms the tripeptide derivative of UDP-NAM-tripeptide. The final two amino acids of the pentapeptide are added as a dipeptide of D-alanine, which is synthesized separately by D-Ala-D-Ala synthase. Fosfomycin is an antibiotic that inhibits the cytosolic phase of phase of peptidoglycan synthesis.

Membrane phase

The membrane carrier used in the membrane phase of peptidoglycan synthesis is the 55-carbon lipid undecaprenyl phosphate (Und-P), comprising 11 isoprene units. The lipid accepts phospho-NAM-pentapeptide from UDP-NAM-pentapeptide in a reaction involving translocase I, releasing UMP into the cytoplasm. These lipid complexes in the membrane then accept NAG from cytoplasmic UDP-NAG in the transfer using translocase II, so that the growing peptidoglycan subunit now contains the NAM-β1:4-NAG disaccharide subunit and pentapeptide. Bacitracin is an antibiotic that inhibits the membrane phase of peptidoglycan synthesis. It binds to Und-P-P and inhibits the membrane pyrophosphatase that releases undecaprenyl phosphate from the pyrophosphate, causing an accumulation of the lipid carrier in the pyrophosphate form.

Cell wall phase

Growing bacteria have lytic enzymes to hydrolyse the mucopeptide locally in order to allow new components to be added at growing points. The peptidoglycan subunit in the membrane is detached from the undecaprenyl pyrophosphate (Und-P-P) carrier and transferred to a growing point in the peptidoglycan by a bond to the NAM-NAG disaccharide. The released undecaprenyl lipid bears pyrophosphate, which must be hydrolysed by a specific pyrophosphatase to form monophosphate that may again accept UDP-NAM-pentapeptide from the cytoplasm. In the cross-linkage of peptidoglycan by transpeptidation, the side-chain amino group of the pentaglycine of one glycan chain reacts enzymatically with the peptide bond between two D-alanine residues of a pentapeptide from another glycan chain. The reaction involves the migration of the peptide bond and the transfer of a proton from the pentaglycine amino group; free D-alanine is released. There is no requirement for energy input because these enzymes work outside the cell on the periplasmic face of the membrane where ATP and other energy sources are not routinely available (Walsh, 2003).

β-lactams

The β-lactams were the first antibiotics to be discovered and used. Without doubt, the β-lactams are the most important group of drugs that inhibit the final stage of peptidoglycan synthesis (Russell and Chopra, 1990). They are favoured because of their efficacy, broad spectra and low toxicity. All β-lactams are bactericidal agents (Walsh, 2003). The penicillins are derived from the fungus *Penicillium* and modifications made upon the parent compound can alter the drug's spectrum of action. The β-lactam antibiotics include the penicillins (oxacillin, ampicillin, carbenicillin, piperacillin etc.) (Table 1.2), where the chemical warhead, the four-membered β-lactam ring, is fused to a five-membered sulphur ring system (Figure 1.5) (Merck, 2007). Penicillins are primarily active against non-β-lactamase-producing, aerobic Gram-negative, some fastidious, aerobic Gram-negative bacteria and some anaerobic bacteria. Aminopenicillins (ampicillin and amoxicillin) are active against additional Gram-negative species, including some members of the *Enterobacteriaceae*. Carboxypenicillins (carbenicillin and ticarcillin) and

ureidopenicillins (mezlocillin and piperacillin) are active against an expanded list of Gram-negative bacteria including many *Pseudomonas* and *Burkholderia* spp. Penicillinase-stable penicillins (cloxacillin, dicloxacillin, methicillin, nafcillin and oxacillin) are active against predominantly Gram-positive bacteria including penicillinases-producing staphylococci (Clinical and Laboratory Standards Institute (CLSI) (M100-S16, 2006).

The cephalosporins (cephalothin, ceftazidime etc.) are β-lactams in which the β-lactam is fused to a sulphur-containing ring expanded system (Walsh, 2003). Different cephalosporins exhibit somewhat different spectrums of activity against aerobic and aerobic Gram-positive and Gram-negative bacteria. The cephalosporins antimicrobial class includes the classical cephalosporins. Cephalosporins are often referred to as "first-", "second-", "third-" or "fourth-generation" cephalosporins (Table 1.2) based on the extent of their activity against the more antimicrobial agent-resistant, Gram-negative aerobic bacteria. All representatives of a specific group or generation do not necessarily have the same spectrum of activity (Clinical and Laboratory Standard Institute (CLSI), M100-S16, 2006).

Other variants of the β-lactam natural products are the penems and monobactams (Table 1.2) (Todar, 2002). The penems (imipenem and meropenem) structure differs slightly from that of the penicillins. They have a broader spectrum of activity against both Gram-negative and Gram-positive bacteria because they are a lot more resistant to β-lactamase hydrolysis. Monobactam antimicrobial agents are monocyclic β-lactams. Aztreonam is the only approved monobactam antimicrobial agent. It only has activity against Gram-negative aerobic bacteria (Clinical and Laboratory Standard Institute, M100-S16, 2006).

There are also antimicrobial agents which are combinations that include a β-lactam and a second agent that has minimal antibacterial activity but functions as an inhibitor of some β-lactamases (Walsh, 2003). Currently, three β-lactamase inhibitors are in use: clavulanic acid, sulbactam and tazobactam. The results of tests of only the penicillin portion of the combination against β-lactamase-producing organisms are

often not predictive of susceptibility to the two-drug combination (Clinical and Laboratory Standard Institute, M100-S16, 2006).

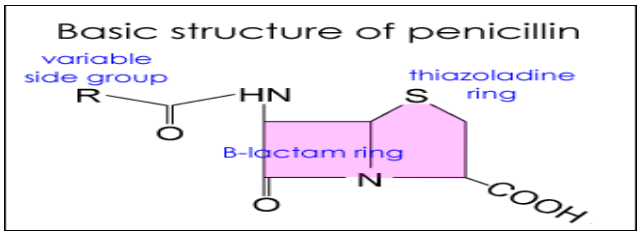


Figure 1.5: Structure of penicillin (Walsh, 2003)

β-lactams stop bacterial cells reproducing by inhibiting the synthesis of a new cell wall, which is essential for the survival of the bacteria. Penicillin, as well as other β -lactams, inhibits the enzyme that places essential cross-links between the individual polymer strings of the cell wall. It does this specifically by using the β-lactam ring to irreversibly block the active site of the enzyme, which catalyzes the reaction, transpeptidase. This inhibition allows the bacteria to newly synthesize a cell wall and to elongate, but not divide. This is due to the lack of cross-linking. The result is disruption of cell wall integrity, making the cell osmotically unstable and susceptible to lysis (Walsh, 2003).

The β-lactams resemble the sequence of the terminal dipeptide of uncrosslinked mucopeptide, D-alanine-D-alanine, the natural substrate for the cross-linking enzyme transpeptidase. The –CO-N- bond of the β-lactam ring is the analog of the peptide bond between the two alanine residues of the natural substrate. Penicillin reacts with the transpeptidase to form a stable acyl intermediate. The β-lactam ring acylates the hydroxyl group of one specific serine residue in the transpeptidase, producing an inactive penicilloyl-enzyme complex (Williams *et al*., 1996).

The transpeptidases “commit suicide” when they start a catalytic cycle with β-lactam antibiotics as substrates, mistaking them for immature peptidoglycans waiting to be cross-linked. The active-site serine adds into the strained four-ring carbonyl and generates an acyl enzyme intermediate in which the β-lactam ring has opened. The enzyme is then stuck in mid-catalytic cycle. The transpeptidases are designed to

exclude water from intercepting the normal acyl enzyme intermediates and, therefore the penicilloyl enzyme forms are very slow to hydrolyse. These covalent penicilloyl enzymes build up and are effectively inactive until slow hydrolysis allows it to recover. It may take between hours and days for hydrolysis to occur (Walsh, 2003). A list of common β-lactam antibiotics is shown in Table 1.2.

Table 1.2: List of common β-lactam antibiotics (adapted from Merck, 2007)

Penicillins	**Cephalosporins**
Amoxicillin	
Amoxicillin/Clavulanate	***1st Generation***
Ampicillin	Cefadroxil
Ampicillin/Sublactam	Cefazolin
Bacampicillin	Cephalexin
Carbenicillin	Cephalothin
Cloxacillin	Cephapirin
Dicloxacillin	Cephradine
Methicillin	
Mezlocillin	***2nd Generation***
Nafcillin	Cefaclor
Oxacillin	Cefamandole
Penicillin G	Cefonicid
Penicillin V	Cefotetan
Piperacillin	Cefoxitin
Piperacillin/Tazobactam	Cefprozil
Ticarcillin	Cefuroxime
Ticarcillin/Clavulanate	Loracarbel
Monobactams	***3rd Generation***
Aztreonam	Cefdinir
	Cefditoren
Carbapenems	Cefixime
Ertapenem	Cefoperazone
Imipenem	Cefotaxime
Meropenem	Cefpodoxime
	Cefsulodin
	Ceftazidime
	Ceftibuten

	Ceftizoxime Ceftriaxone ***4th Generation*** Cefepime Cefozopran

Glycopeptides

Vancomycin and teicoplanin are two glycopeptide antibiotics that have been approved for human use (Moellering, 2006). The structures of both antibiotics can be seen in Figure 1.6. Glycopeptides are only effective against Gram-positive bacteria (Greenwood and Whitley, 2003). They interfere with the glycan unit insertion in peptidoglycan synthesis. Vancomycin, which is a large hydrophilic molecule, undergoes hydrogen bonding to the acyl-D-alanyl-D-alanine terminus of various peptidoglycan precursors. It inhibits the transglycosylation step by which the glycan units are polymerized within the peptidoglycan. It doesn't inhibit the transglycosylase enzyme but the complex of vancomycin with the peptide prevents the substrate from interacting with the active site of the enzyme (Walsh, 2003). Teicoplanin works in a similar way. Their mode of action is not to be confused with that of β-lactams (Russell and Chopra, 1990).

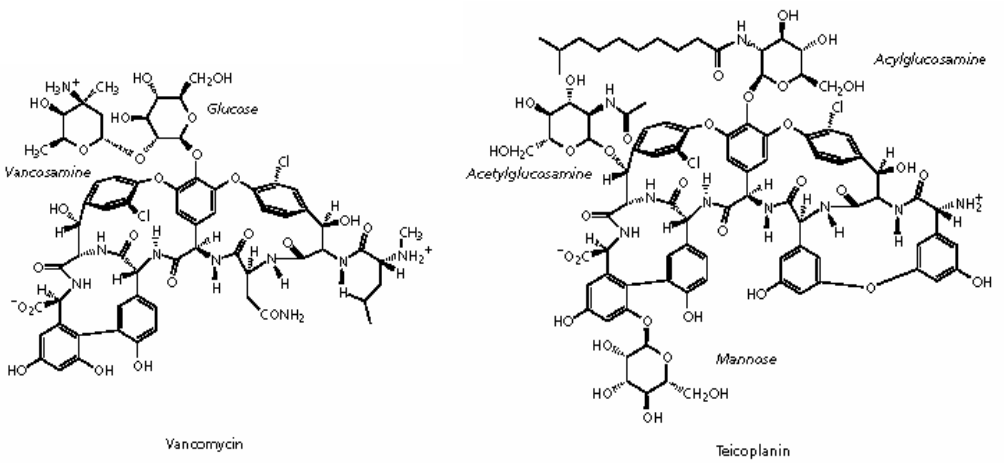


Figure 1.6: Structures of vancomycin (left) and teicoplanin (right) (Available at http://www.chemsoc.org/chembytes/ezine/images/1997/resfig1.gif)

1.2.1.2 Antibiotics that block DNA replication and repair

Biosynthesis of DNA and its repair has been targeted by the quinolone class of antibiotics (Oliphant and Green, 2002). Quinolones are a novel group of synthetic antibiotics that were developed in response to the increasing problem of antibiotic resistance. They are derivatives of nalidixic acid, a synthetic quinolone compound. Quinolones are low molecular weight hydrophilic molecules. Fluoroquinolones such as ciprofloxacin (Figure 1.7), norfloxacin, sparfloxacin and gatifloxacin have a broad spectrum of activity and are widely used in the treatment of both Gram-negative and Gram-positive infections. The quinolones inhibit the replication of DNA without immediately affecting RNA or protein synthesis in sensitive bacteria. These antibiotics inhibit DNA topoisomerases, which are necessary for DNA synthesis. Topoisomerases are essential for cell viability. The DNA topoisomerases change the linking number in supercoiled DNA by making transient cuts in the DNA substrate and then passing the DNA to be relaxed topologically through the transient break, either one strand at a time (Type I) or both strands at a time (Type II). Topoisomerase IV is essential for the separation of interlinked daughter DNA molecules. These antibiotics bind to the complex formed between DNA and DNA gyrase or topoisomerase IV, during the replication process. When the replication fork collides with the quinolones-enzyme-DNA complex, its progress is halted and the reformation of the phosphate diester is prevented. (Walsh, 2003).

Nalidixic acid causes disintegration of DNA and filamentation of bacterial cells but its toxicity in animals is limited to inhibition of mitochondrial DNA replication. These antibiotics display concentration-dependent bactericidal activity. Nalidixic acid is bactericidal to most of the Gram-negative bacteria but is only useful for treatment of urinary tract infections because it does not achieve bactericidal concentrations in any bodily fluid except urine. The second-generation quinolones, norfloxacin and ciprofloxacin are more effective against a wider range of bacteria. New quinolones such as fleroxacin are active against a wide range of Gram-negative aerobes and moderately effective against Gram-positive aerobes (Williams *et al*., 1996).

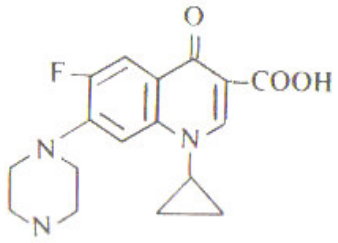


Figure 1.7: Structure of ciprofloxacin (Walsh, 2003)

1.2.1.3 Antibiotics that block bacterial protein biosynthesis

Aminoglycosides were originally isolated from soil bacteria including various species of *Streptomycetes* and *Micromonospora* (Greenwood, 1995). Schatz and Waksmann (1944) reported the first aminoglycoside antibiotic. It was called streptomycin and proved to be the first chemotherapeutic agent that was effective against *Mycobacterium tuberculosis*. Streptomycin is considered to be the parent molecule for the aminoglycosides but there are now two distinct sub families of aminoglycosides based upon the structure of the aminocyclitol ring. The basic chemical structure required for both potency and the spectrum of antimicrobial activity of aminoglycosides is that of one or several aminated sugars joined in glycosidic linkages to a dibasic cyclitol. In most clinically used aminoglycosides (the larger kanamycin/neomycin group) the cyclitol is 2-deoxystreptamine and it is streptidine in the streptomycin group of aminoglycosides. The kanamycin/neomycin group consists of a central aminocyclitol ring (B ring) with two or three substituted aminoglycan rings linked either at the 4 and 5 hydroxyls of the B ring (neomycin, paromomycin, butirosin and lividomycin) or at the 4 and 6 hydroxyls (kanamycin, amikacin, tobramycin and gentamicin) (Figure 1.8) (Smith and Baker, 2002). There have been many more aminoglycoside antibiotics discovered since streptomycin including kanamycin, tobramycin, netilmicin and gentamicin, which have established the aminoglycosides as being very effective against aerobic Gram-negative infections (Gonzalez and Spencer, 1998).

Chemists have developed semisynthetic variants that have broader spectra of activity and that are not susceptible to aminoglycoside resistance enzymes. Aminoglycosides

exhibit activity against a variety of clinically important Gram-negative bacteria such as *Klebsiella* spp., *Serratia* spp., *Citrobacter* spp., *Enterobacte*r spp., *Proteus* spp. and *Pseudomonas* spp. as well as *Staphylococcus aureus* and streptococci, but they have extremely reduced activity against microorganisms growing in an anaerobic environment (Vakulenko and Mobashery, 2003). Aminoglycosides have varying spectra of antimicrobial activity. For example, gentamicin is more active than tobramycin against *Serratia* spp., whereas tobramycin has greater activity against *Pseudomonas aeruginosa* than gentamicin. The widest spectrum of activity of the aminoglycosides belongs to arbekacin, an aminoglycoside that is most commonly used in Japan. It has remarkable activity against MRSA strains that show no susceptibility to other aminoglycosides (Aoki, 1994).

Aminoglycosides are very useful antibiotics as they have relatively predictable pharmokinetics, a postantibiotic effect, synergism with other antibiotics and have concentration-dependent bactericidal activity (Vakulenko and Mobashery, 2003). They are commonly used in combination with antibiotics, which inhibit cell wall synthesis i.e., β-lactams and vancomycin, particularly in the treatment of enterococci, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Gonzalez and Spencer, 1998). The increased permeability afforded by these antibiotics results in an increase in intracellular uptake of aminoglycosides (Eliopoulos and Moellering, 1996). Aminoglycosides exhibit a postantibiotic effect (Craig and Gudmundsson, 1996). That means they continue to kill bacteria after the aminoglycoside has been removed following a short incubation with the microorganism. Aminoglycosides show concentration-dependence (Gonzalez and Spencer, 1998). This means that their bactericidal activity depends more on their concentration than on the duration of bacterial exposure to inhibitory concentrations of antibiotic and is also significantly less dependent on the bacterial inoculum size. The killing potential of aminoglycosides therefore increases with increasing concentration of the antibiotic (Vakulenko and Mobashery, 2003).

Aminoglycosides are one of the commonest causes of drug-induced nephrotoxicity (Walker and Duggin, 1988). Nephrotoxicity induced by aminoglycosides manifests clinically as nonoliguric renal failure (Mingeot-Leclercq and Tulkens, 1999).

Therefore, aminoglycosides are usually not the first antibiotic of choice. Aminoglycosides are usually administered parenterally, although to increase the concentration of the antibiotic at the site of infection or to reduce toxicity, aerosolized tobramycin and gentamicin have been used in cystic fibrosis therapy (Heinzl *et al.*, 2002).

The RNA and protein machinery of the prokaryotic ribosomes is sufficiently distinct from the analogous eukaryotic machinery that there are many inhibitors of protein synthesis, targeting different steps in ribosome action, with selective antibacterial action (Walsh, 2003). Aminoglycoside antibiotics are protein synthesis inhibitors. The ribosome is a complex structure made up of three RNA molecules and more than 50 proteins (Vakulenko and Mobashery, 2003). This complex, along with several GTP-hydrolysing protein factors, catalyses protein synthesis. The bacterial ribosome is made up of two subunits, 50S and 30S. The 50S comprises two further subunits, 5S and 23S rRNAs and 33 proteins, while the 30S is made up of a single 16S rRNA and 20 to 21 proteins (Walsh, 2003). Aminoglycoside antibiotics bind to the 30S ribosomal subunit, which plays a crucial role in providing high-fidelity translation of genetic material (Vakulenko and Mobashery, 2003).

The ribosome has three functionally important tRNA binding sites: A (aminoacyl), P (peptidyl) and E (exit) (Green and Noller, 1997). During protein synthesis, the ribosome decodes information stored in the mRNA and catalyses sequential incorporation of amino acids into a growing polypeptide chain. High fidelity translation is achieved by the ability to discriminate between conformational changes in the ribosome-induced binding of cognate and noncognate tRNAs at the A site. Aminoglycosides that contain the 2-deoxystreptamine ring increase the error rate of the ribosome by allowing incorporation of the noncognate tRNAs. The structure of the 30S subunit indicates that two universally conserved adenine residues (A1492 and A1493) are directly involved in the decoding process during normal translation. In the native structure of the ribosome, these adenine residues are stacked in the interior of helix 44. Binding of the tRNA to the A site flips A1493 and A1492 out from their stacked position. It also flips G530 out from the *syn* to the *anti* conformation. The N1 of adenines interacts with the 2'-OH groups of the tRNA residues that are in the first

and second positions of the codon-anticodon triplet (Vakulenko and Mobashery, 2003).

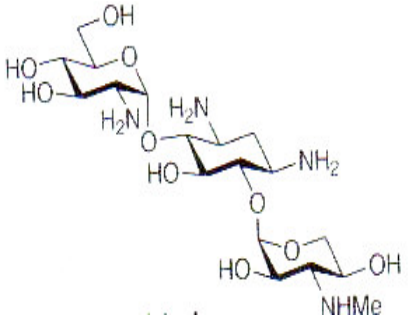


Figure 1.8: Structure of gentamicin (Walsh, 2003)

Aminoglycosides that contain the 2-deoxystreptamine ring bind to the major groove of helix H44 of 16S rRNA. This results in the flipping out of the same conserved A1492 and A1493 residues that are normally displaced upon binding of the cognate tRNA. The conformational changes induced in the 30S subunit by binding of the cognate tRNA are energetically favourable because they allow the ribosome to participate in a greater number of compensating interactions between the codon and anticodon double helixes (Vakulenko and Mobashery, 2003). Because the flipping out of the adenine residues might require energy expenditure, aminoglycoside-induced flipping-out can reduce energetic cost, allowing binding of near-cognate tRNAs and subsequent mistranslation of mRNA (Ogle *et al*, 2001).

Streptomycin has a different structure to that of other aminoglycosides but it binds at the functional centre of the ribosome in close proximity to the binding site of other aminoglycosides. Like other aminoglycosides, it induces misreading of the genetic code, but the mechanism is different. During translation, the 30S subunit switches between two distinct conformations. It has been shown that mutational stabilization of one of the conformations over the other results in two different fidelity phenotypes. One increases fidelity and the other decreases fidelity, i.e., *ram* (ribosomal ambiguity) or error-prone. The interaction of streptomycin with the ribosome is thought to preferentially stabilize the *ram* state. This stabilisation lowers the affinity for tRNAs and allows binding of near-cognate tRNAs, which renders the A-site more promiscuous. It could also affect the proofreading by making transition to the restrictive site more difficult (Vakulenko and Mobashery, 2003).

The passage of aminoglycosides (highly polar molecules) across the outer membrane of Gram-negative bacteria is a self-promoted uptake process involving the drug-induced disruption of Mg^{2+} and Ca^{2+} bridges between adjacent lipopolysaccharide molecules. Penetration through porin channels is unlikely because of the large size of aminoglycosides (Mingeot-Leclercq *et al*., 1999). Uptake of aminoglycoside antibiotics across the bacterial cytoplasmic membrane is essential for antibacterial activity and is similar in Gram-positive and Gram-negative bacteria. Aminoglycoside uptake is multiphasic with three distinct phases, one of which is energy-independent (EIP) and the remainder being energy-dependent (EDPI and EDPII) (Russell and Chopra, 1990).

The EIP phase of uptake occurs very rapidly and represents the initial binding of antibiotic to bacteria. Although, in Gram-negative bacteria, this partially represents interaction with the outer membrane, uptake during EIP also represents binding of drug molecules to the cytoplasmic membrane. EDPI represents a slow, but poorly characterised, energy-dependent uptake of drug molecules across the cytoplasmic membrane. A threshold transmembrane potential generated by a membrane-bound respiratory chain is required for the uptake of aminoglycosides during EDPI. This is why anaerobes are resistant to these antibiotics. EDPII, associated with progressive binding of aminoglycosides to ribosomes within the cell, results in an acceleration of uptake seen towards the end of EDPI. Aminoglycosides virtually irreversibly saturate all ribosomes, causing cell death. It is not known whether the third phase of uptake involves a transport carrier, nor is even the exact nature of the energy source driving EDPII mediated aminoglycoside uptake (Russell and Chopra, 1990).

1.2.1.4 Antibiotics that act on metabolic pathways

Folate is a coenzyme essential for cell growth. However, bacteria cannot transport folate and have to synthesis it *de novo*. Eukaryotes cannot synthesise folate and instead scavenge it from dietary sources and transport it into cells. Therefore selective inhibition can be achieved (Greenwood and Whitley, 2003).

Sulphonamides inhibit the incorporation of p-aminobenzoic acid (pABA) (Figure 1.9) into a precursor of dihydrofolic acid (DHF) that should then be reduced by the enzyme dihydrofolate reductase (DHFR) to tetrahydrofolic acid (THF). THF, which is a derivative of folic acid, is an important coenzyme involved in the transfer of small residues containing a single carbon atom (e.g. methyl, formyl) in intermediary metabolism. THF derivatives are also required for the synthesis of the amino acid methionine and of the nucleic acid bases, including thymine. DHF is synthesized in two stages. Firstly, dihydropteroic acid synthase catalyses the combination of pteridine derivative with pABA. This reaction is inhibited by sulphonamides. This is followed by the condensation of glutamic acid with dihydropteroic acid (Todar, 2002).

Trimethoprim inhibits the conversion of dihydrofolate to tetrahydrofolate. This limits the supply of some amino acids and nucleic acids. There is therefore a lag time between the administration of sulphonamides and the cessation of bacterial growth, which corresponds to the time taken for the bacteria to use up the stocks of biosynthetic components and folic acid already present in the cell. These drugs are bacteriostatic drugs since it may take some generations for the folate pool in the bacteria to decrease (Williams *et al.*, 1996). They are active against both Gram-negative and Gram-positive organisms (Clinical and Laboratory Standard Institute, M100-S16, 2006). These antibiotics are usually administered as co-trimoxazole, which is a combination of the two antibiotics. A diagram showing the sites of action of sulphonamide and trimethoprim in the folic acid synthesis pathway can be seen in Figure 1.10.

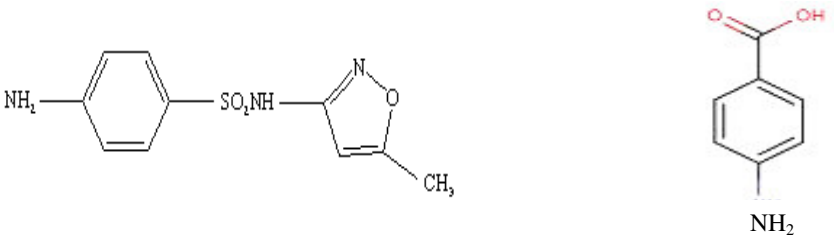


Figure 1.9: Structure of sulphonamide (left) and p-aminobenzoic acid (pABA) (right) (Walsh, 2003)

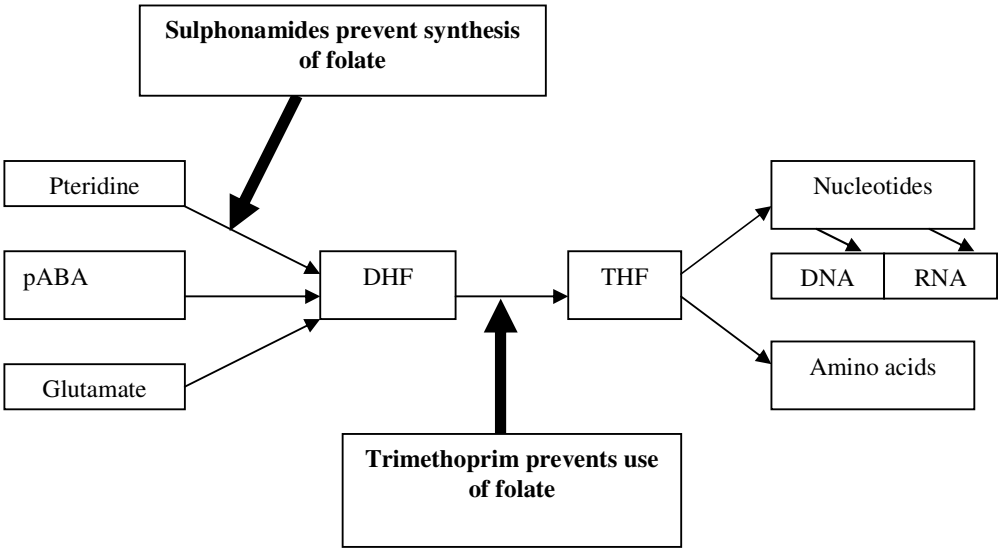


Figure 1.10: Diagram showing the sites of action of sulphonamides and trimethoprim in the folic acid synthesis pathway (Williams *et al*., 1996)

1.3 Antibiotic resistance

For millions of years, bacteria in the environment have been secreting specific compounds toxic to other bacterial cells. Soil bacteria, for example, are extremely prolific secretors of bactericidal chemicals such as aminoglycosides. However, these compounds are not toxic to the microorganisms that produce them. To overcome this threat, the bacteria that secrete these compounds have developed built in self-defence mechanisms, specifically, enzymes that deactivate these compounds. As a result, the bacterium can produce its toxins and, immune from their effects, gain an advantage over its neighbours. However, over time, those neighbouring bacteria have incorporated some of the same enzymes into their genomes, in response, so that they are then able to deactivate the antimicrobials secreted by other bacteria. This has been helped by the fact that the genes encoding such enzymes are generally found on transposons and plasmids. Although this is essential for the survival of the bacteria, it is now seen as an example of acquired resistance (Smith and Baker, 2002).

The past half century was an extraordinarily successful period in medical history during which, most human diseases were brought under control by antibiotics.

However, the increasing number of bacteria that are becoming resistant to antibiotics is now threatening this situation (Mazel and Davies, 1999). Resistance is only just beginning to be considered as a societal issue. Resistance is not a new phenomenon. It was recognised early on as a scientific curiosity and then as a threat to effective treatment. Resistance costs money, livelihoods and lives and threatens to undermine the effectiveness of health delivery programmes. Deaths from acute respiratory infections, diarrhoeal diseases, measles, AIDS, malaria and tuberculosis account for more than 85% of the mortality from infection worldwide. Antimicrobial use is the key driver of resistance (World Health Organisation, 2001).

Every time a patient takes an antibiotic for a bacterial infection, the antibiotic might kill most of the bacteria. But a few tenacious germs may survive by mutating or acquiring resistance genes from other bacteria. These surviving genes can multiply quickly, creating antibiotic resistant strains. The presence of these strains may mean that a patient's next infection will not respond to the first-choice antibiotic therapy. The resistant bacteria may then be transmitted to others in the patient's community (Nordenberg, 1998).

The greatest risk is for patients in hospitals, nursing homes and other settings where people tend to be sick and are taking large quantities of antibiotics, increasing the chance of antibiotic resistant bacteria originating in their own bodies. Hospitalised patients are also in contact with others whose infectious diseases may spread and their immune systems may be weakened and unable to fight infections (Loeb *et al*., 2003).

Antibiotic resistance is not a new problem. Ever since the discovery and subsequent clinical use of antibiotics, resistance to these agents has been observed with a negative impact on the treatment of infectious disease. The growing problem of antimicrobial resistance has become a significant public health concern. It is no longer an isolated problem of a few bacteria; almost all important human pathogens once treatable with antibiotics have developed some resistance (Nordenberg, 1998). It involves almost all types of pathogens, including bacteria, fungi, mycobacteria, viruses and parasites. Antibiotic resistance is now a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community. For example, all

strains of *Neisseria gonorrhoeae* were susceptible to sulphonamides when these were first employed in 1938. By 1948 resistance had built up so much that less than 20% of strains were susceptible (Walsh, 2003). Methicillin was developed to control staphylococci, but there are now increasing cases of methicillin-resistant *S. aureus* (MRSA) in many countries. Resistant strains have normally been controlled by vancomycin but resistance to this antibiotic is beginning to be identified. Some of these strains are still susceptible to other antibiotics but there is concern that a fully resistant strain of *S. aureus* will soon emerge (Patterson, 1999).

These strains are still treatable with other types of antibiotics but another serious problem is beginning to emerge, that of multi-drug resistance. There has been a recent resurgence in Mycobacterial diseases such as tuberculosis. Isoniazid, rafampin, ethambutol and streptomycin are successful in the treatment of tuberculosis but have to be used in multidrug treatments over long periods. This has contributed to the emergence of multidrug resistant strains of *M. tuberculosis* in these patients and, more disturbingly, the spread of these resistant strains into hospitals, as reported by Russell and Chopra (1990).

1.3.1 Causes of resistance to antibacterial agents

In less than two human generations antibiotics have revolutionised medicine, but, by selecting resistance, they carry the seeds of their own obsolescence. Resistance to antibiotics is an increasing global problem and a public health threat. It has resulted in morbidity and mortality from the failure of treatments and the increased costs of treating patients with infections caused by antibiotic resistant bacteria (World Health Organisation, 2001).

The indiscriminate usage of antibiotics in agriculture and particularly in the hospital setting can promote the survival of resistant bacteria and, in addition, impact on other normally innocuous bacteria, encouraging the development of drug-resistant strains of these species (Smith and Baker, 2002). Antibiotics are undoubtedly beneficial when used correctly, but doctors and members of the public frequently use antibiotics inappropriately. Examples of inappropriate use of antibiotics include: doctors

prescribing antibiotics to treat viral infections such as colds and flus; failing to accurately identify the bacteria causing an infection; unnecessarily prescribing expensive, broad-spectrum antibiotics; not following established recommendations for using chemo prophylaxis; the availability of antibiotics over the counter in many countries and the failure of patients to complete their full course of antibiotics. All of these promote the survival of antibiotic-resistant bacteria (World Health Organisation, 2001). It is essential that more stringent controls be placed on the use of antibiotics, since careful prescribing can halt the emergence of resistant strains (Smith and Baker, 2002).

While overuse of antibiotics in human medicine is a major contributor to antibiotic resistance, agricultural use of antibiotics also contributes to the problem. Meat producers use an estimated 70 percent of all U.S. antibiotics and related drugs non-therapeutically, such as in feed additives to promote faster growth of an animal and to compensate for unsanitary and crowded conditions. The amount of antibiotics used non-therapeutically in agriculture is eight times greater than the amount used in all human medicine (Mellon *et al*., 2001). The non-therapeutic use of antibiotics in agriculture is at a low-concentration over a long period of time. Long-term exposure to low antibiotic concentrations is the condition most likely to foster stable maintenance of resistance genes because it gives incoming elements and resistance genes a chance to adapt to their new host (Salyers and Amábile-Cuevas, 1997). Many of the antibiotics used in agriculture are also used to treat humans. The antibiotic resistant bacteria can be transferred to humans via food, contaminated soil and water and through contact with the animals (Mellon *et al*., 2001).

This inappropriate use of antibiotics exerts a selective pressure that acts as a driving force in the development of antibiotic resistance (Barbosa and Levy, 2000). As resistance to these first-line antibiotics occurs, therapy with new, broader spectrum and more expensive antibiotics increases. Increased usage of these antibiotics eventually leads to resistance to the newer drugs (Hart, 1998). However, all antibiotic use whether appropriate or inappropriate exerts selective pressure for the emergence of resistant bacteria (Seppäla *et al*., 1997). Our only means of handling the situation is through prudent use of antimicrobial agents, improved diagnostics and infection control (Fishman, 2006).

Resistance factors, especially those carried on mobile elements, can spread rapidly within human and animal populations. These mobile elements enable resistant bacteria to rapidly spread both locally and globally. Antibiotic resistance patterns vary locally and regionally and so surveillance data is collected from selected sources (Agustín *et al*., 2005). The data obtained from this surveillance can indicate if doctors are appropriately or inappropriately prescribing antibiotics. Although a few studies (Rice *et al*., 1990), (Seppälä *et al*., 1997) have suggested that resistant clones can be replaced by susceptible ones, in general, resistance is slow to reverse or is irreversible (World Health Organisation, 2001).

1.3.2 Mechanisms of antibiotic resistance

Bacterial resistance to antibiotics occurs in many ways, reflecting the different ways in which the various classes of antibiotics have an effect. Antibiotics fall into the following classes: cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors, cytoplasmic membrane function inhibitors and other agents that affect DNA and RNA (Williams *et al*., 1996).

Resistance can be acquired (also known as active) (i.e., the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic) or passive (also known as innate) (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the non-specific barrier afforded by the outer membrane of Gram-negative bacteria) (Wikens and Wade, 2005). Acquired resistance results from: mutations – spontaneous single or multiple changes in the bacterium's chromosomal DNA occurring at a rate of about 10^{-9} to 10^{-5} per cell, per generation cycle (a generation cycle commonly occurs every 20 minutes or so), or by the addition of new DNA – most commonly by the acquisition of plasmids, transposons and integrons (Masterton, 2003).

The major advantage, in terms of dissemination of resistance, of mutations is that the progeny stably inherits them (Courvalin, 1996). Bacterial enzymes that are involved in normal physiological cell metabolism, can, as a result of single or multiple

mutations in their genetic determinants change their substrate spectrum and degrade antibiotics (Martinez and Baquero, 2000). Bacteria can become resistant to many antibiotics by modifying their target structures by single- or multi-step mutations, so that antibiotics cannot bind to them. These mutations usually have little or no influence on the biological activity of the gene products but render them insensitive to the inhibitory activities of a particular antimicrobial agent. Once established in a pathogen, genes encoding enzymes that catalyse covalent modifications of therapeutic agents can undergo mutations that remodel the active site of the enzyme, changing the spectrum of antibiotics that may be modified (Davies, 1994).

The genetic determinants of defence mechanisms may originate from bacteria such as antibiotic producing bacteria. The antibiotic producers possess defence mechanisms against their own antibiotic and these resistance genes are usually found in their chromosomes. These genes can be integrated into mobile genetic elements such as plasmids, transposons and integrons and passed on by horizontal transfer to other bacteria, thus conferring antibiotic resistance to those bacteria (Masterton, 2003).

Plasmids are extrachromosomal, double stranded DNA molecules that have been found in almost all bacterial genera of medical importance. They vary in size from 2 kbp to more than 100 kbp. Plasmids are capable of autonomous replication within the bacterial cell. They encode the mechanism for their own mobilization and are therefore excellent vehicles for transferring genes, not only to their progeny but also from one bacterium to another (Schwarz and Nobel, 1999). Plasmids generally carry genes that play a role in the bacterium's adaptation to a change in its environment, such as those involved in resistance to antibiotics, disinfectants and heavy metals (Dobrindt *et al.*, 2004). However, they also encode metabolic properties such as metabolism of carbohydrates and amino acids, virulence factors such as haemolysins and enterotoxins and conjugal properties such as sex pili production and mobilisation function. Plasmids may be integrated in part or in total into the chromosomal DNA or may represent vectors for transposons via conjugation or transformation (Schwarz and Nobel, 1999).

Transposons are double-stranded DNA elements which, in contrast to plasmids, cannot replicate autonomously. They carry transposition genes along with other

genes. They have to integrate into replication-proficient vector molecules such as plasmids or chromosomal DNA for replication. They can insert more or less at random into plasmids or bacterial chromosomes (Masterton, 2003). They vary in size from 1 kbp to more than 60 kbp (Schwarz and Nobel, 1999). Transposons carry one or more additional genes most of which are genes conferring resistance to antibiotics, which can then 'jump' between the bacterial chromosome and a plasmid and vice versa (Roy, 1999).

Integrons are another vehicle by which antibiotic resistance determinants can be passed on from one bacterium to another. Antibiotic genes are frequently trapped in gene cassettes on integrons, which provide an efficient means for capturing and exchanging various resistance genes (Van Belkum *et al*., 2001). Recruiting exogenous genes represents a rapid adaptation against antimicrobial compounds, and the integron functional platform is perfectly suited for capturing the genes that enable bacteria to survive during multiple antibiotic treatment regimes (Mazel, 2004). Integrons have been almost exclusively found in Gram-negative bacteria (Nešvera *et al*., 1998).

Integrons recognize and capture mobile gene cassettes. Gene cassettes consist of a specific recombination site and one gene that is in most known cases an antimicrobial resistance gene (Hall and Collis, 1995). The resistance integron platforms don't allow for self-transposition. However, this defect is often complemented through association with transposons and conjugative plasmids, which can serve as vehicles for the transmission of genetic material between bacteria (Mazel, 2004).

Integrons possess two essential elements, located at the 5' conserved segment (CS), able to mobilize and insert gene cassettes. These are an *intI* gene encoding a site-specific recombinase belonging to the integrase family and its associated primary recombination site, *attI*. Class 1 integrons also possess a quaternary ammonium compound resistance gene, located at the 3' conserved segment (Collis and Hall, 1995). Captured genes (usually antibiotic resistance genes) are part of discrete mobile cassettes that contain the protein-coding region and a 3'-associated integrase-specific recombination site known as *attC*, belonging to the family of sites known as 59-base elements (Recchia and Hall, 1995). Uncaptured gene cassettes exist in their free form as circular molecules consisting of only one open reading frame and a 59-base

element situated downstream. Integration of these gene cassettes involves IntI-catalysed site-specific recombination between the integron associated *attI* site and the *attC* (59-base element) recombination site associated with the incoming gene cassette (Collis *et al*., 1998). Each gene cassette has a unique 59-base element, which differs in both sequence and length (Collis *et al*., 1998). The *attC* sites vary from 19 bp to 141 bp and their nucleotide sequence similarities are primarily restricted to the inverse core-site and the core-site (Mazel, 2004). The 59-base element family exhibits a common inverted repeat structure and consensus sequences at each end that consists of approximately 25 bases (Collis *et al*., 1998). The outer boundaries of the 59-base element also contain the conserved seven base pair core site GTTRRRY (R = purine, Y = pyrimidine) at the recombinant cross-over point, and an inverse core site RYYYAAC at the 3' end of the inserted gene cassette (Poirel *et al*., 2001 [a]).

Integrons can be divided into two major groups, resistance integrons and super-integrons (Hall and Stokes, 2004). Resistance integrons carry mostly gene cassettes that encode resistance against antibiotics and disinfectants, and can be located either on chromosome or on plasmids (Fluit and Schmitz, 1999). The larger chromosomally–located integrons that contain gene cassettes with a variety of functions belong to the super-integron group. The super-integrons were initially known as class 4 integrons (Shi *et al*., 2006).

There are three different classes of resistance integron, class 1, 2 and 3, defined on the basis of homology of their integrase genes (Norrby, 2005). Each class appears capable of sharing and acquiring the same gene cassettes (Mazel, 2004). Most resistance integrons belong to class 1 and these integrons have been found in many Gram-negative genera including *Pseudomonas* (Fluit and Schmitz, 2004). More than 60 different gene cassettes have been described, the majority of which encode resistance to antibiotics (Lindstedt *et al*., 2003). Class 1 integrons are the most prevalent class in clinical isolates, carrying single or multiple gene cassettes (Weldhagen, 2004). Integron inserted genes encode for various antibiotic resistance mechanisms, conferring resistance to beta-lactams, aminoglycosides, sulphonamides, chloramphenicol, macrolides, rafampin, erythromycin, disinfectants and antiseptics of the quaternary ammonium compound family (Weldhagen, 2004). Class 1 integrons are frequently located on plasmids that can be transferred by conjugation (Girlich *et*

al., 2001). Class 2 integrons are embedded in the Tn7 family of transposons and consist of an integrase gene followed by gene cassettes. Class 3 integrons have been described in some isolates from Japan and are similar in structure to class 2 integrons (Fluit and Schmitz, 2004).

Class 1 integrons are associated with a variety of resistance gene cassettes. Class 1 integrons isolated from bacterial infections in humans often harbour gene cassettes encoding ß-lactam resistance (Fluit and Schmitz, 1999). Several class A β-lactamases are encoded by integron-located gene cassettes. These include VEB, GES, IBC, PSE and CTX-M type enzymes. Two families of class B β-lactamases have been found on integrons. These are the IMP family and the VIM family. Class D β-lactamases found on integron structures belong to the OXA-type family (Fluit and Schmitz, 2004). They commonly occur on Class 1 integrons from *Pseudomonas aeruginosa*, but in rare cases have been found in *Salmonella enterica* and *Enterobacter aerogenes* (Tosini *et al*., 1998), (Ploy *et al*., 1998). OXA-type genes found on gene cassettes within integrons in *Pseudomonas aeruginosa* tend to be the secondary *bla*-gene cassette on the integron, with a class A-type *bla*-gene mostly functioning as the primary integron-borne β-lactamase (Livermore, 2002), (Poirel *et al.*, 2001[b]). This is in contrast to oxacillinase genes found on class 1 integrons from *Enterobacteriacea*e, which tend to be carried as sole β-lactamase gene cassettes on the integron along with other co-resistance genes (Tosini *et al*., 1998), (Ploy *et al*., 1998). In the absence of an antibiotic selective pressure, the integron-borne resistance genes can be lost by the host bacterium (Rosser and Young, 1999).

Resistance to a variety of non-related compounds can be conferred simultaneously by the presence of co-resistance gene cassettes on integrons. Poirel *et al.* (2002) reported that integron-mediated drug resistance tends to favour co-selection of isolates. This allows for widespread dissemination through patients with a wide variety of clinical disciplines. It is common for gene cassettes encoding aminoglycoside-modifying enzymes to co-occur with β-lactamase gene cassettes. On Class 1 integrons these genes occur with the co-resistance genes to quaternary ammonium compounds and sulphonamides at the distal 3' end of the integron (Poirel *et al*., 2002), (Di Conza *et al*., 2002).

The horizontal transfer of genetic material within and between bacteria has been extremely important in the emergence of novel antibiotic resistance traits observed worldwide (Maiden, 1998). Rapid and widespread emergence of resistance and similar patterns of resistance have been encountered in phylogenetically diverse clinical isolates on an increasing scale (Rowe-Magnus *et al.*, 2002).

Bacteria achieve active drug resistance through four major mechanisms: the efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins, modification of the antibiotic target (e.g., through the mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways, such as in resistance to the glycopeptide antibiotics), and via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics (Table 1.3, p. 43). All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics. In general, the antibiotics or their action usually regulate the expression of resistance genes. Therefore, bacteria expend a considerable amount of energy and genetic space to actively resist antibiotics (Wright, 2005).

1.3.2.1 Membrane barrier

Bacteria use several ingenious mechanisms to develop resistance to antibiotics. These include degradation of the antibiotic (β-lactamases), inactivation of the antibiotic by enzymatic modification (aminoglycoside modifying enzymes) and altering the target of the antibiotic (Walsh, 2003). These mechanisms are all specific for a single drug or single class of drugs. There are more general mechanisms of drug resistance, in which access of the unaltered antibiotic to its target is prevented by the barrier and active transport functions of the biological membranes. The organism can surround itself with a barrier of low permeability in order to decrease the influx of the drug into the cell and can also pump out the drug in an energy-dependent fashion (Nikaido, 1994).

Bacteria are unicellular organisms and their internal environment is separated from the external environment by the cytoplasmic membrane. The major permeability barrier in any membrane is the lipid bilayer structure. Some bacteria further protect themselves by making an additional structure that surrounds the cell, outside the cytoplasmic membrane. Gram-positive bacteria surround themselves with a thick layer of peptidoglycan but this offers little protection against most antibiotics. Gram-negative bacteria are surrounded by a second membrane, called an outer membrane, which functions as a very effective barrier (Nikaido, 1994). It is composed of an unusual lipid known as lipopolysaccharide (LPS). The fatty acid chains in this LPS are all saturated, therefore making the interior of the bilayer fluid and preventing the tight packing of the hydrocarbon chains. In general, the larger number of hydrocarbon chains linked to a single head group decreases the fluidity of the lipid interior. Hydrophobic molecules permeate across the outer membrane about one-hundredth the rate through the usual bilayers (Vaara *et al*., 1990) Most clinically important antibiotics show some hydrophobicity. The LPS-containing bilayers therefore act as an efficient barrier against rapid penetration of many antibiotics (Nikaido, 1994).

However, with such an effective barrier, Gram-negative bacteria have developed a separate mechanism to bring essential nutrients into the cell. They do this by means of a special class of proteins known as porins, which produce non-specific aqueous diffusion across the membrane (Nikaido, 1994). These porins also make the influx of antibiotics almost impossible because of their narrow openings and because the openings are lined with a number of charged amino acid residues, which orient the water molecules in a fixed direction. This makes the entrance of lipophilic molecules difficult because it disturbs this energetically favourable orientation of water (Schulz, 1993).

Even with this arrangement, hydrophilic agents such as some of the newer β-lactam antibiotics can penetrate through the porins of enteric bacteria (Yoshimura and Nikaido, 1985). To overcome this, *Pseudomonas aeruginosa* lacks the typical high–permeability porins but instead has low-efficiency porins, which only allow the diffusion of small molecules at about one-hundredth the rate through classical porins (Bellido *et al*., 1992). In order for it to take up nutrients, *P. aeruginosa* has a number

of special channels that enable it to take up a specific class of compound. This makes this bacterium intrinsically resistant to most antibiotics (Hancock *et al*., 1990). However, even the most effective permeability barrier in bacteria cannot completely shut out the influx of small molecules. Even the low permeability membrane of *P. aeruginosa* can only prolong the half-equilibration time of most antibiotics for a few minutes (Nikaido, 1989). Therefore, a second mechanism of antibiotic resistance is usually required in addition to a low permeability barrier.

1.3.2.2 Efflux of the antibiotic

For antibiotics to be effective they must reach their specific bacterial targets and accumulate at the concentrations that can act in some reasonable time frame. For example, antibiotics that act on the ribosome must pass through the cell membranes into the cytoplasm and then accumulate at high enough concentrations that they can block the particular susceptibility step of protein assembly (Walsh, 2000). Both bacterial and eukaryotic cells typically contain an array of cytoplasmic membrane transport systems involved in vital roles such as the uptake of essential nutrients, the excretion of toxic compounds, and the maintenance of cellular homeostasis (Qinghu *et al*., 2006). Increasing numbers of such transport systems are being identified, primarily because of the explosion in the use of cloning and sequencing technology over the last 20 years (Paulsen *et al*., 1996). At least 300 gene products are proposed to transport known substrates effectively, out of which around 20-30 transport antibiotics and other drugs (Van Bambeke *et al*., 2003).

Both Gram-negative and Gram-positive bacteria commonly produce proteins, which act as efflux pumps for antibiotics. If the drug is pumped out faster than it can diffuse in, intrabacterial concentrations of the antibiotic are kept low and ineffectual. Therefore, the bacterial protein synthesis proceeds at largely unimpeded rates. These pumps are variants of membrane pumps that all bacteria possess in order to move lipophilic and amphipathic molecules in and out of the cell. Antibiotic producing microorganisms possess these pumps in order to pump antibiotics out of the cell as fast as they are produced. This acts as a protective mechanism for the microorganism and prevents it from being killed by its own chemical weapons (Walsh, 2000). Most

drug efflux pumps have broad substrate specificity and, therefore may deal with a wide range of drugs of completely unrelated pharmacological classes. Drug efflux decreases the load on efflux mediated detoxification systems, thereby avoiding their saturation, while the chemical modification by enzyme-based systems, which usually increases the amphiphilicity of the drug, provides the pumps with better substrates (Van Bambeke *et al*., 2000).

Four protein families of efflux pumps that can function in antibiotic resistance have been described (Van Bambeke *et al.,* 2000) (Figure 1.11, p.40). The first three couple drug efflux to a counterflow of protons, while the fourth uses the hydrolysis of ATP to provide the energy for active transport of the antibiotic or other foreign compounds out of the cell (Paulsen *et al*., 1996). The pumps driven by proton motive force are categorized in the major facilitator subfamily (MFS), the small multidrug regulator (SMR) family or the RND (resistance/nodulation/cell division) family, based on the projected size and the need for partner proteins and subunits. The second major category of efflux pumps, those hydrolysing ATP, is called the ATP-binding cassette (ABC) family. The ATP-driven pumps predominate in eukaryotes, whereas the proton driven antiporters predominate in bacterial genomes (Walsh, 2003). The genes encoding these pumps can be found on plasmids, transposons or even as part of integrons, which facilitates widespread dissemination of the genes. However, several of these pumps are already encoded in microbial genomes (Van Bambeke *et al*., 2003).

The mechanism of transport and of substrate recognition remains largely unknown in most instances, and many of the current views are based on extrapolations from data obtained with transporters of physiological substrates. SMR, RND, and most MFS transporters use a proton gradient as the driving force. The putative method of drug transport, as established by site-directed mutagenesis of a SMR transporter, could involve the following steps: (i) exchange between the drug and a proton fixed on a charged residue; (ii) translocation of the drug by a series of conformational changes driving it through a hydrophobic pathway; and (iii) replacement of the drug by a proton in the external medium and return to the initial conformational state (Mordoch *et al*., 1999). The overall result of the transport is therefore an exchange between a drug and a proton (antiport). As for proton antiporters, conformational change of the

ABC protein is necessary for drug extrusion and probably is triggered by drug binding and ATP hydrolysis (Van Bambeke *et al.*, 2000).

The exact mechanism for drug transport is still controversial. Among the different models that have been proposed, the two most likely ones present efflux pumps as acting either like hydrophobic 'vacuum cleaners' or like flippases. In the first model, the drug is thought to move freely into the lipid phase of the membrane, then reaching the protein and its central channel, from where it is actively expelled outwardly. In the second model, the drug is also thought to reach the protein from within the membrane, but then would be flipped to the outer layer (Van Bambeke *et al.*, 2000).

It must be emphasised that a given antibiotic may be a substrate for different types of pumps and a given pump may extrude not only different antibiotics within the same class but also different classes of antibiotics. Finally, a single cell may possess a vast and complex arsenal of efflux pumps allowing for the extrusion of a very broad spectrum of drugs (Van Bambeke *et al.*, 2000).

Four different efflux systems dependent on the genes *mexAB-oprM* (β-lactams), *mexXY-oprM* (aminoglycosides), *mexCD-oprJ* and *mexEF-oprN* (carbapenems and quinolones) are known to exist, allowing extrusion of all classes of antibiotics except the polymixins (Poole and Srikumar, 2001). Genes for these efflux systems are found in all strains of *Pseudomonas aeruginosa* but are expressed at relatively low levels, under the control of regulatory genes. Mutations in these regulators can lead to high-level expression and confer enhanced antibiotic resistance (Poole and Srikumar, 2001).

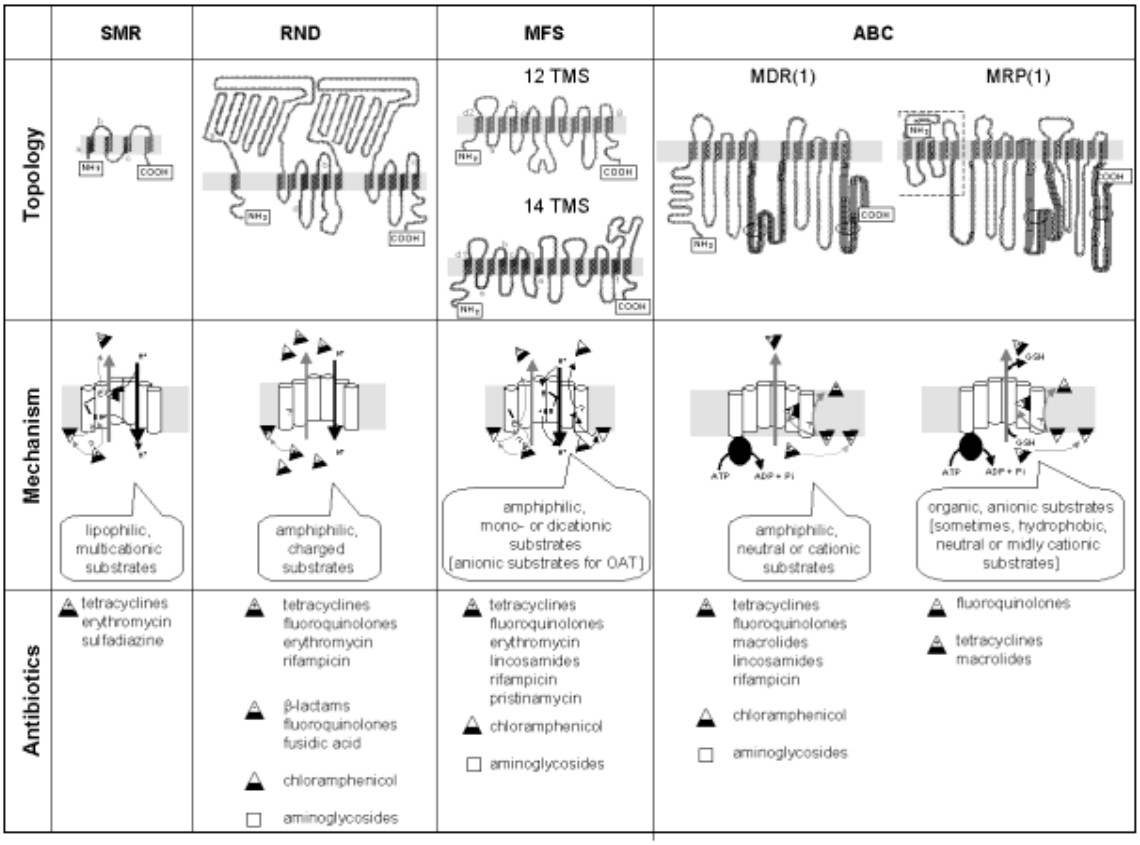


Figure 1.11: Main classes of efflux pumps acting on antibiotics
(Van Bambeke *et al*., 2000)

1.3.2.3 Reprogramming the target structure

This mechanism of antibiotic resistance focuses on reprogramming or camouflaging of the antibiotic target in the bacteria rather than destruction or removal of the antibiotic. Methicillin, with its bulky substitution on the 6'-aminopenicillin scaffold was introduced in 1950 to treat Gram-positive bacterial infections that were resistant to penicillin via inducible β-lactamase hydrolysis of the antibiotic. The side chain slowed the deacylation hydrolytic step and effectively deactivated the β-lactamase. This was effective until methicillin resistant strains of *S. aureus* (MRSA) appeared in 1961. These bacteria had acquired the *mecA* gene, which encodes a new penicillin-binding protein, termed PBP2A. MRSA are resistant to essentially all β-lactam molecules. This insensitivity to β-lactams stems from the low binding affinity of the

mecA-encoded PBP2A, a bifunctional transglycosylase/transpeptidase. In contrast, the normal PBPs, PBP1-PBP4, may remain sensitive to acylation by β-lactams in methicillin sensitive *S. aureus*. The morphology of the peptidoglycan being synthesised by PBP2A in the absence of other PBPs is somewhat altered but sufficient to allow MRSA growth (Walsh, 2003).

In the erythromycin-resistance manifolds, in addition to efflux pumps, resistant bacteria have emerged that have learned to mono- or dimethylate a specific adenine residue, A2058, in the peptidyl transferase loop of the 23S RNA component of the ribosome. A methyl transferase enzyme known as Erm^{27} carries out this modification. It does not impair protein biosynthesis but does lower the affinity of all members of the erythromycin class of drugs for the RNA. This Erm^{27} mechanism is the main resistance mechanism of drug resistant clinical isolates of *S. aureus* and is present in erythromycin-producing strains as a self-immunity mechanism (Walsh, 2000).

The increasing use of vancomycin to treat MRSA has selected for vancomycin resistant enterococci (VRE). These pathogens are less potent than staphylococci, but are opportunistic pathogens and can cause problems in immunocompromised patients. VRE use a reprogramming strategy to avoid vancomycin. In VRE, the *vanHAX* genes encode a new pathway of enzymes that reduces pyruvate to D-lactate (*vanH*), adds D-alanine and D-lactate together to produce D-Ala-D-Lac (*vanA*), and then hydrolyses the normal metabolite D-Ala-D-Ala while sparing D-Ala-D-Lac (*vanX*). In this cell, only the D-Ala-D-Lac accumulates and serves as a substrate to be elongated and presented at the termini of the peptidoglycan strands. This analog is a good substrate for transpeptidase cross-linking, enabling the covalently cross-linked, mechanically sound peptidoglycan to be produced such that VRE are not labile to osmotic lysis. The reprogramming of peptidoglycan to end in D-Ala-D-Lac instead of the normal D-Ala-D-Ala has no effect on the crosslinking by the transpeptidating PBPs but the new terminus lowers the binding affinity for vancomycin by 1000-fold (Walsh, 2003).

1.3.2.4 Cell wall permeability

Aminoglycosides, quinolones, β-lactams and polymixins must cross the bacterial cell wall to reach their targets. Penetration can be reduced by the development of an antibiotic-resistant biofilm with secretion of an anionic exopolysaccharide matrix, which binds cationic antibiotics (Drenkard and Ausubel, 2002). The outer membrane of Gram-negative bacteria may represent a permeability barrier for certain antibiotics. Reduced uptake may be due to the reduced expression, alteration or even loss of porins which allow the antibiotic to enter the bacterial cell. The outer membrane, which excludes large molecules, also limits penetration by small hydrophilic antibiotics, which must pass through aqueous channels in porin molecules (Nikaido, 1994). Aminoglycosides and colistin promote their absorption through the cell wall by binding to the superficial lipopolysaccharide (LPS), allowing penetration and then their active transport via the cytoplasmic membrane antibiotics (Drenkard and Ausubel, 2002). A switch in the charge of the cell wall lipopolysaccharides from a negatively charged into a more neutral form may prevent highly positive charged antibiotics such as aminoglycosides from crossing the outer membrane (Schwarz and Nobel, 1999).

1.3.2.5 Enzymatic strategies of antibiotic inactivation

There are wide ranges of enzymes produced by bacteria, which can inactivate one or more antibiotics. These remarkable proteins use many strategies to confer antibiotic resistance. Some of these enzymes inactivate the antibiotic by hydrolysing susceptible bonds which are central to the antibiotics' biological activities (β-lactamases), whereas others modify the antibiotic resulting in structural alterations that impair target binding (aminoglycoside modifying enzymes) (Walsh, 2003).

Table 1.3: Major antibiotics: structural classes, targets, modes of action and resistance mechanisms (adapted from Walsh, 2003)

Antibiotic	Target	Mode of action	Resistance mechanism
Cell wall			
B-lactams	Transpeptidases/ Transglycosylases (PBPs)	Blockade of crosslinking enzymes in peptidoglycan layer of cell walls	β -lactamases, Penicillin Binding Protein mutants
Glycopeptides	D -Ala- D -Ala termini of peptidoglycan and of lipid II	Sequestration of substrate required for crosslinking	Reprogramming of D-Ala- D – Ala to D -Ala- D -Lac or D-Ala- D –Ser
Protein synthesis			
Aminoglycosides	Ribosome	Blockade of protein synthesis	rRNA methylation Drug efflux Aminoglycoside modifying enzymes
DNA replication / repair			
Fluoroquinolones	DNA gyrase	Blockade of DNA replication	Efflux pumps, gyrase mutations

1.3.3 Antibiotic resistance in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa infections have always been difficult to treat with antibiotics. The completion of the sequence of *P. aeruginosa* revealed why it is such a resistant species (Stover *et al*., 2000). The *Pseudomonas aeruginosa* genome contains about 6.26 Mbp with 5567 open reading frames. Its genome is among the largest genomes in the prokaryotic world. The *Pseudomonas aeruginosa* genome is thus substantially larger than that of *Escherichia coli* (4.64 Mbp, 4279 genes) and of *Staphylococcus aureus* (2.81 Mbp, 2594 genes) (Lambert, 2002). The *P. aeruginosa* genome encodes an unusually high proportion of proteins involved in regulation,

transport and virulence functions. Genes encoding proteins involved in antimicrobial resistance are encoded by 0.3% of the total genes in the genome. Ten percent of the genes are located on pathogenicity islands comprising genes encoding virulence factors. It can also easily acquire large mobile genetic elements such as integrons that encode resistance genes. All this means that the *P. aeruginosa* genome is highly flexible (Mesaros *et al*., 2007).

Chromosomal mutations within its genome can lead to changes in regulation of resistance genes. It can also acquire resistance genes from other organisms via plasmids, transposons and bacteriophages (Lambert, 2002).

P. aeruginosa is noted for its intrinsic resistance to many front-line antibiotics, due mainly to its lower outer membrane permeability and to active efflux of antibiotics. It would appear that, in the course of evolving the functional diversity required to compete with other microorganisms in a variety of environments, it developed mechanisms for resisting naturally occurring antimicrobial compounds. (Stover *et al*., 2000).

Pseudomonas aeruginosa possesses most of the antibiotic resistance mechanisms mentioned in Section 1.3.2. These include resistance conferred from its low-outer membrane permeability (Li *et al*., 1994 [a]), the low permeability of its cell wall (Lambert, 2002), its large array of porins to exclude antibiotics (Bellido *et al*., 1992), its efflux pumps to pump antibiotics from the cell (Köhler *et al*., 1996) (Li *et al*., 1994 [a]), its ability to reprogramme the target of the antibiotic, such as mutating the *gyrA* gene (the target for quinolone antibiotics; Lambert, 2002) and the production of the antibiotic inactivating enzymes, aminoglycoside modifying enzymes and β-lactamases (Walsh, 2003).

1.3.4 Aminoglycoside modifying enzymes

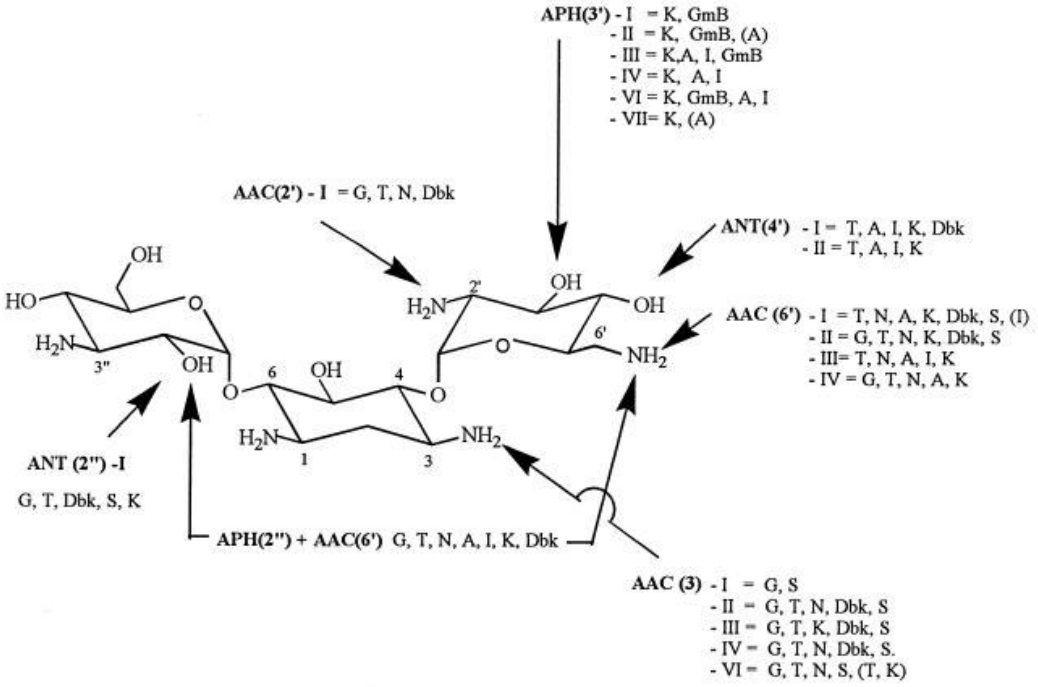
The use of aminoglycosides has reduced potential due to the emergence of aminoglycoside resistant strains of bacteria. Bacterial resistance to aminoglycosides is mainly based on either the modification of the ribosomal target, decreased uptake and

accumulation or enzymatic modification by aminoglycoside modifying enzymes (Schwartz and Noble, 1999).

Alteration of the ribosomal binding sites usually only causes significant resistance to streptomycin and spectomycin. Resistance in this case is caused by mutational changes in ribosomal proteins or 16S rRNA and enzymatic methylation of the rRNA. (Sigmund *et al*., 1984). Another mechanism of drug resistance in bacteria is the active export or efflux of the antibiotic so that lethal concentrations are not attained in the cytoplasm. The antibiotic is not altered or degraded in any way (Vakulenko and Mobashery, 2003). This active efflux is attained and controlled by transmembrane proteins acting as pumps to export the aminoglycoside, often against gradients, both in the cytoplasmic membranes and also in the outer membrane proteins of Gram-negative bacteria (Walsh, 2003). This type of resistance produces low-level broad-spectrum resistance in bacteria (Vakulenko and Mobashery, 2003). Active efflux has been shown to produce resistance to neomycin and kanamycin (Edgar and Bibi, 1997). Decreased drug uptake and accumulation is often due to membrane impermeabilisation. It produces moderate level resistance to aminoglycosides (Mingeot-Leclercq *et al*., 1999).

The most prevalent source of clinically relevant resistance to aminoglycosides is conferred by aminoglycoside modification (Vakulenko and Mobashery, 2003). These antibiotics are enzymatically inactivated by aminoglycoside modifying enzymes. Resistance through enzymatic deactivation is a complex phenomenon involving three different classes of enzyme. Aminoglycoside-modifying enzymes catalyze the covalent modification of specific amino or hydroxyl functions, leading to a chemically modified drug (ester or amide), which binds poorly to ribosomes and for which the EDP-II of accelerated drug uptake fails to occur. The deactivation of the aminoglycoside is catalyzed by bacterial enzymes that transfer an acetyl group, derived from acetyl-CoA, an adenyl group, derived from ATP or a phosphate group, also derived from ATP (Walsh, 2003). These are broadly classified as *N*-acetyltransferases (AACs), *O*-nucleotidyltransferases (ANTs) (or *O*-adenyltransferases) and *O*-phosphotransferases (APHs). Each of the three families is further divided into classes, designated by the site of modification (Figure 1.12), which is indicated in parentheses. These are then further subdivided into enzyme

types, designated by Roman numerals. Finally, the individual enzymes of the same class and type that produce the same phenotype but are encoded by different genes are designated by a lowercase letter (Shaw *et al*., 1993). For example, the AAC(6')-I enzymes AAC(6')-Ia, AAC(6')-Ib, AAC(6')-Ic, etc., are aminoglycoside acetyltransferases that modify the antibiotic at position 6' and produce the same phenotype but are encoded by different genes (Vakulenko and Mobashery, 2003). Shaw *et al*. (1993) listed fifty-seven variants in a comprehensive literature review of aminoglycoside modifying enzymes. These are the largest and most diverse family of resistance enzymes.



A, amikacin; **Dbk**, dibekacin; **Gm**, gentamicin; **I**, isepamicin **Km**, kanamycin; **Lv**, lividomycin; **N**, netilmicin; **S**, sisomicin; **T**, tobramycin.

Figure 1.12: Sites of action of aminoglycoside modifying enzymes on aminoglycoside antibiotics (Avaiable at http://www.md.ucl.ac.be/infect/antiinfectieux /Assets/PLS/AG/AG-res-enz-540.gif).

Aminoglycoside modifying enzymes (AMEs) are often plasmid encoded but are also associated with transposable elements. Plasmid exchange and dissemination of transposons aid the rapid acquisition of a drug resistance phenotype not only within a species but also among a large variety of species (Mingeot-Leclercq *et al*., 1999). A

list of the antibiotic resistance profiles for aminoglycoside modifying enzymes is shown in Table 1.4 (p 51).

1.3.4.1 Aminoglycoside *N*-Acetyltransferases (AACs)

This is the largest of the three AME groups (Shaw *et al*., 1993). They are members of the GCN5 superfamily of proteins (Dyda *et al*., 2000). This group comprises four classes of enzyme; AAC(1), AAC(3), AAC(2') and AAC(6'). They use acetyl-CoA as the donor of the acetyl group in modifying aminoglycosides at position 1 and 3 of the 2-deoxystreptamine ring and positions 2' and 6' of the aminohexose ring (Shaw *et al*., 1993).

AAC(6')s are the largest group of aminoglycoside acetyltransferases and are the most common mechanism of aminoglycoside acetyltransferase-mediated aminoglycoside resistance in clinical isolates of bacteria. They are capable of modifying almost all of the clinically important aminoglycosides (Vakulenko and Mobashery, 2003). The AAC(6')-I produces resistance to amikacin, tobramycin, kanamycin, netilmicin, isepamicin, dibekacin and sisomicin (but not to gentamicin) (Shaw *et al*., 1993). At least 26 AAC(6')-I enzymes have been identified (Smith and Baker, 2002). Two AAC(6')-II enzymes, AAC(6')-IIa and AAC(6')-IIb have been discovered and they confer resistance to gentamicin, tobramycin, dibekacin, netilmicin and sisomicin (but not to amikacin). The AAC(6') enzymes are classified into three subfamilies by comparing their amino acid sequences. The first includes AAC(6')-Ib, AAC(6')-Ie, AAC(6')-IIa and AAC(6')-IIb. The second group contains AAC(6')-Ia, AAC(6')-Ii, AAC(6')-Ip, AAC(6')-Iq AAC(6')-Id. The third family contains AAC(6')-Ic, -Id, -If, -Ig, -Ih, -Ij, -Ik, -Il, -Ir, -Is, -It, -Iu, Iv, -Iw, -Ix and –Iz (Vakulenko and Mobashery, 2003).

AAC(6')-Ib is the most commonly found AAC(6') in bacteria. Shaw *et al*. (1993) found that among those Gram-negative strains producing the AAC(6') resistance profile, 70.6% possess the AAC(6')-Ib gene. The reason why the AAC(6)-Ib is so widely distributed among bacteria is that the gene for the enzyme is commonly found on mobile genetic elements, which facilitates its rapid transfer (Vakulenko and Mobashery, 2003). The aminoglycoside resistance profile of AAC(6')-IIa and

AAC(6')-IIb has only been observed in *Pseudomonas* strains. The *aac(6)-IIa* gene shows a 74% sequence identity with *aac(6)-Ib.* The presence of AAC(6)-II enzymes with the presence of low permeability and efflux pumps are now a major mechanism of resistance to aminoglycosides in *Pseudomonas aeruginosa* (Shaw *et al*., 1993)

The AAC(3) enzymes are the second largest group of aminoglycoside acetyltransferases (Shaw *et al*., 1993). AAC(3)-I enzymes, AAC(3)-Ia and AAC(3)-Ib, produce a narrow spectrum of resistance including sisomicin, gentamicin and fortimicin (Vakulenko and Mobashery, 2003). AAC(3)-II enzymes produce a wide range of resistance to tobramycin, sisomicin, gentamicin, netilmicin and dibekacin (Shaw *et al*., 1993). Less common AAC(3) enzymes include AAC(3)-III, AAC(3)-IV and AAC(3)-VI. The AAC(3)-III acetylates sisomicin, neomycin, dibekacin, gentamicin, tobramycin, kanamycin, lividomycin and paromomycin. AAC(3)-IV modifies netilmicin, tobramycin, gentamicin, apramycin and dibekacin but is rarely found among clinical strains. AAC(3)-VI produces resistance to gentamicin but is also rarely encountered in clinically important bacteria (Shaw *et al*., 1993). AAC(3)-VII, AAC(3)-VIII, AAC(3)-IX and AAC(3)-X have also been identified but are only found in aminoglycoside-producing actinomycetes (Vakulenko and Mobashery, 2003).

AAC(1) acetylates apramycin, paromomycin, lividomycin and ribostamycin. However, AAC(1) has not been found in any clinically important strains (Vakulenko and Mobashery, 2003). AAC(2')-I produces resistance to gentamicin, tobramycin, dibekacin and netilmicin (Shaw *et al*., 1993). It is primarily restricted to the *Providencia* group of organisms but a few cases of *Pseudomonas* strains with this enzyme have been observed. It is universally present in all clinical strains of *Providencia stuartii* (Shaw *et al*., 1993).

1.3.4.2 Aminoglycoside *O*-Nucleotidyltransferases (ANTs)

This group is the smallest of the three groups of acetyltransferases. It comprises five classes of enzymes; ANT(2"), ANT(3"), ANT(4'), ANT(6) and ANT(9). These enzymes use ATP as the second substrate and modify aminoglycoside antibiotics by

transferring AMP to their hydroxyl group at position 2'', 3', 4', 6 and 9 (Shaw *et al*., 1993).

ANT(2") is widespread among Gram-negative bacteria. The gene for this enzyme is found on plasmids, transposons and integrons. It produces resistance to gentamicin, tobramycin, dibekacin, sisomicin and kanamycin (Cameron *et al*., 1986). ANT(3")-I confers resistance to streptomycin by modifying its 3"-hydroxyl group and to spectomycin by modifying it at position 9. There are at least eight *ant(3")-I* genes and they show between 59% and 95% amino acid sequence identity (Partridge *et al*., 2002) (Shaw *et al*., 1993). ANT(4')-I confers resistance to tobramycin, amikacin, isepamicin and dibekacin. It is the main mechanism of aminoglycoside resistance in Japanese strains of *S. aureus* and has been found in 50% of MRSA strains in Europe (Schmitz *et al*., 1999). ANT(4')-II is only found in Gram-negative bacteria such as the Pseudomonads (Vakulenko and Mobashery, 2003). ANT(6)-I is found in enterococcal and staphylococcal isolates. It confers resistance to streptomycin. ANT(9)-I is only found in *Staphylococcus aureus*. It has limited clinical importance as it only produces resistance to spectinomycin (Vakulenko and Mobashery, 2003).

1.3.4.3 Aminoglycoside *O*-Phosphotransferases (APHs)

This is the second largest group of aminoglycoside modifying enzymes. Aminoglycoside *O*-phosphotransferases (kinases) use ATP as the second substrate and are able to phosphorylate specific hydroxyl groups in all classes of antibiotics (Vakulenko and Mobashery, 2003). There are seven classes of APH enzymes, APH(3'), APH(2"), APH(3"), APH(4), APH(7"), APH(6) and APH(9). Most of the enzymes belong to the APH(3') subfamily, of which there are eight types, APH(3')-I to APH(3')-VI (Shaw *et al*., 1993). These enzymes modify the hydroxyl groups of aminoglycosides at the 3' position. APH(3')-I confers resistance to kanamycin, neomycin, lividomycin, paromomycin and ribostamycin (Shaw *et al*., 1993). It is generally found on plasmids and transposons in Gram-negative bacteria, although it has been found in a Gram-positive strain, *Corynebacterium* (Ouellette *et al*., 1987). The ability of APH(3)-I to produce resistance to kanamycin has resulted in the clinical obsolescence of this antibiotic (Vakulenko and Mobashery, 2003).

APH(3)-II produces resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin and gentamicin. Although it has an impressive aminoglycoside resistance profile, it is rarely encountered in clinical isolates (Shaw *et al*., 1993). APH(3')-III confers resistance to kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, butirosin gentamicin, amikacin and isepamicin (Shaw *et al*., 1993). The gene for this enzyme is found in both Gram-negative and Gram-positive bacteria. APH(3')-IV and APH(3')-V are only found in antibiotic-producing microorganisms (Vakulenko and Mobashery, 2003). APH(3')-VI is responsible for resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin gentamicin, amikacin and isepamicin and is found primarily in *Acinetobacter* strains. APH(3')-VII confers resistance to kanamycin and neomycin (Shaw *et al*., 1993).

The next largest group consists of the APH(2")-I enzymes, of which there are four different types, APH(2")-Ia to APH(2'')-Id (Smith and Baker, 2002). These enzymes modify the 2" hydroxyl group of gentamicin, tobramycin and kanamycin. APH(2")-Ib produce mid-level resistance to the above antibiotics whereas APH(2")-Id confers higher levels of resistance (Shaw *et al*., 1993). Activity of these enzymes is restricted to enterococci (Vakulenko and Mobashery, 2003).

APH(3") modify the 3" group of streptomycin. There are two types APH(3")-Ia and APH(3")-Ib. APH(6) enzymes modify the 6-hydroxyl group of streptomycin. There are four types of these enzymes APH(6)-Ia to APH(6)-Id. APH(4) and APH(7") confer resistance to hygromycin and APH(9) produces resistance to spectinomycin. However APH(4), APH(7") and APH(9) have not been discovered in any clinically important strains (Vakulenko and Mobashery, 2003).

Table 1.4: Antibiotic resistance profiles for aminoglycoside modifying enzymes (modified from Shaw *et al.*, 1993)

Enzyme	Resistance Profile
Acetyltransferase	
AAC(1)	Apr, Lv, Prm, But, Neo
AAC(3)-I	Gm, Siso
AAC(3)-II	Gm, Tob, Ntl, Siso
AAC(3)-III	Gm, Tob, Siso, Km, Neo, Prm, Lv
AAC(3)-IV	Gm, Tob, Ntl, Apr, Siso
AAC(3)-VI	Gm, Siso, Tob, Ntl, Km
AAC(6')-I	Tob, Ntl, Amk, Siso
AAC(6')-II	Gm, Tob, Ntl, Siso
AAC(6')-APH(2'')	Gm, Tob, Ntl, Amk
AAC(2')-I	Gm, Tob, Ntl
Nucleotidyltransferase	
ANT(2'')-I	Gm, Tob, Siso, Km
ANT (3'')-I	Sm, Sp
ANT (4')-I	Tob, Amk
ANT(4')-II	Tob, Amk
ANT(6)-I	Sm
ANT(9)-I	Sp
Phosphorylase	
APH(3')-I	Km, Neo, Prm, Lv, Gm
APH(3')-II	Km, Neo, Prm, But, Gm
APH(3')-III	Km, Neo, Prm, Lv, But, Gm, Amk
APH(3')-IV	Km, Neo, Prm, But
APH(3')-V	Neo, Prm,
APH(3')-VI	Km, Neo, Prm, But, Gm, Amk
APH(3')-VII	Km, Neo, Amk
APH(3'')-I	Sm
APH(6)-I	Sm
APH(4)-I	Hyg

Amk, amikacin; **Apr**, Apramycin, **But**, butirosin; **Gm**, gentamicin; **Hyg**, hygromycin B; **Km**, kanamycin; **Lv**, lividomycin; **Neo**, neomycin; **Ntl**, netilmicin; **Sm**, streptomycin; **Prm**, paromomycin; **Siso**, sisomicin; **Sp**, spectinomycin; **Tob**, tobramycin.

1.3.5 GCN5-Related N-Acetyltransferases (GNAT)

One of the fundamental biochemical processes is the transfer of an acetyl group from one molecule to another. GCN5-related *N*-acetyltransferases (GNAT) catalyze the transfer of the acetyl group from acetyl coenzyme A to a primary amine. There are several members of the GNAT superfamily (Dyda *et al*., 2000). Histone *N*-acetyltransferase (HAT) enzymes are involved in the acylation of histones at specific lysine residues. This is a process that is required for transcriptional activation and that has been implicated in chromatin assembly and DNA replication. The serotonin N-acetyltransferase family catalyzes the penultimate step in the synthesis of the circadian neurohormone melatonin from serotonin. The circulating levels of melatonin are correlated with the light-dark cycle. They play a role in coordinating the sleep-wake cycle and adaptations to seasonal changes.

However, the most relevant member of the GNAT superfamily for this study is the bacterial aminoglycoside *N*-acetyltransferase (AAC). These enzymes are responsible for the chemical modification of aminoglycoside antibiotics, which results in a decreased affinity of the antibiotic for its target, the 30S ribosome. These N-acetyltransferases can be further divided into different subclasses depending on the regiospecificity of acetyl transfer (Dyda *et al*., 2000).

The three-dimensional structures of several of these GNAT enzymes have been determined (Tercero *et al*., 1992) and (Neuwald and Landsman, 1997). There are four conserved motifs, C, D, A and B. The most highly conserved motifs are A and B, with C being the least conserved. These four regions comprise the *N*-acetyltransferase domain. Although there is functional variation across this family of enzymes, the protein similarity is almost identical (Dyda *et al*., 2000). The structure of the aminoglycoside N-acetyltransferase is illustrated in Figure 1.13.

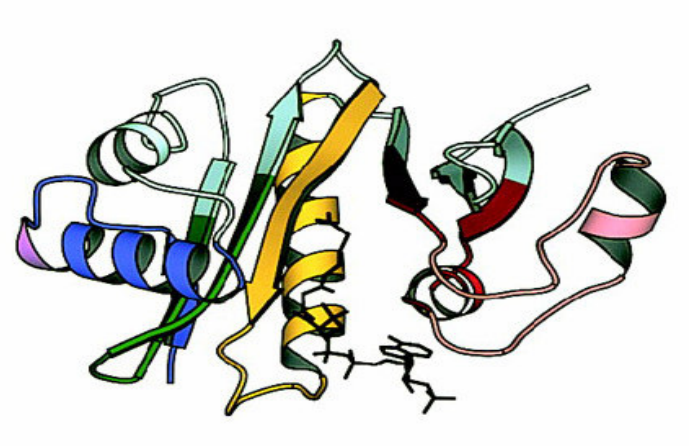


Figure 1.13: Three–dimensional structure of GCN5-related *N*-acetyltransferase. The four conserved motifs of the GNAT superfamily, C, D, A and B are shown in purple, green , yellow and red, respectively. The black lines indicate acetyl coenzyme A (Dyda *et al*., 2000).

Dyda *et al*. (2000) described the structure and function of the GNAT enzymes. The *N*-acetyltransferase domain folds around a central, mixed β-sheet that is built up of antiparallel strands. AAC(6') has seven strands in the β-sheet (Wybenga-Groot *et al*, 1999). The β-sheet that forms the core of the N-acetyltransferase domain is made up of two parts, one encompassing the first four strands and second, the last three strands. In the loop connecting the first and second strands, two helices run antiparallel to each other and almost perpendicular to the first four strands.

There are many interactions (primarily hydrophobic) between helices α1 and α2 and the four-stranded sheet below. These contribute to the rigidity of the first four strands of the β-sheet. There are a lot of hydrophobic interactions between the helices. These interactions, together with the hydrogen bonding between the antiparallel strands, result in a rigid and compact subdomain in this region of the molecule (Dyda *et al*., 2000).

Motif C includes the first helix. The second helix (α2) is shorter than α1. This part of the enzyme is subject to conformational changes, shortening or extending depending on whether the acetyl coenzyme A (AcCoA) binding site is occupied or not (De

Angelis *et al*, 1998). The polypeptide chain after α2 completes the first four strands of the sheet running through sequence motif D. Motif D includes most of strands β2 and β3 and turns into β4 where motif A, the longest and most highly conserved motif starts. Motif C and D interact with each other, forming the rigid subdomain that comprises the first half of the molecule. β4 is crucial for AcCoA binding and catalytic activity of the enzyme. Strand β4 and the structure following it (α3) form the essence of the AcCoA binding site. Motif A starts just before the parallel stretch of β4 and extends to α3. The parting of these two parallel strands results in a wedge-like opening in the centre of the protein where AcCoA binds (Dyda *et al*., 2000).

The β bulge is a characteristic feature of the GNAT family (Richardson, 1981). It is located in the middle of β4. This bulge is thought to break the parallel segment between β4 and β5, forming the AcCoA binding site. Downstream of the β bulge are three residues that form main-chain hydrogen bonds with the AcCoA substrate. The acetyl and pantetheine moieties of AcCoA (Figure 1.14) project carbonyl and amides groups to both sides and are separated at the correct distance to hydrogen bond with an adjacent β strand (Dyda *et al*., 2000).

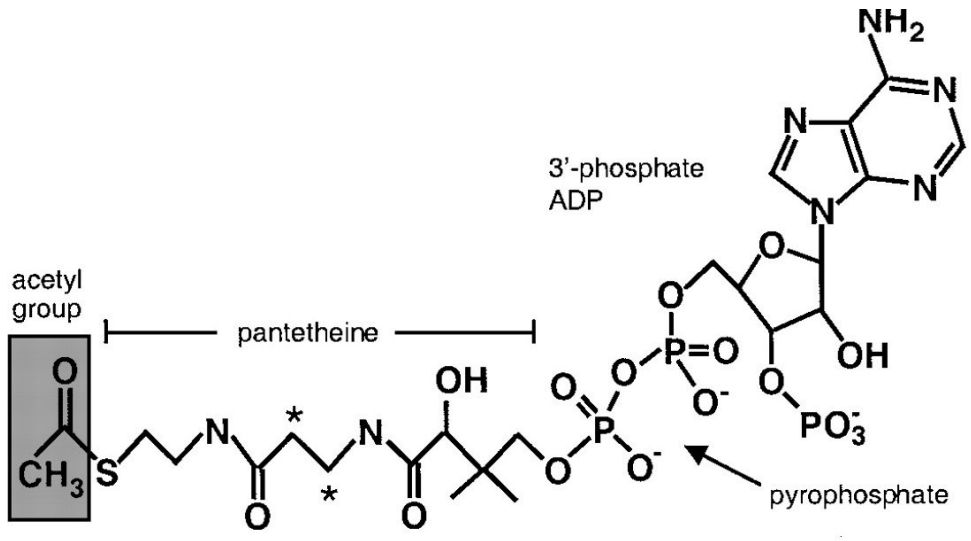


Figure 1.14: The chemical structure of acetyl coenzyme A (Dyda *et al*., 2000)

The pyrophosphate-binding pocket is found at the end of α3, the longest helix in the structure. Its helix dipole contributes to phosphate binding. α3 consists of five turns, which leads to β5. The turn between these two structures is where motif A ends. Motif B starts at β5. Although β5 and α4, the helix that follows it, lack direct hydrogen bonds, they do make interactions with the AcCoA substrate. There are many large hydrophobic residues located on α4 that contact the substrate, stabilizing this bottom part of the donor substrate-binding site. α4 also plays a role in correctly positioning the acetyl group of AcCoA for the transfer action to occur. Motif B ends at the end of α4. In AAC(6') there is then a long insertion between β5 and α4 between here and the C-termini (Dyda *et al*., 2000).

AcCoA binds in the opening formed between the diverging strands β4 and β5, contacting protein atoms from β4 and α4, through main-chain interactions. The carbonyl of the acetyl group is hydrogen bonded to the main-chain amine of a residue just downstream of the β bulge (Dyda *et al*., 2000).

Structure determinations have not yet been carried out that include aminoglycoside *N*-acetyltransferase but structure determinations have been carried out for arylalkylamine *N*-acetyltransferase (AANAT) (Hickman *et al*., 1999) and the *Tetrahymena* GCN5 *N*-histone acetyltransferase (HAT) (Rojas *et al*., 1999). In both cases the *N*-acetyltransferase domain uses the same regions to make key interactions with the amine-containing substrate. α1 and α2 are critical for interaction. This two-loop region is located on the surface of the molecule and defines a cleft located directly above the acetyl group of AcCoA. Both serotonin (substrate for AANAT) and the histone H3 (substrate of HAT) lie in this cleft. It seems that the formation of a GNC5/AcCoA complex is required prior to binding (Dyda *et al*., 2000).

There are two possible ways that acetyltransferases could catalyze the transfer of the acetyl group. The first is a ping-pong mechanism, where the acetyl group is transiently transferred to a suitably located cysteine residue of the enzyme, forming a covalently bound acetylated enzyme intermediate (Figure 1.15). The enzyme then catalyses the transfer of the acetyl group from the cysteine residue to the acceptor substrate. The second, and more likely, mechanism is the transfer of the acetyl group

directly from AcCoA to the acceptor via direct nucleophillic attack by the primary amine on the acyl-carbon. This mechanism requires the formation of a ternary complex between the enzyme, AcCoA, and the acceptor substrate. Kinetic experiments, the failure to identify covalently bound intermediates and the inability to inactivate the acceptor using reagents that block thiol groups favour the direct acetyl transfer mechanism.

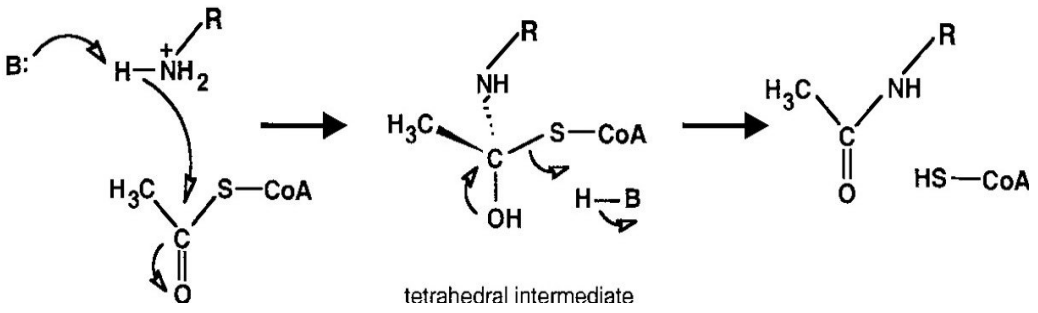


Figure 1.15: The reaction catalysed by GCN5-related *N*-acetyltransferases, showing the presumed tetrahedral intermediate that results from nucleophillic attack of a primary amine on the acyl carbon of the acetyl group.
(Dyda *et al*., 2000)

For direct nucleophillic attack to occur, the primary amine must be in an uncharged form. Therefore, it is likely that GNAT enzymes provide some sort of deprotonation, probably involving an amino acid near the active site, which acts as a general base (Dyda *et al*., 2000).

1.3.6 β-lactamases

β-lactamases are enzymes which catalyse the hydrolysis of an amide bond in the beta-lactam ring of antibiotics belonging to the penicillin/cephalosporin family to create ineffective antimicrobials (Figure 1.16) (Abraham and Chain, 1940). β-lactamases have attracted a lot of attention because of their clinical importance and their ecological and evolutionary interest. β-lactamases are thought to share a common ancestory with the DD-peptidases, which form peptide cross-links with the peptidoglycan network during cell wall synthesis (Kelly *et al.*, 1986). Mechanistically the β-lactamases are similar to the DD-peptidases in the acylation step (Pratt, 2002). However, the acyl intermediate of the β-lactamases undergoes deacylation much more rapidly and is therefore very efficient at destroying the antibiotic and protecting the peptidoglyacan synthesis (Sun *et al.*, 2003).

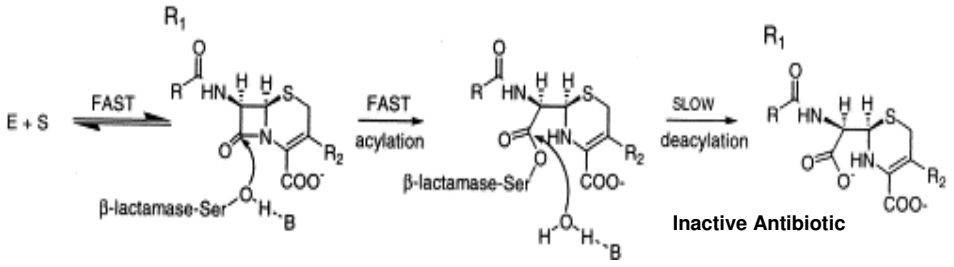


Figure 1.16: The inactivation of a β-lactam antibiotic by a β-lactamase (Caselli *et al.*, 2001)

β-lactamases are produced by both Gram-positive and Gram-negative bacteria and protect the organisms against the action of β-lactam antibiotics (Massova and Mobashery, 1998). The destruction of β-lactams by β-lactamases is the most important resistance mechanism in Gram-negative bacteria (Babic *et al.*, 2006). β-lactamases of Gram-positive species are largely extracellular, although, depending on the growth conditions, some enzymes may adhere to the cytoplasmic membrane. By contrast, the β-lactamases of Gram-negative species are largely periplasmic, although some extracellular release may occur, mediated by leakage rather than secretion (Livermore, 1995).

Originally, β-lactamases were named after substrates, biochemical properties, peculiarities of sequence, location of their discoveries, location of the gene on the chromosome, strains of bacteria, the patient providing the sample and the investigators who described them. In 1975, the application of isoelectric focusing for β-lactamases characterisation allowed many more enzymes to be distinguished (Jacoby, 2006). β-lactamases have become families of closely related enzymes. To date, over 530 β-lactamase enzymes have been reported. 150 TEM, 88 SHV, 88 OXA, 53 CTX-M, 22 CMY, 22 IMP, 12 VIM and smaller numbers in other enzyme families have been described by Jacoby and Bush (http://www.lahey.org/studies). The TEM and SHV families are closely related, with individual members differing by only one to seven amino acids. Other families (CTX-M and IMP) differ among themselves by as much as 20% in amino acid composition, while members of the OXA family can have almost 80% difference, because they have been grouped by activity on oxacillin and related substrates and not by primary structure (Babic *et al*., 2006).

β-lactamases can be broadly divided into enzymes with a serine residue at the active site, similar to bacterial penicillin-binding proteins, from which they probably evolved, and metalloenzymes with zinc ion as a cofactor and with a separate heritage (Sun *et al*., 2003). Both are ancient enzymes. Analysis of β-lactamase sequences allows them to be divided into four molecular classes according to their amino acid content. Class A, C and D use an active-site serine in their mechanism of action, whereas class B enzymes require divalent metal cations like zinc to catalyse β-lactam hydrolysis (Ambler, 1980). Another less commonly used classification system is based on substrate and inhibitor profile (Bush *et al*., 1995). The three classes of serine β-lactamases are evolutionarily related and belong to a superfamily that also includes DD-peptidases and a variety of other penicillin-binding proteins (PBPs) (Joris *et al*., 1988). All these proteins contain a Ser-X-X-Lys motif, where the serine is the active site residue. Although clearly homologous, the sequences of the three classes of serine beta-lactamases exhibit a large degree of variability and only a small number of residues are conserved in addition to the catalytic serine.

β-lactamases do not consist of many components. They are globular proteins that characteristically have alpha helices, β-pleated sheets and share similar structural

features (Herzberg, 1991). In a short time, infectious bacteria have acquired β-lactamases to counteract the effects of traditional β-lactams. They have also acquired variant forms of these enzymes to hydrolyse newly developed extended-spectrum β-lactams (Petrosino *et al*., 1998).

Class B β-lactamases

Metallo-β-lactamases are classified as group 3 enzymes because they hydrolyse penicillins, cephalosporins and carbapenems but are resistant to almost all conventional β-lactam inhibitors (Bush *et al*., 1995). They do not hydrolyse aztreonam. Ambler (1980) classified metallo-β-lactamases as class B enzymes. Class B β-lactamases require divalent cations, primarily zinc, for activity and are inhibited by metal chelators. The metalloenzymes are further categorised into three functional subgroups, B1, B2 and B3, based on metal requirements. In subclass B1, which includes most metallo-β-lactamases, Zn1 is tightly coordinated and Zn2 is loosely coordinated (Fabiane *et al*., 1998). The B2 functional subgroup possesses two zinc sites each with similar binding affinity (Crowder *et al*., 1996). The B3 subgroup has a Zn1 site that tightly binds zinc and is sufficient for maximal enzymatic activity (Hernandez-Valladares *et al*., 1997).

Class B metallo-β-lactamases have been identified in both Gram-positive and Gram-negative bacteria. They are found in many clinically important species such as *Acinetobacter* spp. and *Pseudomonas aeruginosa*. The most clinically important Class B β-lactamases belong to the IMP and VIM families (Majiduddin *et al*., 2002). These β-lactamases confer resistance to all β-lactams except monobactams and their activity is not inhibited by β-lactamase inhibitors (Babic *et al*., 2006). The production of these enzymes is primarily constitutive (Majiduddin *et al*., 2002). The genes for class B β-lactamases can be chromosomal or plasmid-borne and are found on integrons. This facilitates the dissemination of these genes by horizontal transfer (Laraki *et al*., 1999).

Class C β-lactamases

Class C enzymes (originally called cephalosporinases) can be distinguished on the basis of their primary structure and active site serine (Ambler, 1980). Bush *et al*. (1995) defined class C enzymes as group 1 β-lactamases. Their phenotype is characterised by resistance to a variety of penicillins, β-lactamase inhibitors, cefoxitin, cefotetan, ceftazidime and most importantly, carbapenems (Stapleton *et al*., 1999). They are only produced by Gram-negative bacteria, most commonly by the *Enterobacteriaceae*, but have also been found in *Pseudomonas* and *Aeromonas*. The genes encoding these enzymes are usually chromosomally encoded (Sanschagrin *et al*., 1995). The main representative of this group is AmpC (Majiduddin *et al*., 2002).

Class A β-lactamases

Class A, C and D β-lactamases share structural similarities with the target of β-lactam antibiotics, the DD-peptidases, and are thought to have emerged from the same ancestral enzyme (Koch, 2003). Class A enzymes include the β-lactamases TEM, SHV, OHIO, CARB/PSE, ROB-1 and PC1. According to the Bush classification scheme (Bush *et al*., 1995), based on biochemical parameters such as substrate profiles and reactions with inhibitors, these enzymes preferentially hydrolyse penicillins, cephalosporins or carbenicillins. Genetic dissemination of Class A enzymes occurs via plasmids and transposable elements (Sanschagrin *et al*., 1995).

Four motifs, S^{70}-X-X-K, S^{130}-X-N, K^{234}-T/S-G and the Ω-loop, are commonly found in the vacinity of the active-site pocket of Class A β-lactamases. The S^{70}-X-X-K motif includes the active site serine at position 70 and a lysine at position 73 (Ambler, 1980). The mechanism of Class A enzymes involves the nucleophillic attack by Ser^{70} after activation by Lys^{73} or Glu^{166} on the carbonyl carbon of the β-lactam ring resulting in the acyl enzyme intermediate (Majiduddin *et al*., 2002). Mutagenic studies clearly indicate that Ser^{70} is involved in the hydrolysis of the β-lactam antibiotic (Chen *et al*., 1996). Lys^{73} is proposed to have a role in acylation of penicillin and deacylation of cephalosporins. Glu^{166} located in the Ω-loop, is proposed

to play a role as a general base in both acylation and deacylation (Majiduddin *et al*., 2002).

Back donation of the carbonyl proton occurs via a hydrogen bond network involving a water molecule, Lys^{73} and Lys^{234}, a component of the K-T/S-G motif. Lys^{234} stabilises Ser^{130} (a component of the S-X-N motif) through hydrogen bonding (Matagne *et al*., 1998). Deacylation and regeneration of the β-lactamase would occur through the activation of a water molecule by Glu^{166}. The activated water molecule would then attack the carbonyl carbon of the acyl enzyme intermediate, and back donation of a proton to the Ser^{70} would occur resulting in a regenerated enzyme (Majiduddin *et al*., 2002).

Class D β-lactamases

The DNA sequence similarity between the class D β-lactamase and class A and C is limited and restricted to three main regions around the active site (Majiduddin *et al*., 2002). Class D enzymes belong to the group 2d of the Bush functional classification scheme of β-lactamases (Bush *et al*., 1995). OXA-2 was the first class D enzyme to be discovered (Dale *et al*., 1985). The first characterised class D β-lactamases were referred to as oxacillinases because they commonly hydrolyse the isoxazolylpenicillins, oxacillin and cloxacillin, two to four times faster than classical penicillins such as penicillin G (Sun *et al*., 2003). The eponym, OXA, of the class D β-lactamases, thus, refers to their preferred penicillin substrate (Walther-Rasmussen and Høiby, 2006). Currently, 121 different variants of class D β-lactamases have been identified on the protein level, and 45 of these exhibit carbapenems-hydrolysing activities, which is in contrast to other class D β-lactamases (Walther-Rasmussen and Høiby, 2006). They are designated OXA-1, OXA-2 etc. and fall into at least five different subgroups on the basis of phylogeny analysis (Barlow and Hall, 2002).

Class D β-lactamases differ significantly from Class A and C enzymes by the utilisation of a carboxylated lysine side chain for catalysis (Golemi *et al*., 2001) and exhibiting biphasic burst kinetics (Ledent *et al*., 1993). Biphasic kinetics is when the initial hydrolysis rate of a substrate declines more rapidly than is explicable by

substrate depletion, before stabilising at at steady-state rate (Danel *et al.*, 1997). The Class D deacylation step of catalysis is much slower than the acylation step because the oxacillinases less effectively activate a water molecule for hydrolysis (Sun *et al.*, 2003). A major clinical problem with Class D β-lactamases is that inhibitors for these enzymes are lacking (Huovinen *et al.*, 1988). Unlike Class A enzymes, Class D enzymes are not inhibited by the β-lactamase inhibitor, clavulanic acid (Page, 2000). Class D β-lactamases have three highly conserved active-site elements in common. The first is the tetrad, Ser^{67}-X-X-Lys (where X represents a variable residue containing the active site serine). The second, the Ser^{115}-X-Val/Ile, is equivalent to the invariable Ser-Asp-Asn motif in Class A β-lactamases and Tyr-Ala/Ser-Asn in AmpC β-lactamases. The Lys^{216} and Tyr/Ser-Gly element is common to most of the serine-active β-lactamases. Other conserved motifs in Class D β-lactamases are the triad Tyr/Phe^{144}-Gly-Asn and the tetrad Trp^{232}-X-X-Gly. These have no analogues in other β-lactamase classes (Walther-Rasmussen and Høiby, 2006). The active site serine in Class D enzymes reacts with substrate to form an acyl-enzyme intermediate. A common feature among Class A and D enzymes is a reactive serine and an oxyanion hole to stabilize tetrahedral transition states in both acylation and deacylation. Two conserved lysine residues exist in the binding site. However, it is only in Class D enzymes that the lysine in the Ser^{67}-X-X-Lys^{70} is modified by CO_2 in a pH-dependent manner to provide full activity (Sun *et al.*, 2003).

Class D β-lactamases are the least understood of the β-lactamases because few structures of Class D β-lactamases have been solved (Maveyraud *et al.*, 2000, Pernot *et al.*, 2001, Sun *et al.*, 2003). OXA-10 is one of the few enzymes that has been crystalized and its structure solved by Maveyraud *et al.*, 2000. Its structure is very similar to the structures of OXA-1 (Sun *et al.*, 2003) (Figure 1.17, p. 64) and OXA-13 (Pernot *et al.*, 2001). Each OXA-10 monomer folds as a two-domain protein. The first domain includes a seven stranded antiparallel β-sheet and the N- and C-terminal helices. The second domain is made of six α-helices connected by loop regions. The catalytic site is located at the interface of the two domains. The binding site for β-lactam substrates in OXA-10 is delineated by strand β5 on one side and by the $Ser^{115}/Ala^{116}/Val^{117}$ loop that connects α4 and α5 on the other side. Ser^{67}, the catalytic serine, which is acylated during substrate turnover, is found on the N-terminal portion

of α3. Ser^{67} along with Lys^{70}, Ser^{115}, Lys^{205} and Gly^{207} are strictly conserved among Class D β-lacamases. These are also the only invariant amino acids between Class A and Class D enzymes (Maveyraud *et al*., 2000).

The Glu^{166} in the Ω-loop region of Class A β-lactamases is essential for catalysis and is responsible for the activation of the water molecule that promotes efficient deacylation of the acyl-enzyme intermediate. There is no acidic residue in Class D enzymes that would be a counterpart to Glu^{166}. The Ω-loop in OXA-10 is four residues shorter than in Class A enzymes and its conformation is not related to those in Class A and C enzymes. The residues in the Ω-loop are hydrophobic and include the invariant tryptophan residue in Class D enzymes (Maveyraud *et al*., 2000). The same case is true for the β-lactam binding site of OXA-1 which is much more hydrophobic than binding sites in Class A and Class C β-lactamases (Sun *et al*., 2003). Both Class A, C and Class D enzymes have a conserved hydrophobic side chain on the Ω-loop in the binding site. This residue is Leu^{169} in Class A enzymes and Leu/Ile^{161} in Class C enzymes. This hydrophobic group in Class D enzymes has replaced a critical hydrophillic array (Glu^{166}-H_2O-Asn^{170} triad, which activates the catalytic water molecule) in the class A catalytic site (Sun *et al*., 2003). In OXA-1 the β9 strand is connected to the β10 strand by a loop. The size of this loop varies in length between oxacillinases and is thought to be a determinant in the specificity of oxacillinase variants for various β-lactams. Oxacillinases, unlike Class A enzymes, have large hydrophillic aromatic residues at position 215 just after the conserved K-T-G motif on the β9 strand (Sun *et al*., 2003).

The geometry of the OXA-10 active site is different to that of Class A enzymes, in which Lys^{73} is at polar interaction with Glu^{166} (general base) and hydrogen bonded to Asn^{132}. This residue involved in substrate binding in Class A enzymes is always substituted for Val^{117} in Class D β-lactamases. Carboxylation of Lys^{70}, which converts a neutral or positive site into an anionic one is thought to act as a base to activate the Ser^{67} hydroxyl group for enzyme acylation. It therefore is believed to have the same function in Class D β-lactamases that Glu^{166} has in Class A enzymes, i.e., to act as a general base to activate a water molecule for hydrolysis of the acyl-enzyme intermediate (Maveyraud *et al*., 2000). A water molecule is thought to bridge from

Lys^{70} to Trp^{160} and is claimed to be the water molecule for deacylation. A hydrogen bond exists between the carboxylate group and the side chain amide group of the Ω-loop, an interaction that is common to all known carbamylated oxacillinase structures. The carboxylate group forms two other hydrogen bonds with the reactive Ser^{67} and Ser^{120} (Sun *et al*., 2003).

It is not yet known how this serine is activated. Unlike Lys^{73} and Glu^{166} of class A β-lactamases, or Tyr^{150} of the class C enzymes, the general base for the Class D enzymes has not been identified. Lys^{70}, which is the structural equivalent of Lys^{73} in class A β-lactamases and Lys^{67} of class C β-lactamases, appears to be in the optimal position to activate a catalytic water molecule for nucleophilic attack of the active site serine. (Maveyraud *et al*., 2000), (Pernot *et al*., 2001). The Trp^{154}, which positions a catalytic water molecule, appears to be an important residue in the substrate specificity of oxacillinase enzymes (Poirel *et al*., 2001 [a]).

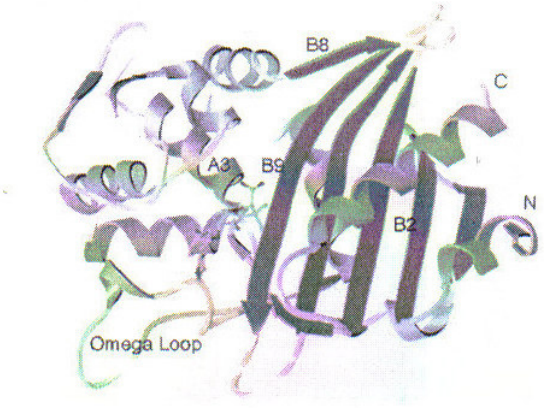


Figure 1.17: Tertiary stucture of OXA-1 β-lactamase
(Sun *et al*., 2003)

Most of the OXA-type β-lactamases have been discovered in clinical isolates of *Pseudomonas aeruginosa* but some have also been obtained from other Gram-negative bacteria such as *Acinetobacter baumannii* and *Salmonella typhimurium* (Dale *et al*., 1985). Some OXA-type β-lactamases are encoded by chromosomal genes that appear to be resident in some microbial genomes such as those in some *Aeromonas* spp., *Ralstonia picketti* and *Pseudomonas aeruginosa* (Giuliani *et al*., 2005). Many of these enzymes, however, are encoded by genes associated with mobile elements,

integrons in particular (Naas and Nordmann, 1999). These secondary OXA-type β-lactamase genes have been reported to occur in several pathogenic species, including members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, where they can confer β-lactam resistance (Crowley *et al*., 2002).

Although many oxacillinases have a narrow substrate spectrum, some are also capable of degrading extended-spectrum cephalosporins or carbapenems. As with many β-lactamases, OXA-2 and OXA-10 have evolved variants, which confer resistance to extended-spectrum cephalosporins including ceftazidime and cefotaxime (Huovinen *et al*., 1988). These variants arise from single amino acid substitutions, which alter the substrate specificity of the enzyme (Paetzel *et al*., 2000). The number of amino acid substitutions ranges from one in OXA-15 to nine in OXA-28 (Majiduddin *et al*., 2002).

1.4 Aims of the project

Pseudomonas aeruginosa is difficult to treat in a clinical environment because it is resistant to many antibiotics. A number of isolates, presumptive *Pseudomonas aeruginosa* strains, were obtained from two Irish hospitals, the Adelaide and Meath National Children's Hospital, Tallaght, Dublin 24 and the Waterford Regional Hospital. It was of interest to study these isolates in order to:

- identify and characterise the strains
- create an antibiotic resistance profile for each isolate
- investigate the mechanisms of antibiotic resistance in the multidrug resistant strain, *P. aeruginosa* PA13.

2.0 Materials and methods

2.1 Bacterial Cultures

2.1.1 Source

2.1.1.1 Clinical isolates

Eighteen bacterial isolates were obtained from two Irish hospitals, the Adelaide and Meath National Children's Hospital, Tallaght, Dublin 24 and the Waterford Regional Hospital. They were given the codes PA1-PA18. They were received on nutrient agar slants and were transported under cooled conditions.

2.1.1.2 Control Strains

Table 2.1: Characterisics and sources of control strains

Strain	Characteristic	Source
Bacillus stearothermophilus	Spore forming control	DCU Culture Collection
Enterococcus faecalis	Non-motile control	DCU Culture Collection
Escherichia coli ATCC® 25922	β-lactamase negative control	Microbiology Laboratory, Coombe Womens's Hospital
Escherichia coli ATCC® 35218	β-lactamase positive control	Microbiology Laboratory, Coombe Womens's Hospital
Escherichia coli	Non-spore forming contol, oxidase negative control	DCU Culture Collection
Pseudomonas aeruginosa ATCC® 27853	API 20NE control, aminoglycoside resistance control	Microbiology Laboratory, Coombe Womens's Hospital
Pseudomonas aeruginosa PAO1	Gram –ive control, motile control, oxidase positive, Tween 80 positive control	DCU Culture Collection
Serratia marcescens	Fermentation control, arginine hydrolysis negative control	DCU Culture Collection
Staphylococcus aureus	Gram +ive control, catalase positive control	DCU Culture Collection
Streptococcus pyogenes	Catalase negative control, β-haemolysis control	DCU Culture Collection
Streptococcus viridans	α-haemolysis control	DCU Culture Collection

2.1.1.3 *Escherichia coli* strains used for molecular studies

Table 2.2: Source and genotype of *E. coli* strains used in molecular studies

Strain	Genotype	Source
E. coli BL21(DE3)	F^-, *omp*T, *hsd*S_B (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3), Tet^r, Lon, ompT	Novagen
E. coli XL10-Gold	Tet^r, Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac* Hte [F'*pro*AB, *lacI*^{q}ZΔM15, Tn10 (*Tet*^{r}), Cam^r]	Dr. Michael O' Connell, DCU
E. coli Rosetta™ Blue	*end*A1, *hsd*R17(r_{K12}^-, m_{K12}^+), *sup*E44, *thi*-I, *rec*A1, *gyr*A96, *rel*A1, *lac*[F' *pro*A^+B^+ *lacI*^{q}ZΔM15::Tn10(tet^R)] pRARE(argU, argW, ileX, glyT, leuW, proL) (Cm^R), Lon, ompT	Novagen

2.1.2 Maintenance of strains

Protect Beads™

The clinical isolates were stored on Protect Beads™ (Technical Service Consultant, UK). The beads were supplied in tubes containing 0.5 ml cryopreservation fluid. To prepare the beads from a solid culture, colonies were picked off using a sterile loop and were used to inoculate the cryopreservation fluid containing the beads to create a thick suspension in the tube. The tube was capped and the suspension was inverted six times. The tube was then allowed to stand for 30-40 seconds. As much excess suspension as possible was removed from the tube using a sterile pipette leaving the culture-coated beads in situ. The tubes were labelled and stored at -80°C. These cells can be stored for up to ten years. To resuscitate the cultures, the vial was removed from the freezer and a bead removed aseptically and placed on a plate of nutrient agar.

The isolates were grown on nutrient agar plates (Section 2.4.6) and stored at 4°C for short-term storage.

Glycerol Stocks

Glycerol stocks were prepared for storage of control strains and *E. coli* strains used in molecular studies. For glycerol stock preparation, 2 ml of an overnight culture grown in nutrient broth (*E. coli* strains used in molecular studies were grown in the presence of 10 µg/ml tetracycline, *E. coli* ATCC®35218 was grown in the presence of 20 µg/ml ampicillin and *P. aeruginosa* ATCC®27853 was grown in the presence of 10 µg/ml gentamicin) was added to 1 ml of sterile 80% (v/v) glycerol solution and mixed in a bijoux tube. The glycerol stocks were stored at -80°C immediately, for up to three years. The strains were stored at 4°C on LB agar plates for short-term storage.

2.2 Source of Chemicals

Chemicals were obtained from Sigma Aldrich Chemical Company (UK) unless otherwise stated.

2.3 Buffers and solutions

2.3.1 Destaining solution

Destaining solution used for destaining SDS-PAGE gels was made by adding 100 ml acetic acid and 450 ml methanol to 450 ml distilled water.

2.3.2 6X DNA Loading Dye

The loading dye was prepared by dissolving bromophenol blue (0.25 g) and sucrose (40 g) in 100 ml dH_2O and used as required.

2.3.3 0.5M EDTA

Ethylenediaminetetraacetic acid disodium salt (93.05 g) was dissolved in 400 ml deionised H_2O and brought to pH 8 with NaOH pellets. The volume was brought to 500 ml with distilled H_2O and the solution was autoclaved at 121°C for 15 minutes.

2.3.4 1M IPTG (Isopropyl-β-D-thiogalactopyranoside)

IPTG (0.238g) was dissolved in 1ml dH_2O. The solution was filter sterilised (using a 0.2 µm syringe filter) and stored in 5 ml volumes at -20°C. For a working concentration of 1mM, 100 µl was added to 100 ml media.

2.3.5 Lysis buffer

Lysis buffer was prepared by dissolving 40mM Tris-acetate (pH 7.8), sodium acetate (20mM), EDTA (1mM) and 1% (w/v) SDS in distilled water.

2.3.6 Plasmid Preparation Solutions (Birnboim and Doly, 1979)

Solution 1

This solution was prepared by adding 10 ml 0.5M glucose, 10 ml Na_2-EDTA and 2.5 ml 1M Tris-HCl to 77.5 ml distilled water.

Solution 2

This solution was prepared by adding 20 ml 1M NaOH and 10 ml SDS (10% w/v) to 70 ml distilled water. This solution was prepared fresh every month and stored at room temperature

Solution 3

Solution 3 was 3M Potassium Acetate (pH 4.8). Glacial acetic acid (11.5 ml) and 28.5 ml distilled water was added to 60ml of 5M potassium acetate. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

2.3.7 Ringers Solution

Ringers solution was prepared by adding one tablet (Sigma) to 500 ml of distilled water and autoclaving at 121°C for 15 minutes.

2.3.8 5X Running Buffer

5X Running buffer was made by dissolving 15 g Tris base, 72 g glycine and 5 g SDS in 1L distilled water. The pH was not adjusted and the solution was stored at room temperature.

2.3.9 Sample Buffer

Sample buffer was prepared by adding 1.25 ml 0.5M Tris HCl (pH 6.8), 5ml glycerol, 2ml SDS (10% w/v), 0.5ml β-mercaptoethanol and 0.5 mg bromophenol blue to 1ml of distilled water. All samples were diluted 1:4 and heated to 95°C for 5 minutes, prior to loading SDS-PAGE gel.

2.3.10 Sodium Phosphate Buffer

Sodium phosphate buffer (10 mM and 100 mM) was prepared by dissolving 1.55 g/L (10 mM) or 15.47g/L (100 mM) Na_2HPO_4 and 0.584 g/L (10 mM) or 5.84 g/L (100 mM) NaH_2PO_4 in distilled water. The pH of the resulting solution was then adjusted to pH 7.0 using 1M NaOH.

2.3.11 Staining solution

The staining solution for staining SDS-PAGE gels was prepared by adding 100 ml acetic acid, 450 ml methanol and 0.25 g coomassie blue to 450 ml distilled water.

2.3.12 50X TAE

Tris-acetate buffer was prepared by dissolving Tris (2M) and ethylenediaminetetra acetic acid (0.05M) in distilled water and adjusting the pH to 8.0 with glacial acetic acid. The buffer was stored at room temperature and diluted as required.

2.3.13 1X TAE – Working Buffer

Dilute 10 ml of 50X TAE to 500ml with 490 ml of dH_2O and add 20µl of ethidium bromide 10 mg/ml.

2.3.14 TE Buffer

Tris-acetate buffer was prepared by dissolving Tris-HCl (10mM) and Na_2-EDTA (1mM) in distilled water and adjusting the pH to 8.0 with HCl. The buffer was stored at room temperature and diluted as required.

2.3.15 TFB 1 Buffer

TFB 1 buffer was prepared by dissolving rubidium chloride (100 mM), magnesium chloride (50 mM), calcium chloride (30 mM) and 15% (v/v) glycerol in ultrapure water. The pH was adjusted to 5.8 using KOH and the buffer was filter sterilised.

2.3.16 TFB 2 Buffer

TFB 2 buffer was prepared by dissolving MOPS (10 mM), rubidium chloride (10 mM), calcium chloride (75 mM) and 15% (v/v) glycerol. The pH was adjusted to 6.8 using KOH and the buffer was filter sterilised.

2.4 Media

All microbiological media unless otherwise stated were obtained from Oxoid.

2.4.1 Agarose Gel Preparation

An agarose gel (0.8% w/v) was prepared by the addition of 0.4 g agarose (Sigma) to 50 ml of 1X TAE buffer and boiled until the agarose was sufficiently dissolved. This was allowed to cool to ~50°C and 2 µl (10 mg/ml) ethidium bromide (final concentration 0.4 µg/µl) was added. The gel was poured and a comb was inserted to make the wells. When the gel was set, the comb was removed and the gel was placed

in the gel box and immersed in 1X TAE buffer. Samples were prepared by taking 10 µl of the DNA, 2 µl of loading dye and 10 µl dH_2O. 10µl of the λ DNA size standard was loaded into the first well of the agarose gel, followed by 20 µl of DNA samples in subsequent wells. The nucleic acid ladder (125-21,226 bp) used in all agarose gels was obtained from Sigma (Cat. No.: D9281). The gels were run for 60 minutes at 130V and 100mA using the Hybaid Electrophoresis system (M.A., U.S.). The gels were viewed and automatically photographed using the Imagemaster VDS image analysis system (Amersham Pharmacia Biotech, N. J., U.S.)

2.4.2 Arginine Agar

Table 2.3: Components of arginine agar

Component	**Amount/L**
Peptone	1g
NaCl	5g
K_2HPO_4	0.3g
Phenol red 1.0%	1 ml
L(+) arginine hydrochloride	10g
Agar	3g
Distilled water	1000 ml

The medium was dissolved, the pH adjusted to 7.2 and distributed in 3.5 ml volumes into glass universals before sterilization (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

2.4.3 Blood agar

Blood agar was made by adding 50 ml of sterile defibrinated horse blood to 950 ml of nutrient agar. Nutrient Agar was sterilised by autoclaving and allowed cool to 50°C. Defibrinated blood was added aseptically. The agar was mixed and distributed into

plates. Blood should never be added before autoclaving (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

2.4.4 Cetrimide Agar

Dehydrated cetrimide agar powder (Merck) (4.45g) was dissolved in 100 ml of distilled water. Glycerol (10 ml/L) was added and the agar was autoclaved at 121°C for 15 minutes. The medium was supplemented with 15 µg/ml naldixic acid to select for *Pseudomonas aeruginosa*.

2.4.5 Hugh and Liefsons' Medium

Table 2.4: Components of Hugh and Liefsons' medium used for the oxidation-fermentation test

Component	Amount/L
Peptone	2g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
Distilled Water	1000 ml
Bromothymol blue (0.2% w/v)	15 ml

The solids were dissolved by heating in water and the pH was adjusted to 7.1 with 2M NaOH. The indicator dye was added and the medium was sterilised. Following sterilisation, a sterile solution of glucose was added aseptically to give a final concentration of 1% v/v. The medium was mixed and distributed asceptically in 10 ml volumes into sterile test tubes (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

2.4.6 LB, Mueller-Hinton and Nutrient Media

Table 2.5: Amount of dehydrated powder (or tablets) used to make LB, Mueller-Hinton and Nutrient media

Medium	Amount/L dH_2O
LB agar (Sigma)	37g
LB broth (Sigma)	20 tablets
Mueller-Hinton agar	38g
Mueller-Hinton broth	21g
Nutrient agar	20g
Nutrient broth	13g

All of these media were sterilized by autoclaving for 15 minutes at 121°C.

2.4.7 Preparation of Mueller-Hinton Agar for disk susceptibility test

Mueller-Hinton agar was prepared as follows. The agar was prepared according to the manufacturer's instructions to a pH of 7.2-7.4. Immediately after autoclaving, the agar was allowed to cool to 45-50°C. The freshly prepared agar was poured into plastic, flat bottomed petri dishes on a level surface to give a uniform depth of approximately 4 mm (this corresponded to 25-30 ml of agar in a plate with a diameter of 100 mm). The plates were allowed to cool to room temperature and, unless the plates were used in the same day, they were stored at 2-8°C in a refrigerator. All plates were used within seven days of preparation. A representative sample of each batch of plates was examined for sterility by incubating at 37°C for 24 hours.

2.4.8 Preparation of cation adjusted Mueller-Hinton Broth for broth dilution method

In order to determine the MIC values of aminoglycosides the correct concentrations of the divalent cations Ca^{++} and Mg^{++} (25mg of Ca^{++}/L and 12.5mg/L of Mg^{++}/L) were added to the Mueller-Hinton broth otherwise the MICs of aminoglycosides *for P. aeruginosa* would be much different from those MICs obtained on Mueller-Hinton agar. The cation concentration of the Mueller-Hinton broth was adjusted as follows:

(a) A stock solution of magnesium was prepared by adding 8.36g of $MgCl_2 \bullet 6H_2O$ to 100 ml of deionised water. This solution contains 10 mg of Mg^{++}/ml.

(b) A stock solution of calcium was prepared by adding 3.68g of $CaCl_2 \bullet 2H_2O$ to 100 ml of deionised water. This solution contains 10 mg of Ca^{++}/ml.

These stock solutions were filter sterilised using a 0.2 μm syringe filter and stored at 2-8°C.

(c) Mueller-Hinton broth was prepared as directed by the manufacturer, and chilled overnight at 2-8°C before cation addition. The starting concentration of the cations in the media was accounted for when calculating the amount of Ca^{++} and Mg^{++} to add to the medium.

(d) 0.1 ml of chilled Ca^{++} or Mg^{++} stock solution per litre of broth was added with stirring for each desired increment of 1 mg/L in the final concentration in the adjusted broth.

2.4.9 *Pseudomonas* Isolation Agar F

4.64g of dehydrated PIA F agar powder (Difco) was dissolved in 100 ml of distilled water. 1g glycerol was added and the medium was autoclaved at 121°C for 15 minutes.

2.4.10 *Pseudomonas* Isolation Agar P

Dehydrated PIA P agar powder (Difco) (3.8g) was dissolved in 100 ml of distilled water. Glycerol (10 g/L) was added and the agar was autoclaved at 121°C for 15 minutes.

2.4.11 SDS-PAGE resolving gel (12% w/v)

The resolving gel used in SDS-PAGE was prepared by adding 2.5 ml 1.5M Tris HCl (pH 8.8), 50 µl SDS (20% w/v), 4 ml acrylamide bisacrylamide (30%/0.8% w/v) and 50 µl ammonium persulphate (10% w/v) to 3.39 ml distilled water. 10 µl of Temed was mixed with the solution to catalyse the polymerization of acrylamide. The ammonium persulphate was freshly prepared on the day of use.

2.4.12 SDS-PAGE stacking gel (4% w/v)

The stacking gel used in SDS-PAGE was prepared by adding 1.25 ml 0.5M Tris-HCl (pH 6.8), 25 µl SDS (20% w/v), 0.67 ml acrylamide bisacrylamide (30%/0.8% w/v) and 25 µl ammonium persulphate (10% w/v) to 3.02 ml distilled water. 10 µl Temed was mixed with the solution to set the gel. The ammonium persulphate was freshly prepared on the day of use.

2.4.13 Tween 80 agar

Table 2.6: Components of Tween 80 agar

Component	Amount/L
Tween 80	10g
Peptone	10g
NaCl	5g
$CaCl_2.2H_2O$	0.1g
Agar	20g
Distilled Water	1000 ml
pH	7.4

The solids were dissolved in water and sterilised (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

2.5 Measurement of pH

The pH was measured using an Orion Triode^{TM} pH electrode Model 91-57BN connected to an Orion benchtop pH/ISE meter (model 920A).

2.6 Tests used to identify bacterial isolates

2.6.1 Cell and colony morphology characteristics

The cell and colony morphology of the isolates were observed following growth of the organisms on nutrient agar (Section 2.4.6) and incubation for 24 hours at 37°C according to Cowan and Steel's Manual for the identification of Medical Bacteria, 1993.

2.6.2 Gram reaction

The Gram stain was carried out on 18-24 hour cultures according to the Hucker method (Collins and Lyne, 1985). A loopful of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed of with a gentle stream of water. Grams iodine (0.4% w/v) was added and allowed to stand for 30 seconds before being rinsed off. The stain was washed with ethanol (95% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one minute. This was then washed with water for 5 seconds. If the bacteria was Gram-negative, it appeared pink under the microscope. If the cell was Gram-positive, it appeared purple under the microscope.

Controls: *Stapylococcus aureus* - positive
Pseudomonas aeruginosa - negative

2.6.3 Spore stain

A smear of the organism was prepared from a 48-hour nutrient broth culture and heat fixed. The smear was stained with 5% (w/v) aqueous malachite green and kept

steaming for over 5 minutes, renewing the stain as it evaporated. It was washed for 30 seconds with distilled water and counterstained with 0.25% (w/v) safranin for 1 minute (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: positive - *Bacillus stearothermophilu*s
negative - *Escherichia coli*

2.6.4 Motility test

An overnight culture of the organism was examined in "hanging drop" preparations, using a 100x magnification and reduced illumination. A "hanging drop" slide was prepared by placing a loopful of the bacterial suspension onto the centre of a coverslide. A depression slide onto which a ring of Vaseline had been spread around the concavity was lowered onto the coverslip, with the concavity facing down over the drop. When a seal had formed, the hanging drop slide was turned over and examined under a microscope (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: motile- *Pseudomonas aeruginosa*
non-motile - *Enterococcus faecalis*

2.6.5 Catalase activity

A loopful of culture was emulsified with a loopful of 3% (v/v) hydrogen peroxide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated a positive result (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: positive - *Staphylococcus aureus*
negative - *Streptococcus pyogenes*

2.6.6 Oxidase activity

(a) Filter paper was impregnated with a 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine (with 0.1% (v/v) ascorbic acid to prevent auto-oxidation). Bacterial cultures were smeared across the filter paper with a glass rod. The formation of a purple colour within 5-10 seconds indicated oxidase positive

cultures (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993)

(b) Oxoid oxidase identification sticks were used to take up some bacterial culture. A positive reaction was recorded when purple coloration formed within 30 seconds.

Controls: positive – *Pseudomonas aeruginosa*
negative – *Escherichia coli*

2.6.7 Oxidation-Fermentation test

Two tubes of Hugh and Liefson's medium (Section 2.4.5) were stab inoculated with the test culture. One tube was covered with sterile mineral oil and both tubes were incubated at 37ºC for up to 14 days. Acid production was indicated by a change in the colour of the medium from blue-green to yellow. Fermentative organisms produced acid in both tubes and oxidative organisms produced acid in only the open tube and usually only at the surface (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: *Pseudomonas aeruginosa* - oxidation
Serratia marcescens - fermentation

2.6.8 Haemolysin production

A plate of Blood agar (Section 2.4.3) was inoculated by streaking once across the surface. Plates were incubated at 37ºC for 24 hours. There were three possible outcomes: α-haemolysis (green zones, cell envelopes intact), β-haemolysis (clear, colourless zone, cell envelopes disrupted) or γ-haemolysis (no action on red cells). γ-haemolysis describes a negative result (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: α-haemolysis – *Streptococcus viridans*
β-haemolysis – *Streptococcus pyogenes*
γ-haemolysis – uninoculated medium

2.6.9 Tween 80 hydrolysis

Tween 80 agar plates (Section 2.4.13) were inoculated by streaking once across the surface. Plates were incubated at 37ºC for 1-7 days. Plates were checked each day. An opaque halo of precipitation around the growth indicated hydrolysis of the Tween. Opaque zones surrounding the inoculum consisted of calcium salts of free fatty acids and were indicative of Tween 80 hydrolysis (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: positive – *Pseudomonas aeruginosa*
negative – uninoculated medium

2.6.10 Arginine hydrolysis

Arginine agar (Section 2.4.2) was stab-inoculated and a layer of sterile mineral oil on the surface was pipetted onto the surface to a depth of about 1 cm. The tubes were incubated at 30ºC for 5 days. A positive reaction was shown by a colour change of the indicator to red (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: positive – *Enterococcus faecalis*
negative – *Serratia marcescens*

2.6.11 Pigment Production

In order to identify general pigment production, the isolates were streaked on Nutrient agar plates (Section 2.4.6). Cultures were streaked onto Difco *Pseudomonas* isolation agar F (PIA F) (Section 2.4.9) to detect fluorescein production and Difco *Pseudomonas* isolation agar (PIA P) (Section 2.4.10) to detect pyocyanin production. The isolates were also grown on cetrimide agar (Section 2.4.4) to detect pigment production. All of the plates were incubated at 37ºC for 1-2 days. Any colours produced by the isolates were recorded. An ultraviotet lamp was used to detect for fluorescein production the PIA F (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

2.6.12 Temperature profiles

The organisms were grown on nutrient agar (Section 2.4.6) and incubated aerobically as follows:

5°C for 7-14 days

37°C for 1-2 days

42°C for 1-2 days

(Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

2.6.13 API Tests

The API identification system API20NE (bioMérieux, Marcy-l'Etoile, France) for non-enteric Gram-negative rods was used for identification of the clinical isolates. The identification system was used according to the manufacture's instructions. The inocula were prepared as follows: an overnight nutrient broth culture (10 ml) was harvested in a Eppendorf 5810R bench-top centrifuge (4000 X g for 10 minutes) and washed once with sterile 10 mM sodium phosphate buffer. The pellet was resuspended in 0.85% (w/v) NaCl (10 ml) and the density adjusted to 0.5 McFarland. This suspension was used to inoculate a portion of the tests. For assimilation tests, 200 µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was used to inoculate the remaining tests. *Pseudomonas aeruginosa* ATCC 27853 was used as a control strain. The strips were read and interpreted after incubation at 30ºC for 24 hours. Identification was obtained using the Analytical Profile Index: the pattern of the reactions obtained was coded into a numerical profile. On a results sheet the test were separated into groups of three and a number 1, 2 or 4 was indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number was obtained which constituted the numerical profile. Identification was then obtained using the identification software (API 20NE V6.0 database) by manually entering the 7-digit numberical profile. The profile was listed along with the percentage of identification – an estimate of how closely the profile corresponded to the taxon relative to all the other taxon in the database and the T index – an estimate of how closely the profile corresponded to

the most typical set of reactions for each taxon (value between zero and one which was inversely proportional to the number of atypical tests).

2.6.14 Biolog Test

Identification was carried out by a Biolog microlog system (BIOLOG Inc., Hayward, Calif., USA) according to the manufacturers' instructions. Biolog GN is a commercially available identification kit, recommended for the identification of Gram negative bacterial isolates. Biolog GN establishes identification based on the exchange of electrons generated during respiration, leading to tetrazolium-based colour changes. It tests the ability of an organism to oxidize a panel of 95 different carbon sources. GN microplates for Gram-negative organisms were used. An isolated colony was transferred to Biolog inoculating fluid using sterile swabs to give the correct cell density required by the system. Cell densities were measured using the Biolog turbidometer and were compared against GN-NENT (Gram-negative non-enteric) turbidity standards supplied by the company. A transmittance level of between 53%-59% was required to inoculate Biolog GN microplates. 150 µl of the resulting inoculum was added to each of the 96 test wells in the microtitre plate. The plates were incubated for 16-24 hours at 37ºC. All purple wells were scored as positive and all colourless cells were scored as negative. Cells with an extremely faint colour were scored as borderline. The results were interpreted using Biolog automated microlog computer software (MicroLog^{TM} 3). The Biolog identification system used a multiplicative measure of probability to identify the unknown isolate. This measured the goodness of match between a given pattern for the unknown isolate and the known pattern for a reference strain. It also allowed for experimental error. A figure for similarity was also given. This figure is calculated by multiplying the probability of a match given that the unknown organism is one of the organisms in the database by the probablity that the unknown organism is in the database. The similarity value went from zero to one. The higher the value, the better the identification.

2.7 Antimicrobial susceptibility testing

2.7.1 Preparation of antibiotics

The antibiotics were weighed (taking their purity into account), dissolved with distilled water and syringe filtered using a 0.2µm filter. They were stored at 20°C until needed (for no longer than one month).

2.7.2 Antimicrobial disk susceptibility tests

Mastring multidisks

Preliminary antibiotic susceptibility testing was performed in triplicate with Mastring-S M14 antibiotic multidisks (Mast Diagnostics, Merseyside, U.K.). These disks contained eight antibiotics including ampicillin (10 µg), cephalothin (5 µg), colistin sulphate (25 µg), gentamicin (10 µg), streptomycin (10 µg), sulphatriad 200 µg, tetracycline (25 µg) and cotrimoxazole (25 µg). Mueller-Hinton agar was inoculated with overnight culture of the strain to be tested. A multidisk was aseptically laid on the surface of the agar using a sterile tweezers. The plates were incubated for 24 hours. The zones of inhibition were observed.

Antibiotic susceptibility testing according to Clinical and Laboratory Standard Institute

The tests were conducted according to the approved method of the Clinical and Laboratory Standards Institute (CLSI) [M2-A9] (2006). Mueller-Hinton agar (Section 2.4.7) was used as the growth medium in all the antibiotic disk susceptibility tests. All tests were done in triplicate.

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard was used. The 0.5 McFarland standard was prepared as follows: a 0.5 ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant

stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.08 and 0.1 for the 0.5 McFarland standard. The $BaSO_4$ suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. These tubes were tightly sealed and stored in the dark at room temperature. The $BaSO_4$ turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appeared, the standard was replaced. The barium sulphate standards were replaced or their densities verified monthly.

The direct colony suspension method is the most convenient method for inoculum preparation. The inoculum was prepared by making a direct saline suspension of isolated colonies selected from an 18- to 24-hour agar plate. This resulted in a suspension containing approximately 1 to 2 x 10^8 CFU/ml. To perform this accurately, the inoculum tube and the 0.5 McFarland standard were compared visually. A Unicam 8625 spectrophotometer (Cambridge, U.K.) was then used to confirm that the inoculum tube was at the required turbidity of between 0.08 and 0.1 at $OD600_{nm}$.

A sterile cotton swab was dipped into the adjusted suspension, optimally within 15 minutes after adjusting the turbidity of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess inoculum from the swab. The dried surface of the Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The rim of the agar was then swabbed as a final step. The lid was left ajar for three to five minutes to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

The predetermined battery of antimicrobial disks (Oxoid, Hampshire, U.K.) was dispensed onto the surface of the inoculated agar plate using a dispensing apparatus.

They were distributed evenly so that they were no closer than 24 mm from centre to centre. No more than five disks were placed on a plate at a time to avoid overlapping of zones. Once placed on the plate, the disks were not relocated because some of the antibiotic diffuses almost instantaneously. The plates were then inverted and placed in a 37°C incubator within 15 minutes after the disks were applied. The plates were examined after 16-18 hours of incubation. The zones were measured to the nearest millimetre using a ruler held on the back of the inverted petri dish. The standard error of the ruler was ± 0.5 mm. The zone margin was considered to be the area showing no obvious, visible growth that can be detected with the unaided eye. The sizes of the zones of inhibition were interpreted by referring to CLSI standards (Table 2.7) and were reported as being susceptible, intermediate or resistant to the agents that were tested. The zones of inhibition diameters of each antibiotic were also obtained for the control strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 35218 to ensure the method was being performed correctly. Their zones of inhibition diameters were measured and compared to the expected diameters according to CLSI (Table 2.8).

Table 2.7: Zone Diameter Interpretive Standards and Equivilent Minimal Inhibitory Concentration (MIC) Breakpoints for *Pseudomonas aeruginosa* (Clinical and Laboratory Standards Institute M100-S16, 2006)

Antimicrobial Class	Antimicrobial Agent (Concentration)	Zone Diameter Nearest whole mm			Equivalent MIC Breakpoints (μg/ml)	
		R	I	S	R	S
PENICILLINS	Ticarcillin (75 μg)	≤14	-	≥15	≥128	≤64
β-LACTAM/β-LACTAMASE COMBINATION	Ticarcillin-clavulanic acid (75/10 μg)	≤14	-	≥15	≥128/2	≤64/2
	Pipercillin-taobactam (100/10 μg)	≤17	-	≥18	≥128/4	≤64/4
CEPHEMS	Ceftazidime (30 μg)	≤14	15-17	≥18	≥32	≤8
	Cefepime (30 μg)	≤14	15-17	≥18	≥32	≤8
	Cefotaxime (30 μg)	≤14	15-22	≥23	≥64	≤8
	Ceftriaxone (30 μg)	≤13	14-20	≥21	≥64	≤8
	Ceftizoxime (30 μg)	≤14	15-19	≥20	≥32	≤8
	Moxalactam (30 μg)	≤14	15-22	≥23	≥64	≤8
CARBAPENEMS	Imipenem (10 μg)	≤13	14-15	≥16	≥16	≤4
	Meropenem (10 μg)	≤13	14-15	≥16	≥16	≤4
MONOBACTAMS	Aztreonam (30 μg)	≤15	16-21	≥22	≥32	≤8
FLUOROQUINOLONES	Ciprofloxacin (5 μg)	≤15	16-20	≥21	≥4	≤1
FOLATE PATHWAY INHIBITORS	Co-trimoxazole* (25 μg)	≤19	-	≥20	≥32	<32
TETRACYCLINES	Tetracycline (30 μg)	≤14	15-18	≥19	≥16	≤4

R = Resistant; **I** = Intermediate resistance; **S** = sensitive

* Those antibiotics for which CLSI have not yet established MIC breakpoints have are represented by an asterisk

Table 2.8: Actual and expected zones of inhibition for Quality Control Strains used to monitor accuracy of disk diffusion testing (Clinical and Laboratory Standards Institute M100-S16, 2006)

Antimicrobial Agent	Disk Content	*Escherichia coli* ATTC 25922		*Pseudomonas aeruginosa* ATCC 27853		*Escherichia coli* ATCC 35218	
		Expected Zone diameter (mm)	Actual Zone diameter (mm)	Expected Zone diameter (mm)	Actual Zone diameter (mm)	Expected Zone diameter (mm)	Actual Zone diameter (mm)
Aztreonam	30µg	28-36	29	23-29	23	-	-
Cefepime	30µg	31-37	34	24-30	26	-	-
Cefotaxime	30µg	29-35	33	18-22	19	-	-
Ceftazidime	30µg	25-32	32	22-29	27	-	-
Ceftizoxime	30µg	30-36	31	12-17	17	-	-
Ceftriaxone	30µg	29-35	31	17-23	21	-	-
Ciprofloxacin	5µg	30-40	38	25-33	25	-	-
Imipenem	10µg	26-32	32	20-28	22	-	-
Meropenem	10µg	28-34	31	27-33	31	-	-
Moxalactam	30µg	28-35	29	17-25	24	-	-
Piperacillin/ tazobactam	100/10µg	24-30	27	25-33	32	24-30	24
Ticarcillin	75µg	24-30	24	21-27	23	6	6
Ticarcillin/ clavulanic acid	75/10µg	24-30	27	20-28	26	21-25	22

2.7.3 MIC Determination by Microtitre Broth Dilution Method

In vitro susceptibility testing of all strains was determined by a microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [M7-A7], 2006). This method is called "microdilution" because it involves the use of small volumes of broth dispensed in sterile, plastic microdilution trays that have round or conical bottom wells. Cation adjusted Mueller-Hinton broth (Section 2.4.8) was the growth medium used in all the microdilution tests. All tests were done in triplicate.

The procedure for the microbroth dilution method is illustrated in Figure 2.1. The antimicrobial dilutions were prepared at double the desired final concentrations in cation adjusted Mueller-Hinton broth to allow for a 1:2 dilution of the antibiotics when an equal volume of inoculum was added. 50 µl of 2X the desired antibiotic solution was then added to each well. Each tray included a growth control well (no antibiotic) and a sterility (uninoculated) well. If the trays were not being used immediately, they were sealed in plastic bags and placed in a freezer at -20°C. All antibiotics were tested in triplicate.

Within 15 minutes of preparation, a standardised inoculum was prepared using the direct colony suspension method as described in Section 2.7.2. The adjusted inoculum suspension was diluted in Ringers solution (Section 2.3.7) so that each well would contain approximately 5 x 10^5 CFU/ml. Each well of the microdilution tray was then inoculated with 50 µl of the prepared inoculum using a micropipette. A purity check of the inoculum suspension was performed by subculturing an aliquot onto a nutrient agar plate. The microdilution trays were incubated at 37°C for 16-20 hours. The microdilution trays were never stacked more than four high to maintain the same incubation temperature for all cultures.

The MIC is the lowest concentration of the antimicrobial agent that completely inhibited growth of the organism in the microdilution wells as detected by the unaided eye. The amount of growth in the wells containing the antibiotic was compared to the amount of growth in the growth-control wells used in each set of tests. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) had to occur

in the growth-control well. The MICs were interpreted by referring to CLSI interpretive standards (Table 2.9) and were reported as being susceptible, intermediate or resistant to the agents that were tested. The validity of the method was checked according to the method of the CLSI (Clinical and Laboratory Standards Institute [M7-A7], 2006) using the control strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The MICs were compared to the CLSI expected MICs in Table 2.10.

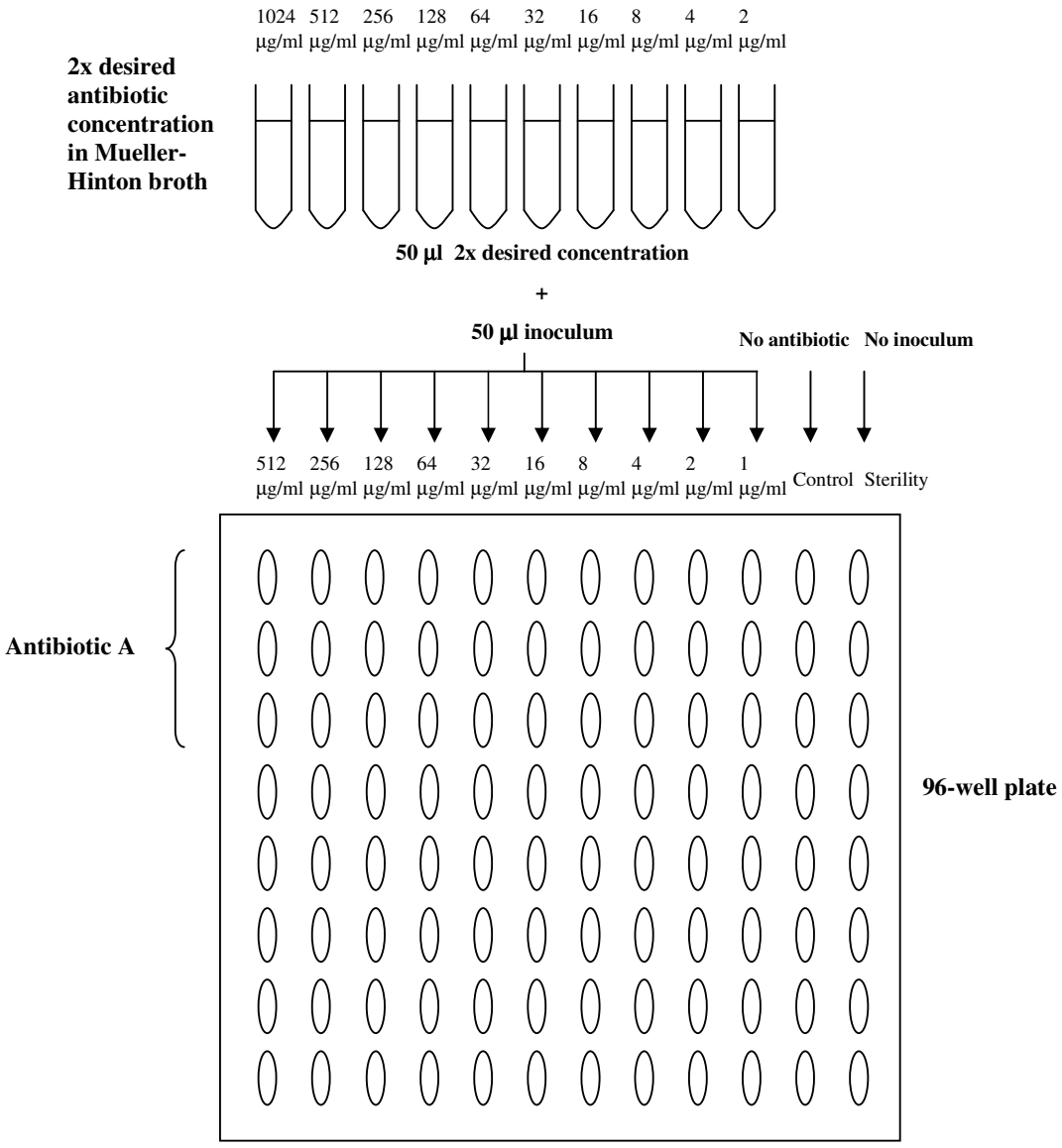


Figure 2.1: Schematic of microbroth dilution method

Table 2.9: MIC Interpretive Standards (µg/ml) for Breakpoints for *Pseudomonas aeruginosa* (Clinical and Laboratory Standards Institute M100-S16, 2006)

Antimicrobial Class	Antimicrobial Agent	MIC (µg/ml) Interpretive Standard		
		S	I	R
PENICILLINS	**Penicillin G***	≤128	-	≥512
	Ampicillin*	≤128	-	≥512
	Amoxicillin*	≤128	-	≥512
	Cloxacillin*	≤128	-	≥512
	Oxacillin*	≤128	-	≥512
	Carbenicillin	≤128	-	≥512
	Pipercillin	≤64	-	≥128
CEPHEMS	**Cephalothin***	≤8	16	≥64
	Cefsulodin*	≤8	16	≥64
	Ceftazidime	≤8	16	≥32
AMINOGLYCOSIDES	**Gentamicin**	≤4	8	≥16
	Amikacin	≤16	32	≥64
	Tobramycin	≤4	8	≥16
	Netilmicin	≤8	16	≥32
	Sisomicin*	≤8	16	≥32
	Apramycin*	≤4	8	≥16
	Butirosin A*	≤4	8	≥16
	Kanamycin*	≤16	32	≥64
	Lividomycin A*	≤4	8	≥16
	Hygromycin B*	≤4	8	≥16
	Neomycin*	≤4	8	≥16
	Paramomycin*	≤4	8	≥16
	Spectinomycin*	≤4	8	≥16
	Streptomycin*	≤4	8	≥16
FLUOROQUINOLONES	**Ofloxacin**	≤2	4	≥8
PHENICOLS	**Chloramphenicol**	≤8	16	≥32
TETRACYCLINES	**Tetracycline**	≤4	8	≥16

R = Resistant; **I** = Intermediate resistance; **S** = sensitive

* Those antibiotics for which CLSI have not yet established MIC breakpoints have are represented by an asterisk

Table 2.10: Expected and Actual Minimum Inhibitory Concentrations (MICs) (μg/ml) of Quality Control strains used to monitor accuracy of MIC testing (Clinical and Laboratory Standards Institute M100-S16, 2006)

Antimicrobial Agent	*Escherichia coli* ATTC 25922		*Pseudomonas aeruginosa* ATCC 27853	
	Expected MIC (μg/ml)	**Actual MIC (μg/ml)**	**Expected MIC (μg/ml)**	**Actual MIC (μg/ml)**
Amikacin	0.5-4	3.9	1-4	1.9
Ampicillin	2-8	3.9	-	-
Aztreonam	0.06-0.25	0.23	2-8	7.81
Carbenicillin	4-16	7.81	16-64	31
Ceftazidime	0.06-0.5	0.475	1-4	3.9
Chloramphenmicol	2-8	3.9	-	-
Ciprofloxacin	0.004-0.015	0.11	0.25-1	0.95
Gentamicin	0.25-1	0.95	0.5-2	1.9
Netilmicin	0.5-1	0.95	0.5-8	1.9
Ofloxacin	0.015-0.12	0.11	1-8	3.9
Piperacillin	1-4	1.9	1-8	1.9
Tetracycline	0.5-2	0.95	8-32	15.6
Tobramycin	0.25-1	0.475	0.25-1	0.95

2.8 Vectors used for cloning and expression

Four vectors were used in this study. Two of these, pDrive and pCR®2.1 were cloning vectors and two, pET-28a and pPC were expression vectors. The pDrive cloning vector was used to clone an amplified 2.2 kbp insert prior to sequencing and for the cloning of the *bla-$_{OXA}$* gene from *P. aeruginosa* PA13 prior to its insertion into the pPC expression vector. The pCR®2.1 cloning vector was used to clone the *bla-$_{OXA}$* gene from *P. aeruginosa* PA13 prior to its insertion into the pET-28a expression vector.

2.8.1 pDrive cloning vector

The pDrive cloning vector was obtained from Qiagen, UK. The vector provides highly efficient cloning of PCR products through UA hybridisation. It contains a large number of unique restriction enzyme sites, universal sequencing primer (M13) sites, and promoters for in vitro transcription and also allows both kanamycin and ampicillin selection as well as blue/white screening of recombinant colonies (Figure 2.2). It is supplied in a linear form with a U overhang at each 3' end, which hybridises with high specificity to the A overhang of PCR products generated by Taq and other non-proofreading DNA polymerases.

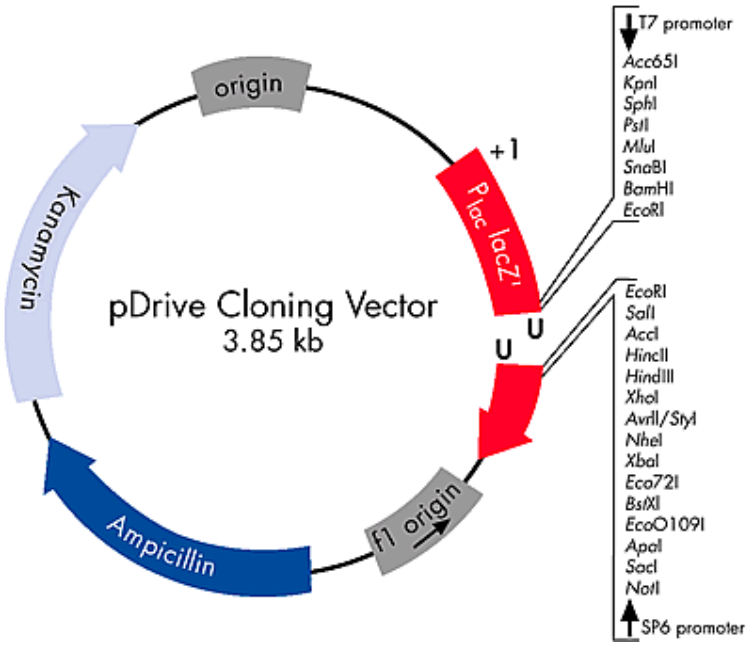


Figure 2.2: pDrive cloning vector map (Available at www.qiagen.com)

2.8.2 pCR®2.1 cloning vector

The pCR®2.1 cloning vector was obtained from Invitrogen, UK. It contains a large number of unique restriction enzyme sites and promoters for in vitro transcription and also allows both kanamycin and ampicillin selection as well as blue/white screening of recombinant colonies (Figure 2.3). It is supplied in a linear form with a T overhang at each 3' end, which hybridises with high specificity to the A overhang of PCR products generated by Taq and other non-proofreading DNA polymerases.

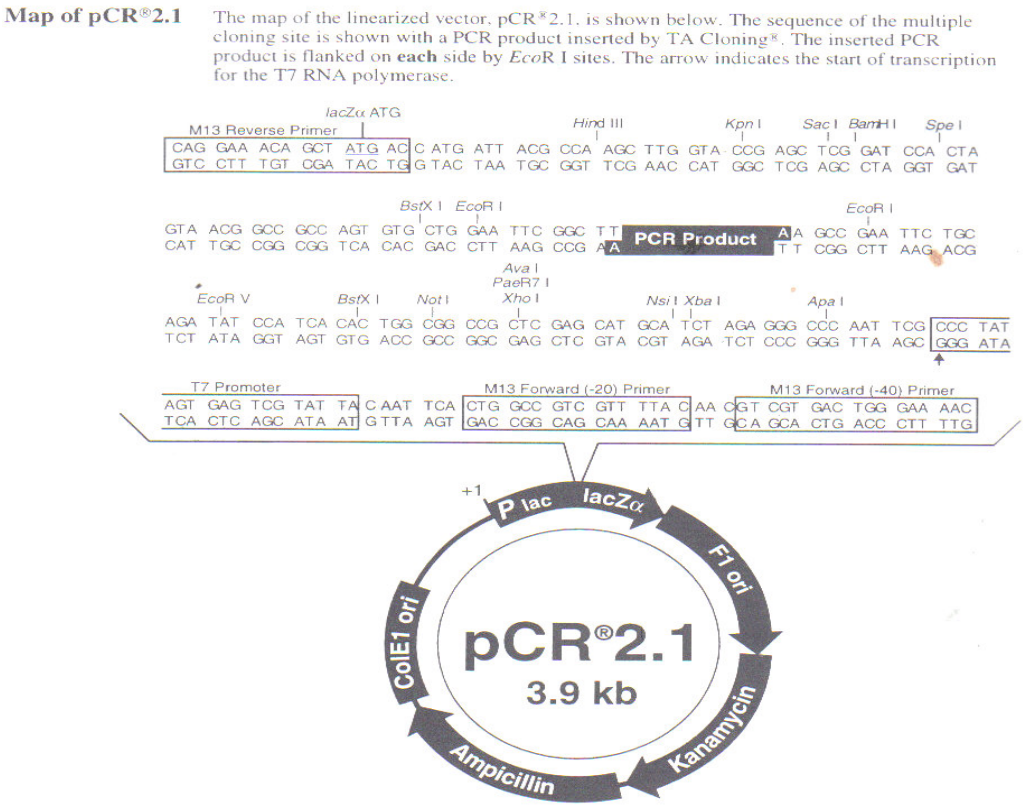


Figure 2.3: pCR®2.1 TA cloning vector map (Available at www.invitrogen.com)

2.8.3 pET-28a expression vector

The pET-28a was obtained from Novagen, UK. The vector contains a strong bacteriophage T7 promoter. It requires a source of T7 RNA polymerase to induce expression and therefore has to be introduced into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control such as *E. coli* BL21 (DE3). The pET-28a vector contains a multiple cloning site with many unique restriction enzyme recognition sites including *NcoI* and *XhoI* restriction sites. It also contains a kanamycin resistance gene for the selection of recombinant colonies (Figure 2.4).

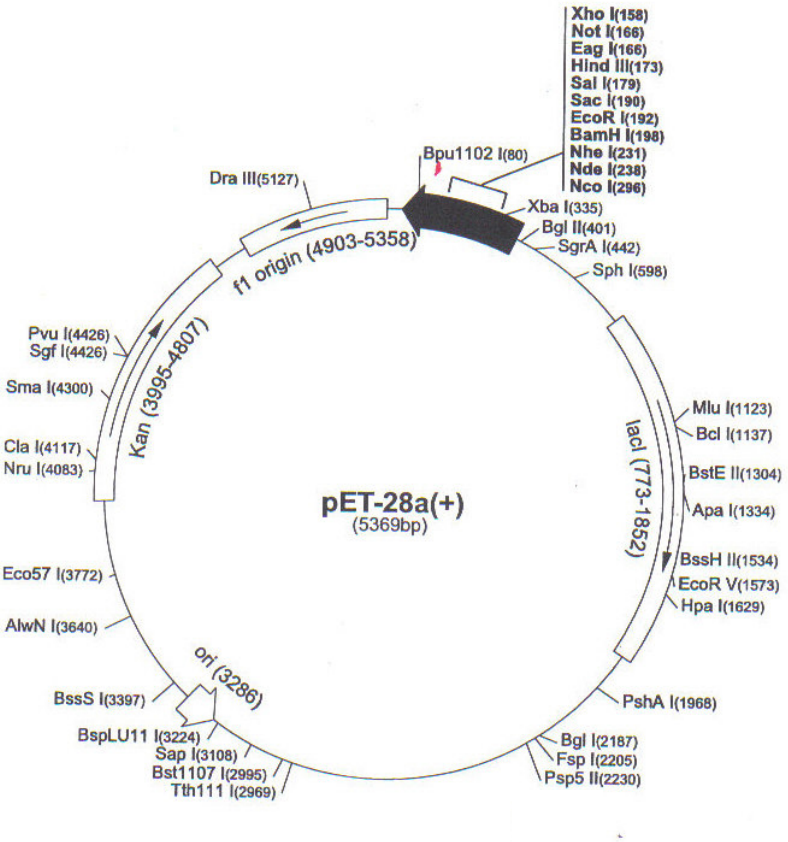


Figure 2.4: pET-28a expression vector map (Available at www.emdbiosciences.com)

2.8.4 pPC expression vector

The pPC expression vector was obtained from Dr. Michael O'Connell, DCU. It was constructed from a pQE-60 expression vector. It features the *tac* transcriptional promoter (P*tac*). P*tac* is a hybrid promoter consisting of the –35 region from the *trp* promoter fused to the –10 region (Pribnow box), operator and RBS (Shine-Dalgarno sequence) from P*lac*. The LacI protein can repress Ptac. pPC also contains an ampicillin resistance gene for the selection of recombinant colonies and a multiple cloning site containing *NcoI* and *BglII* restriction sites (Figure 2.5).

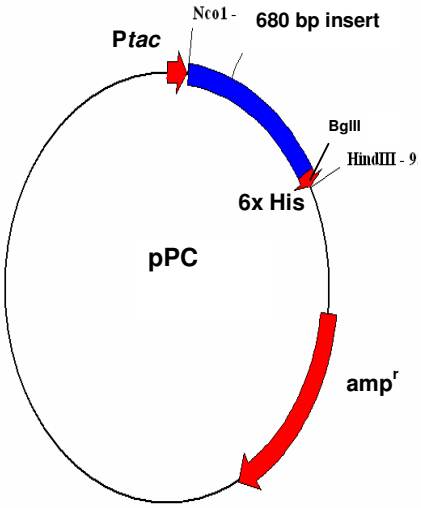


Figure 2.5: pPC expression vector map.
Generated using pDraw32.

2.9 Preparation of DNA

DNA was prepared using three methods. Genomic DNA was prepared using the method described in 2.9.1. Plasmid DNA was prepared using either a commercially available kit or by the Birnboim and Doly method (1979). These methods are described in Section 2.9.2 and 2.9.3 respectively

2.9.1 Rapid preparation of Gram-negative bacterial genomic DNA

This method was modified from that described by Chen and Kuo (1993) and used to prepare genomic DNA. A 1.5 ml aliquot of bacterial culture was pelleted at 13,000 X g in a Biofuge 13 microfuge (Heraeus Instruments) and the supernatant was removed. The cell pellet was resuspended in 200 µl of lysis buffer (Section 2.3.5) and lysed by vigorous pipetting. Then 66 µl of a 5M NaCl solution was added, the tube was mixed by inversion and the viscous mixture was centrifuged at 13,000 X g for 10 min at 4°C. The supernatant was transferred into a fresh tube and an equal volume of phenol chloroform was added and mixed gently by inversion 50 times. After centrifugation at 13,000 X g for 5 min, the supernatant was removed to a fresh tube and double the volume of chloroform was added. The tube was again mixed gently by inversion a further 50 times. Following centrifugation at 13,000 X g for 3 min, the extracted supernatant was transferred to a fresh tube and precipitated with 2.5 volumes of 95% (v/v) ice-cold ethanol. The pellet was washed twice with 70% (v/v) ethanol, dried in a vacuum dryer and redissolved in 50 µl of TE buffer (2.3.14). 1µl of RNase (5µg/ml) was added to the tube and incubated at 37ºC for 1 hour. Genomic DNA was stored at 4°C.

2.9.2 Plasmid DNA Purification

2.9.2.1 Gen Elute Plasmid DNA extraction kit

Plasmid DNA was isolated using the Genelute Plasmid mini-prep kit (Sigma-Aldrich). This kit is designed specifically for the efficient isolation of recombinant *E. coli* plasmid DNA.

The procedure is a modification of the alkaline-lysis method (Birnboim and Doly, 1979) and uses a silica membrane contained in a spin column. The silica membrane binds up to 20 µg of high copy plasmid DNA per ml of overnight culture. All the necessary components are supplied with the kit for isolation of the DNA.

Recombinant *E. coli* cells were grown overnight at 37°C in 5 ml LB or nutrient broth containing the appropriate antibiotic. Cells were harvested by centrifugation and resuspended in a resuspension solution containing RNase. Cells were then treated with a lysis solution to lyse the bacterial cell walls. A neutralisation step followed, to remove contaminants and debris. The DNA binding step followed, in the presence of a high salt concentration. The bound DNA was then washed thoroughly with alcohol. Plasmid DNA was then recovered by elution with sterile distilled water or TE buffer (Section 2.3.14) for further analysis. The recovered plasmid was predominantly in its supercoiled form.

2.9.2.2 Plasmid Preparation Method (Birnboim and Doly, 1979)

This method, used for the screening of large numbers of transformants, was described by Birnboim and Doly (1979). A 1.5 ml aliquot of a bacterial culture grown in selective media was pelleted at 4000 X g in a Biofuge 13 microfuge (Heraeus Instruments) and the supernatant removed. The pellet was resuspended by vortexing in 200 µl of solution 1 (Section 2.3.6) and was then left for 5 minutes at room temperature. Then 200 µl of solution 2 (Section 2.3.6) was added and the tube was mixed by inversion and placed on ice for 5 minutes. Then 200 µl of solution 3 (Section 2.3.6) was added and the tube was mixed by inversion and placed on ice for 10 minutes. The supernatant (600 µl) was placed into a fresh tube and 400 µl of phenol chloroform isoamylalcohol (25:24:1) was added and mixed by vortexing. After centrifugation at 13,000 X g for 5 minutes the aqueous layer was removed to a fresh tube and an equal volume of isopropanol was added. After 10 minutes at room temperature the tube was centrifuged for 10 minutes at 13,000 X g to pellet the plasmid DNA. The pellet was washed twice with 70% (v/v) ethanol, dried briefly in a vacuum dryer and resuspended in 50 µl of TE buffer (Section 2.3.14). 1µl of RNase

(5µg/ml) was added to the tube and incubated at 37ºC for 1 hour. Plasmid preps were stored at 4°C.

2.9.3 DNA concentration determination

DNA was diluted 1:50 in Milli-Q water and absorbance readings taken at A_{260nm}. One A_{260nm} unit (1cm light path) is equal to 50µg/ml DNA (Sambrook *et al*., 1989).

2.10 Primers and primer design

Primers for amplifying the 16S rRNA gene were designed by Marchesi *et al*. (1998). Primers were designed for the amplification of all genes encoding aminoglycoside modifying enzymes conferring resistance to gentamicin, which were described in Shaw *et al*. (1993). Nucleotide sequences for each of these genes were obtained from GenBank. Primer design was achieved by a mixture of manual and computer based examination. The primers were designed manually and an analysis of their homology was performed using the Megalign package (Dnastar Inc., Madison, WI). All of the primers used were synthesised by MWG Biotech, Germany.

2.10.1 Primers for the amplification of 16S rRNA

The universal primers in Table 2.11 were used to amplify the 16S rRNA genes from the clinical isolates.

Table 2.11: PCR amplification primers for 16S rRNA

Primer name	Primer sequence	Reference
63f	5'-CAG GCC TAA CAC ATG CAA GTC-3'	Marchesi *et al.*, 1998
1387r	5'-GGG CGG (AT)GT GTA CAG GC-3'	Marchesi *et al.*, 1998

2.10.2 Primers used for screening the clinical isolates for aminoglycoside modifying enzyme genes

Genes for enzymes which confer resistance to gentamicin were chosen from Shaw *et al*. (1993). Seventeen pairs of primers (Table 2.12) were designed to screen for the presence of aminoglycoside modifying enzyme genes in all of the isolates. In most cases the primers were designed in the laboratory from gene sequences available in Genbank. Other primers were attained from Heuer *et al*. (2002).

Table 2.12: PCR amplification primers to screen for genes encoding aminoglycoside modifying enzymes in clinical isolates

Primer name	Primer sequence	Reference
aac(2')-Ia F	5'-ATG ATT TTA TTA TTT TCC CTA CAC-3'	Shaw *et al*., 1993
aac(2')-Ia R	5'-ACA GCA TCG CCA GTC AC-3'	Shaw *et al*., 1993
aac(3)-Ia F	5'-CAT CAG CGG ACG CAG GGA GGA C-3'	Shaw *et al*., 1993
aac(3)-Ia R	5'-ACG GCC ACA GTA ACC AAC AAA-3'	Shaw *et al*., 1993
aac(3)-Ib F	5'-CCG CAG TTG CAA ACC CTC AC-3'	Shaw *et al*., 1993
aac(3)-Ib R	5'-AAT TGT TAG GTG GCG GTT CTT-3'	Shaw *et al*., 1993
aac(3)-IIa F	5'-ATG CGC GTG AGC TTC TTG GTC-3'	Shaw *et al*., 1993
aac(3)-IIa R	5'-GGT GCA CGA GCG TCA TTG G-3'	Shaw *et al*., 1993
aac(3)-IIb F	5'-CGC GTG TCG GCT TCA TCG TC-3'	Shaw *et al*., 1993
aac(3)-IIb R	5'-GAT CCG ATA GGG TCA ACG CC-3'	Shaw *et al*., 1993
aac(3)-IIc F	5'-ATA CCC TTT TGA GTT CGT TTT TGT-3'	Shaw *et al*., 1993
aac(3)-IIc R	5'-TAA AAA GCA GGG TAT GGG GAA-3'	Shaw *et al*., 1993
aac(3)-IIIa F	5'-TGT TGC ACT TGA TTA TTG ATT C-3'	Shaw *et al*., 1993
aac(3)-IIIa R	5'-ACC GCT GTG GGA TGA CTG T-3'	Shaw *et al*., 1993
aac(3)-IIIb F	5'-GCC GGC GCT CCC AGT CG-3'	Shaw *et al*., 1993
aac(3)-IIIb R	5'-TGG ATG CGG ACG ATC AAC CCT-3'	Shaw *et al*., 1993
aac(3)-IIIc F	5'-TGC GCG GCC GCT TCC TAC C-3'	Shaw *et al*., 1993
aac(3)-IIIc R	5'-AAG CCA ACC GGC GAT CAC ACG A-3'	Shaw *et al*., 1993
aac(3)-IVa F	5'-CTC CGC GTT CAG CCA GCA TC-3'	Shaw *et al*., 1993
aac(3)-IVa R	5'-GAG CTG CAT CAG GTC GGA GA-3'	Shaw *et al*., 1993
aac(3)-VIa F	5'-CCA AGG TAG TCG GCA AAT AAT GT-3'	Shaw *et al*., 1993
aac(3)-VIa R	5'-CTT GAG GTC CAT CGG GTT C-3'	Shaw *et al*., 1993
aac(3)-Xa F	5'-ACG ATC CGA GCG GTG TTT-3'	Shaw *et al*., 1993
aac(3)-Xa R	5'-TCC GCG TGA CCG TTG TGC-3'	Shaw *et al*., 1993

aac(6)-IIa F	5'-TTG CCC TCC CGC ACG ATG A-3'	Shaw *et al*., 1993
aac(6)-IIa R	5'-GCT AGA TTT TAA TGC GGA TGT TGC-3'	Shaw *et al*., 1993
aac(6)-IIb F	5'-AAC GAG CAA TTG CAG CCC CA-3'	Shaw *et al*., 1993
aac(6)-IIb R	5'-GGC AGG TCG GAG AGC AGG ATG-3'	Shaw *et al*., 1993
ant(2'')-I F	5'-TGG GCG ATC GAT GCA CGG CT(AG) G-3'	Heuer *et al.*, 2002
ant (2'')*-I* R	5'-AAA GCG GCA CGC AAG ACC TC(AC) AC-3'	Heuer *et al.*, 2002
aph(3')-1 F	5'-GCC ACA AAT GTT AAG GCA ATG A-3'	Heuer *et al.*, 2002
aph(3')-I R	5'- GAA TCT CCA AAA TC(AG) AT(AT) AT(GT) CC-3'	Heuer *et al.*, 2002
aac(6')-aph(2") F	5'-AGT AAA AAG GCC ATA TAA CAG TCC-3'	Shaw et al., 1993
aac(6')-aph(2") R	5'-GAA GTA CGC AGA AGA GAA AAG AT-3'	Shaw et al., 1993

2.10.3 Primers used for cloning the *bla-$_{OXA}$* gene into expression vectors

The primers in Table 2.13 were used to amplify the *bla-$_{OXA}$* with restriction sites for cloning and expression. The cloning of *bla-$_{OXA}$* for expression in the pET-28a vector required a *NcoI* restriction site at the 5' end and a *XhoI* restriction site at the 3' end of the gene. The cloning of *bla-$_{OXA}$* for expression in the pPC vector required a *NcoI* restriction site at the 5' end and a *BamHI* restriction site at the 3' end of the gene.

Table 2.13: PCR primers to amplify the *bla-$_{OXA}$* gene with restriction sites for cloning and expression in expression vectors pET-28a and pPC

Primer Name	**Primer sequence**
pETOXA F	5'-gaa act tac cat ggc aat ccg att cct cac cat-3' *NcoI*
pETOXA R	5'-gat tga ctc gag gtt ggg cgg caa tgc gtc-g3' *XhoI*
pPCOXA F	5'-gaa act tac cat ggc aat ccg att cct cac cat-3' *NcoI*
pPCOXA R	5'-gat tga gga tcc gtt ggg cgg caa tgc gtc g-3' *BamHI*

2.10.4 Primers used for sequencing

Primers were designed to sequence the amplified 16S rRNA from the clinical isolates, the integron from *P. aeruginosa* PA13 and the oxacillinase (*bla-$_{OXA}$*) gene from *P. aeruginosa* PA13 cloned into the pET-28a and pPC expression vectors.

2.10.4.1 Primers used to sequence 16S rRNA gene from all of the isolates

Table 2.14: Primers used to sequence 16S rRNA from all of the isolates

Primer name	Primer sequence	Reference
63f	5'-CAG GCC TAA CAC ATG CAA GTC-3'	Marchesi *et al.*, 1998
1387r	5'-GGG CGG (AT)GT GTA CAG GC-3'	Marchesi *et al.*, 1998

2.10.4.2 Primers used to sequence the integron

Table 2.15: Primers used to sequence the integron

Primer Name	Primer sequence
AAC6F	5'- ttg ccc tcc cgc acg atg a - 3'
AAC6R	5'- gct aga ttt taa tgc gga tgt tgc - 3'
AAC6intF	5'- gca aga gtc cgt cac tcc - 3'
AAC6intR	5'- ata tca tga aag gct ggc t - 3'

2.10.4.3 Primers used to sequence the *bla-$_{OXA}$* gene in the expression vectors

Table 2.16: Primers used to sequence the *bla-$_{OXA}$* gene in both directions in the expression vectors

Primer Name	Primer sequence
OXAseq F	5' caa gac ttg cga tca gcg atg cga aat 3'
OXAseq R	5' cca gta atc gcc ctt gat tgt cga agg 3'

2.11 PCR Amplification

PCR was used to amplify 16S rRNA from all the isolates, to screen for genes encoding aminoglycoside modifying enzymes, and to amplify the oxacillinase gene (*bla*-$_{OXA}$) for cloning into vectors. In all cases the amplified DNA products were resolved by conventional electrophoresis through horizontal 0.8% w/v agarose gels at 130V for 60 minutes, stained with ethidium bromide and the results were visualised and photographed using an Imagemaster VDS image analysis system (Amersham Pharmacia Biotech, N. J., US).

2.11.1 PCR protocol for the amplification of the 16S rRNA gene using universal primers

The 16S rRNA gene was amplified using the universal primers in Table 2.11. A total volume of 50µl was used for the amplification. Each reaction contained 5µl of template DNA, 10 *p*mol/µl of each primer forward-63f and reverse-1387r, 5µl 10X PCR reaction buffer, 2.5mM of each dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$ and 1µl 10X *Taq* DNA polymerase. All reactions were amplified in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA) using the following temperature profile: Denaturation at 95°C for 2 minutes, followed by 25 cycles of 95°C for 1 minute, 55.8°C for 1 minute, 72°C for 1.5 minutes and a final extension step at 72°C for 5 minutes.

2.11.2 PCR protocol for the amplification of the integron DNA from *P. aeruginosa* PA13

The integron in *P. aeruginosa* PA13 was amplified using the primers in Table 2.12. A total volume of 50µl was used for the amplification. Each reaction contained 5µl of template DNA, 10 *p*mol/µl of each primer forward-*aac(6')-IIa*-F and reverse-*aac(6')-IIa*-R, 5µl 10X PCR reaction buffer, 2.5mM of each dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$ and 1µl 10X *Taq* DNA polymerase. All reactions were amplified in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA) using the following temperature profile: Denaturation at 95°C for 3 minutes, followed by 30 cycles of

95°C for 30 seconds, 63.4°C for 30 seconds, 72°C for 30 seconds and a final extension step at 72°C for 10 minutes

2.11.3 PCR protocol for amplification of *bla-$_{OXA}$* gene from *P. aeruginosa* PA13 for cloning and expression

The *bla-$_{OXA}$* gene was amplified using the primers in Table 2.13. A total volume of 50µl was used for the amplification. Each reaction contained 5µl of template DNA, 10 *p*mol/µl of each primer pETOXA-F and pETOXA-R, 5µl 10X PCR reaction buffer, 2.5mM of each dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$ and 1µl 10X *Taq* DNA polymerase. All reactions were amplified in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA) using the following temperature profile: Denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 70°C for 30 seconds, 72°C for 30 seconds and a final extension step at 72°C for 10 minutes.

2.12 Preparation of competent cells

Two methods were used for the preparation of competent cells for transformation. These were the calcium chloride method and the rubidium chloride method. These procedures are described in Section 2.12.1 and 2.12.2 respectively.

2.12.1 Preparation of competent cells for transformation (calcium chloride method)

A modified method of Dagert and Ehrlich (1979) was used for the preparation of competent cells for transformation. *E. coli* XL1-Blue cells required treatment with a salt solution, to facilitate the uptake of the pDrive plasmid harbouring the amplified PCR product. By treating the cells with calcium chloride solution, the permeability of the bacterial cell wall is increased allowing the proficient passage of the plasmid DNA across the cell wall into the host vector.

An overnight culture of *E. coli* XL1-Blue was prepared by suspending a colony from a fresh LB agar plate in 100 ml of LB broth. The culture was incubated in a shaking

incubator at 200 rpm and 37°C for 24 hours. 10 ml of this culture was then suspended in 90ml fresh nutrient broth and grown for 90 minutes to an OD 600nm of approximately 0.3. 10 ml aliquots were centrifuged at 4000 X g in a Eppendorf 5180R bench top centrifuge for 10 min to pellet the cells. The supernatant was discarded and the cells were washed in 5ml ice-cold 10mM NaCl and re-centrifuged.

The supernatant was discarded and cells were resuspended in $CaCl_2$ and recentrifuged, twice. Following a third washing with 5 ml 30mM $CaCl_2$, the cells were suspended in another 5 ml $CaCl_2$ and chilled in ice for 20 minutes. Cells were then recentrifuged and resuspended in 1 ml volumes of ice-cold 30mM $CaCl_2$ + 15% (v/v) glycerol. 200 µl aliquots were prepared in pre-chilled microfuge tubes. The cells could be used immediately for transformation or stored at -80°C. They were quickly frozen by immersion in ethanol that had been cooled to -80°C prior to storage at -80°C.

2.12.2 Preparation of competent cells for transformation (rubidium chloride method)

The method of Hanahan (1985) was used to prepare competent cells for transformation. An overnight culture of *E. coli* XL10-Gold was prepared by suspending a colony from a fresh LB agar plate in 100ml of LB broth. The culture was incubated in shaking incubator at 200 rpm and 37°C for 24 hours. 1 ml of this culture was then added to 100 ml of pre-warmed LB broth and grown at 200 rpm and 37°C until the O.D. 600_{nm} reached 0.5. It is important that the cells are growing actively in the mid-exponential phase to achieve good competence.

The culture was cooled on ice for 20 minutes and then transferred to a cooled sterile centrifuge tube. The cells were harvested by centrifugation for 5 minutes at 4000 X g in a cooled rotor at 4°C in a Sorval RC-5B centrifuge (Du Pont Instruments, Delaware, U.S.). The cells were always kept cold by keeping them on ice. The supernatant was discarded carefully and the cells returned to ice quickly. The cells were then washed in 50 ml ice-cold 10mM $MgCl_2$ and collected by centrifugation for 5 minutes at 4000 X g in a cooled rotor at 4°C. The cells were resuspended by gently

swirling them in 30 ml of ice-cold TFB1 buffer (Section 2.3.15) and the resuspended cells were kept on ice for 90 minutes. The cells were collected by centrifugation for 5 minutes at 4000g in a cooled rotor at 4°C and then resuspended in 4 ml of ice-cold TFB 2 buffer (Section 2.3.16). 200 µl aliquots were prepared in pre-chilled microfuge tubes.

The cells could be used immediately for transformation or stored at -80°C. They were quickly frozen by immersion in ethanol that had been cooled to -80°C prior to storage at -80°C.

2.13 Cloning and ligation

The cloning and ligation of genes was performed using three methods. These were cloning using the pDrive cloning vector, cloning using the pCR®2.1 cloning vector and ligation using T4 DNA ligase.

2.13.1 Cloning using the Qiagen pDrive vector

The Qiagen pDrive cloning vector was chosen for the cloning and subsequent sequencing of the integron amplified from *P. aeruginosa* PA13. It was also used for cloning the *bla-$_{OXA}$* gene from *P. aeruginosa* PA13 prior to insertion into the pPC expression vector. The pDrive vector was supplied with a ligation master mix (2x) providing all the reagents and cofactors required for efficient ligation of the PCR product to the pDrive vector with high specificity. Ligations were prepared as in Table 2.17.

Table 2.17: pDrive ligation mix

Component	Volume
pDrive cloning vector	1µl
PCR product	4µl
Ligation master mix	5µl
dH_2O	variable

Following the manufacturer's instructions, the master mix was added last into the reaction. The ligation was mixed and incubated at 4°C for 2 hours. A longer incubation time resulted in an increase in the number of recombinants yielded. Ligations were then used immediately after incubation or stored at -20°C, until required.

2.13.2 Cloning using the pCR®2.1

The pCR®2.1 cloning vector (Invitrogen) was used for cloning the *bla-$_{OXA}$* gene from *P. aeruginosa* PA13 prior to its insertion into the pET-28a expression vector. The pCR®2.1 vector was supplied with a ligation buffer (10x) and T4 DNA ligase providing all the reagents and cofactors required for efficient ligation of the PCR product to the pCR®2.1 vector with high specificity. Ligations were prepared as in Table 2.18.

Table 2.18: pCR®2.1 ligation mix

Component	Volume
PCR product	2 µl
pCR®2.1 cloning vector	2 µl
10X Ligation Buffer	1 µl
T4 DNA Ligase	1 µl
dH_2O	variable

Following the manufacturer's instructions, the ligation reaction was mixed and incubated at 14°C for a minimum of 4 hours. A longer incubation time resulted in an increase in the number of recombinants yielded. Ligations were then used immediately after incubation or stored at -20°C, until required.

2.13.3 Ligation of *bla-$_{OXA}$* gene into expression vectors

The *bla-$_{OXA}$* gene amplified from *P. aeruginosa* PA13 was ligated into both the pET-28a and pPC expression vectors using T4 ligase (New England Biosciences, UK). The components in Table 2.19 were mixed and incubated at 4°C overnight.

Table 2.19: Components used in ligation reactions

Component	Volume (µl)
DNA insert	4
Expression vector	4
T4 ligase	1
T4 ligase buffer	1
Total	10

2.14 Transformation

200 µl of *E. coli* competent cells were thawed on ice. Once thawed, 2 µl of the ligation mixture (Table 2.19) was added to the tube and mixed gently. The tubes were then left on ice for 30 minutes. The tubes were then incubated for 30 seconds at 42°C in a waterbath and placed on ice for a further 5 minutes. 800 µl of LB broth was added to the ligations and the tubes were incubated at 37°C, while shaking at 200 rpm for 1 hour. After this time, the cells were centrifuged at 6000 X g for 1 minute. The pellet was then resuspended in 200 µl of LB broth. 100µl of the cells were plated out on LB agar with IPTG (1mM), ampicillin (100µg/ml) and X-gal (50µg/ml). Plates were incubated at 37°C for 24 hours. Post-incubation, the plates were screened for recombination white colonies, which were selected and streaked onto fresh LB/IPTG/agar plates for plasmid purification.

2.15 Restriction digests

Restriction digests were used to confirm the insertion of the amplified 2.2 kbp product into the pDrive cloning vector prior to sequencing, to remove the cloned *bla-$_{OXA}$* gene from both the pCR®2.1 and pDrive cloning vectors, to prepare the pET-28a expression vector for ligation, to remove the control insert from the pPC expression vector, preparing it for ligation, and to confirm the correct orientation of the cloned *bla-$_{OXA}$* gene in both expression vectors. Both single and double restriction digests were used.

2.15.1 Single restriction digests

Single restriction digests were used to confirm the insertion of the amplified 2.2 kbp product from *P. aeruginosa* PA13 in the pDrive cloning vector and also to confirm the correct orientation of the *bla-$_{OXA}$* gene in both the pET-28a and pPC expression vectors. The single restriction digests were performed using the components in Table 2.20. The restriction enzymes used were *Eco*R I (for insert confirmation in pDrive), *Xho* I (for confirmation of correct orientation of insert in pET-28a) and *Bgl* II (for confirmation of correct orientation of insert in pPC).

Table 2.20: Components used in single digest restriction reactions

Component	Volume (µl)
Plasmid DNA	4
Restriction enzyme	1
Reaction Buffer H	2
dH_2O	13

All restriction digests were performed in a heating block at 37°C for either 4 hours or overnight.

2.15.2 Double restriction digests

Double restriction digests were used to remove the cloned *bla-$_{OXA}$* gene from both the pDrive and pCR®2.1 cloning vectors, to prepare the pET-28a expression vector for ligation and to remove the control insert from the pPC expression vector, preparing it for ligation. The double digests were performed using the components in Table 2.21. The restriction enzymes used were *Nco* I/*Xho* I (to remove *bla-$_{OXA}$* from pCR®2.1 and to prepare pET-28a for ligation), *Nco* I/*BamH* I (to remove *bla-$_{OXA}$* from pDrive) and *Nco* I/*Bgl*II (to remove the control insert from pPC, preparing it for ligation).

Table 2.21: Components used in double digest restriction reactions

Component	Volume (µl)
Buffer E	6
Restriction enzyme 1	3
Restriction enzyme 2	3
BSA (x10)	6
DNA	30
dH_2O	12
Total	**60**

The restriction reaction was incubated at 37°C for 4 hours.

2.16 Excision and purification of DNA fragments from agarose gel

Restricted DNA fragments from agarose gels were exised and purified using two methods. One method used a commercially available kit to exise and purify the fragment (Section 2.16.1) and the other is described in Section 2.16.2.

2.16.1 Excision of DNA fragments from agarose gel using extraction kit

The agarose gel slice containing the relevant DNA fragment was excised from the gel with a scalpel while viewing the band on a U.V. transilluminator (Vilber Lourmat, Tourcy, France). The fragment was then recovered from the gel using a HiYield™

Gel/PCR DNA fragments extraction kit (Real Biotech Corp., Taiwan) by following the manufacturer's instructions.

The extraction kit is designed to recover or concentrate DNA fragments (50bp-10kb) from agarose gels, PCR or other enzymatic reactions. The method uses a chaotropic salt, guanidine thiocyanante to dissolve agarose gel and denature enzymes. DNA fragments in chaotropic salt solution bind to the glass fibre matrix of the spin column. After washing off the contaminants, the purified DNA fragments are eluted by addition of low salt elution buffer or water. Salts, enzymes and unincorporated nucleotides are effectively removed from reaction mixtures without phenol extraction or alcohol precipitation. Typical recoveries are 60-80%.

2.16.2 Excision and purification of restricted DNA fragments from agarose gel

The DNA fragment insert was removed from the agarose gel using the method of O'Cuív (2003). The fragment was excised using a scalpel while viewing the band on a U.V. transilluminator (Vilber Lourmat, Tourcy, France). The DNA fragment was placed in a microfuge tube containing glass wool which was then placed within a larger microfuge tube. A hole was made in the bottom of the inner microfuge tube. The microfuge tubes were centrifuged at 13,000 X g for 20 minutes in a Biofuge 13 microfuge (Heraeus Instruments) to liquefy the DNA.

The sample containing the DNA to be precipitated was brought up to 400 µl with distilled water. 400 µl of phenol chloroform isoamylalcohol (25:24:1) was added and mixed by brief vortexing (5 seconds). Upon centrifugation at 13,000 X g for 5 min the mixture is divided into an upper aqueous and lower organic layer. The aqueous layer was removed to a new microfuge tube with an equal volume of chloroform and mixed by brief vortexing. The tube was centrifuged at 13,000 X g for 5 min and the aqueous layer was transferred to a new microfuge tube. A 1/10 volume of 3 M sodium acetate was added followed by an equal volume of isopropanol and mixed by inversion. The tube was left at room temperature for 60 min and then centrifuged at 13,000 X g for 20 min to pellet the DNA. The pellet was washed with 70% (v/v) ethanol and then dried. The DNA was resuspended in 20-50 µl of TE buffer.

2.17 Antarctic Phosphatase treatment

Both expression vectors were treated with antarctic phosphatase before ligation. Antarctic Phosphatase (New England Biolabs) catalyzes the removal of 5' phosphate groups from DNA and RNA. Since phosphatase-treated fragments lack the 5'-phosphoryl termini required by ligases, they cannot self-ligate (Sambrook *et al.*, 1989). This property was used to decrease the vector background in cloning strategies.

Table 2.22: Components used in the antarctic phosphatase treatment of expression vectors

Component	Volume (µl)
Antarctic phosphatase	6
Antarctic Reaction Buffer	6
DNA	48
Total	60

The reaction (Table 2.22) was incubated at 37°C for 2 hours and then heated to 65°C for 5 minutes inactivated the enzyme.

2.18 DNA sequencing

DNA gene sequencing was performed by both Qiagen and MWG Biotech sequencing services. ABI automated sequence technology was used e.g. the ABI PRISM® 377 DNA Sequencer, which automatically analyses DNA molecules labelled with multiple fluorescent dyes based on the principle of the Sanger sequencing method (Sanger *et al*., 1977), which involves chain termination of the nucleotides.

2.19 Bioinformatic sequence analysis

Sequences were verified using the online BLAST searches on the NCBI website (www.ncbi/nlm/nih/gov). Amino acid alignments were performed using the ClustalW (Thompson *et al*., 1994) program on the European Bioinformatics Institute website (www.ebi.ac.uk). Open reading frames were identified using an open reading frame

finder program on the NCBI website. Nucleotide sequences were translated using the ExPASy online translation tool (http://www.expasy.ch/tools/dna.html). The 16S rRNA phylogenetic tree was drawn using Multiphyl programme (Keane *et al.*, 2007) available online at http://distributed.cs.nuim.ie/multiphyl.php and were viewed using Treeview software (Version 1.6.6) available online at: http://taxonomy.zoology.gla.ac.uk/rod/rod.html.

2.20 Graphics for construct maps

The maps of vector constructs were drawn using pDRAW32 software (AcaClone software, US) available at http://www.acaclone.com

2.21 Induction and extraction of target protein

5 ml aliquots of LB broth containing 100 µg/ml of selective antibiotic (kanamycin for pET-28a and ampicillin for pPC) and 1% (w/v) glucose were inoculated with single colonies of the transformed cells. These cultures were incubated overnight at 37°C while shaking at 250 rpm.

The following morning, 1 ml of the overnight culture was added to 100 ml of LB broth containing the selective antibiotic and 1% (w/v) glucose. The cultures were incubated at 37°C while shaking at 250 rpm for 2-3 hours until they reached an OD_{600nm} of 0.3-0.4. Once this OD was achieved the culture was divided into two 50 ml cultures. 50-500 µM IPTG (a non-hydrolyzable lactose analogue) was added to the culture to be induced. The other 50 ml culture had no IPTG added to it and was used as the non-induced control. The cultures were then incubated while shaking for 4 hours.

The cells from each culture were then decanted into pre-cooled centrifuge tubes and centrifuged at 5000 X g at 4°C in a Sorvall RC-5B centrifuge (Du Pont Instruments, Delaware, U.S.) for ten minutes. The supernatant was removed and the cells were resuspended in ice-cold 100 ml 100mM sodium phosphate buffer (pH 7) (Section 2.3.10). The cells were then centrifuged at 5000 X g and 4°C for a further 10 minutes.

The supernatant was then removed and the cells were resuspended in 10 ml ice-cold 100 mM sodium phosphate buffer. The cells were sonicated (Vibracell, Sonics and Materials Inc.) (pulses of 2.5 s at amplitude 40) on ice for 30 seconds. The suspension was then centrifuged at 4000 X g for 20 minutes. The supernatant (soluble fraction) was removed to a sterile universal and stored at 2-8°C until the sample was analysed using SDS-PAGE. The pellet (insoluble fraction) was resuspended in 100mM sodium phosphate buffer and stored at 2-8ºC until the sample was analysed using SDS-PAGE.

2.22 SDS-Polyacrylamide gel electrophoresis

Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), which was performed using 12% (w/v) gradient gels (Section 2.4.11) overlaid with a 4% (w/v) stacking gel (Section 2.4.12). The gel plates were cleaned prior to use with hot soapy water, rinsed with distilled water, rinsed with ethanol and allowed to dry. Seals were placed between the gel plates and the plates were clamped together. The components for both the separating gels and stacking gels were mixed. The resolving gel was poured first, about three-quarters of the way up the gel. This gel was overlaid with isopropanol (to prevent air bubbles) and allowed to set for 30 minutes. The isopropanol was removed and the stacking gel was poured above the resolving gel. A comb was then inserted into the stacking gel and the gel was allowed to set for 60 minutes.

Samples (25 µl) from induced and uninduced strains were prepared by boiling in sample buffer (Section 2.3.9) were electrophoresed at 15 mA until the tracking dye entered the resolving gel, at which time the mA was increased to 30 mA for 3-4 hours. A wide range molecular weight standard (6,500-205,000 Da) was obtained from Sigma (Cat. No.: M4038) was used as a marker on all SDS-PAGE gels.

Coomassie brilliant blue staining solution (Section 2.3.11) was used to stain the SDS-PAGE gels. The gels were stained for 3-4 hours at room temperature with gentle shaking. The gels were rinsed in milli-Q water and transferred into destaining solution (Section 2.3.1), then gently shaken at room temperature until blue bands and a clear background were obtained. Fresh destaining solution was added if required. The gels were kept in Milli-Q water overnight after destaining.

3.0 Results

3.1 Identification of clinical bacterial isolates

It was of interest to study clinical isolates of *Pseudomonas aeruginosa*, obtained from two Irish hospitals, the Adelaide and Meath National Children's Hospital, Tallaght, Dublin 24 and Waterford Regional Hospital. Preliminary identification tests were performed on all the isolates (Gram stain, oxidase and catalase tests). Twelve isolates were choosen for further identification. These were PA1, PA3, PA5, PA7, PA8, PA9, PA10, PA11, PA12, PA13, PA16 and PA17. *Pseudomonas aeruginosa* PAO1 was used as a control in all tests. The isolates were identified using a variety of techniques. These included morphological characteristics, biochemical testing, pigment production, API 20NE, Biolog GN and 16S rRNA analysis.

3.1.1 Morphological characteristics

The cell and colony characteristics of the isolates are outlined in Tables 3.1 and 3.2 respectively. The cell characteristics were noted following cultivation on nutrient agar at 37°C. All the strains were identified as being Gram-negative, non-spore forming, motile rods as seen in Table 3.1. With the exception of colour, which was either white or creamy, the colony characteristics of twelve of the strains were identical. One isolate, PA12, was the only mucoid strain (Table 3.2).

Table 3.1: Cell characteristics of the clinical isolates

Strain	Cell Characteristic			
	Gram +/-	**Shape**	**Spores +/-**	**Motility**
PA1	-	Rod	-	+
PA3	-	Rod	-	+
PA5	-	Rod	-	+
PA7	-	Rod	-	+
PA8	-	Rod	-	+
PA9	-	Rod	-	+
PA10	-	Rod	-	+
PA11	-	Rod	-	+
PA12	-	Rod	-	+
PA13	-	Rod	-	+
PA16	-	Rod	-	+
PA17	-	Rod	-	+
PAO1	-	Rod	-	+

Table 3.2: Colony characteristics of the clinical isolates strains

Strain	Colony Characteristic				
	Colour	**Shape**	**Edge**	**Elevation**	**Texture**
PA1	Cream	Round	Flat	Raised	Non-mucoid
PA3	Cream	Round	Flat	Raised	Non-mucoid
PA5	White	Round	Flat	Raised	Non-mucoid
PA7	Cream	Round	Flat	Raised	Non-mucoid
PA8	White	Round	Flat	Raised	Non-mucoid
PA9	Cream	Round	Flat	Raised	Non-mucoid
PA10	White	Round	Flat	Raised	Non-mucoid
PA11	White	Round	Flat	Raised	Non-mucoid
PA12	Cream	Round	Flat	Raised	Mucoid
PA13	White	Round	Flat	Raised	Non-mucoid
PA16	White	Round	Flat	Raised	Non-mucoid
PA17	Cream	Round	Flat	Raised	Non-mucoid
PAO1	White	Round	Flat	Raised	Non-mucoid

3.1.2 Identification of isolates using biochemical tests

A variety of biochemical tests were performed on all the strains to enable their identification and the results are shown in Table 3.3. These tests were selected as conventional phenotypic tests used to identify *Pseudomonas aeruginosa* (Cowan and Steel's manual for the identification of medical bacteria, 1993).

Table 3.3: Biochemical properties of the bacterial isolates

Strain	A	B	C	D	E	F	G	H	I	J
PA1	Ox	+	+	+	+	+	+	-	+	+
PA3	Ox	+	+	+	+	+	+	-	+	+
PA5	Ox	+	+	+	+	+	+	-	+	+
PA7	Ox	+	+	+	+	+	+	-	+	+
PA8	Ox	+	+	+	+	+	+	-	+	+
PA9	Ox	+	+	+	+	+	+	-	+	+
PA10	Ox	+	+	+	+	+	+	-	+	+
PA11	Ox	+	+	+	+	+	+	-	+	+
PA12	Ox	+	+	+	+	+	+	-	+	+
PA13	Ox	+	+	+	+	+	+	-	+	+
PA16	Ox	+	+	+	+	+	+	-	+	+
PA17	Ox	+	+	+	+	+	+	-	+	+
PAO1	Ox	+	+	+	+	+	+	-	+	+

A – Hugh and Leifson, Oxidative (Ox) and Fermentative; **B** – Oxidase; **C** – Catalase; **D** – Haemolysis; **E** – Growth on Cetrimide agar; **F** – Tween 80 hydrolysis; **G** – Thornley Arginine Dihydrolase; **H** – Growth at 5°C; **I** – Growth at 37°C; **J** – Growth at 42°C

The isolates were all oxidase and catalase positive and were shown to be oxidative organisms when grown on Hugh and Liefson's medium. The strains gave positive results for arginine hydrolysis and growth on cetrimide agar. The isolates also were all β-haemolytic on blood agar. All strains possessed lipolytic activity (Tween 80 hydrolysis). The isolates grew well at both 37°C and 42°C but were unable to grow at 5°C (Table 3.3). On the basis of these results the isolates were identified as *Pseudomonas aeruginosa*.

3.1.3 Colour production by the isolates when grown on selective agars

The isolates were grown on a variety of media to investigate pigment production. The colours produced by the isolates when they were grown on selective media at 37°C were noted. All isolates were grown on *Pseudomonas* Isolation Agar F, a medium that stimulates the production of fluorescein, *Pseudomonas* Isolation Agar P, a medium that stimulates the production of pyocyanin and also on cetrimide agar, a medium that stimulates the production of pyocyanin. These pigments are typical of *Pseudomonas aeruginosa*. The isolates were also grown on nutrient agar, which is not a selective medium. The colours that diffused into the media from each isolate are described in Table 3.4.

The isolates produced yellow/green, indicative of fluorescein production, blue/green, indicative of the pyocyanin production, red, indicative of pyorubin and brown, indicative of pyomelanin production (Table 3.4). The isolates universally produced the fluorescent yellow/green pigment associated with the production of pyoverdin (fluorescein) on PIA F agar. Nine of the isolates produced the blue/green pigment that is associated with the production of the blue/green pigment, pyocyanin on PIA P agar. These same strains also produced this blue/green pigment on cetrimide agar. Three isolates (PA3, PA11 and PA12) produced a red pigment associated with the production of pyorubin on nutrient agar. One isolate, PA9, produced a brown pigment associated with the production of pyomelanin on all four media. This strain also produced pyoverdin on PIA F agar and pyocyanin on PIA P agar and cetrimide agar, however these pigments were produced along with the brown pyomelanin pigment.

Table 3.4: Growth of isolates on agar at 37°C to demonstrate pigment production

Medium	PIA F	PIA P	Cetrimide Agar	Nutrient Agar
Strain	**Colour produced**			
PA1	Yellow	Blue/Green	Blue/Green	None
PA3	Yellow	Blue/Green	Blue/Green	Red
PA5	Yellow	Blue/Green	Blue/Green	Blue
PA7	Yellow	None	Yellow	None
PA8	Yellow	Blue/Green	Blue/Green	Blue
PA9	Yellow/ Brown	Brown/ Blue	Yellow/ Brown	Brown
PA10	Yellow	Blue/Green	Yellow	None
PA11	Yellow	Blue/Green	Blue/Green	Red
PA12	Yellow	None	Yellow	Red
PA13	Yellow	None	Yellow	None
PA16	Yellow	Blue/Green	Blue/Green	Blue
PA17	Yellow	Blue/Green	Blue/Green	Blue
PAO1	Yellow	Blue/Green	Blue/Green	Blue

PIA = Pseudomonas isolation agar

Yellow = fluorescein (pyoverdin)

Blue/Green = pyocyanin

Red = pyorubin

Brown = pyomelanin

3.1.4 Identification of isolates using API 20NE

In addition to the biochemical tests, identification was performed using the commercially available identification kit, API 20NE (BioMerieux). The API 20NE system, which was recommended for the identification of Gram-negative bacterial isolates, consisted of twenty-one enzymatic and carbon compound assimilation tests which were performed in cupules on a plastic strip where desiccated contents were reconstituted with a suspension of the test organism. The reaction for each of the assimilation tests for each of the isolates is shown in Table 3.5.

All of the isolates were identified as *Pseudomonas aeruginosa* by the API 20NE identification kit. The identifications were excellent (9 strains and control strain), very good (3 strains) and good (1 strain), with % i.d. ranging from 91.4% to 99.99% (Table 3.5). The results of the API 20NE tests showed that although there is great similarity among all the strains, they do vary in their ability to produce various enzymes and utilize the various carbon sources in the kit. All thirteen strains had the same reaction in thirteen of the twenty tests on the API 20NE test strip. However, there were some exceptions. PA3 was able to produce indole, it could acidify glucose and it was positive for β-galactosidase. PA8, PA9, PA10 and PA16 were all negative for urease. PA10 and PA16 could not assimilate N-acetyl-glucosamine, PA10 and PA17 could not assimilate adipate and PA10 could not assimilate citrate. The results obtained for the *Pseudomonas aeruginosa* ATCC 27853 control strain were as expected (BioMerieux, 1997).

Table 3.5: Results of API 20NE identification for *Pseudomonas aeruginosa* strains

Test	Strain (*P. aeruginosa*) PA													
	1	**3**	**5**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**16**	**17**	**PAO1**	**Control**
Reduction of nitrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Glucose acidification	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Arginine Dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	-	-	-	+	+	+	-	+	+	-
Esculin Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-galactosidase	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Glucose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-Glucosamine assimilation	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Maltose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caprate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adipate assimilation	+	+	+	+	+	+	-	+	+	+	+	-	+	+
Malate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate assimilation	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Phenyl-acetate assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Strain	PA 1	PA 3	PA 5	PA 7	PA 8	PA 9	PA 10	PA 11	PA 12	PA 13	PA 16	PA 17	PAO1	Control
i.d. (%)	99.99	99.9	99.99	99.9	99.6	99.99	91.4	99.99	99.99	99.99	99.9	99.99	99.9	99.99
T	0.90	0.58	0.90	0.90	1.00	0.90	0.60	0.90	0.90	0.90	0.88	0.74	0.90	0.90
i.d.	Ex	V.G.	Ex	Ex	V.G.	Ex	G	Ex	Ex	Ex	Ex	V.G.	Ex	Ex

Ex = Excellent = %id ≥ 99.9% and T ≥0.75; **V.G.**=Very good=%id ≥99% and T ≥0.5;

G = Good = %id ≥ 90% and T ≥0.25

3.1.5 Identification of isolates using Biolog GN

All of the isolates were identified as *Pseudomonas aeruginosa* by the Biolog GN identification system. The reactions of each isolate to the various carbon sources are shown in Table 3.6.All the strains were identified with a 100% probability of being *P. aeruginosa* except for PA9, which had a 99% probability. The similarity (SIM) values of the isolates varied from 0.52 to 0.99. The higher the value the better the identification. The SIM value is used as a calling criterion to judge the reliability and confidence of the identification. The Biolog results showed that all of the isolates have very similar biochemical properties. All of the isolates possessed the same ability to utilize or inability to utilize sixty-two of the ninety-six carbon sources on the Biolog plates. However, there were a number of exceptions. These exceptions show that no two of the isolates are the same.

PA11 and PA12 were able to use α-cyclodextrin. PA3, PA5, PA7, PA8, PA16, PA17 were unable to assimilate dextrin. PA5, PA16 and PA17 were unable to use N-acetyl-D-glucosamine or D-fructose. PA5, PA9, PA11, PA12, PA16 and PA17 were unable to utilize L-arabinose. PA5, PA9, PA12, PA13, PA16, PA17 were not able to use D-arabitol. PA12 had the ability to utilize cellobiose, melibiose and D-glucosaminic acid. PA1, PA7, PA11 and PA12 could use β-methyl D-glucoside. PA3, PA5, PA9 and PA17 were unable to utilize D-psicose. PA7 was able to use L-rhamnose and both PA7 and PA8 were able to use sucrose. PA1, PA9 and PA11 had the ability to utilize trehalose. PA1 was unable to use α-ketovaleric acid, D, L lactic acid, propionic acid, or succinic acid and had the ability to use D-saccharic acid. PA9 was able to utilize L-phenylamine and unable to utilize D, L carnitine. Neither PA1 nor PA9 were able to use quinic acid. Both PA1 and PA3 had the ability to use uridine. PA17 was unable to use L-ornithine or L-threonine. PA1, PA7, PA8, PA16 and PAO1 were able to use sebacic acid. PA5, PA9, PA13 and PA17 were not able to utilize L-leucine. Both PA7 and PA10 were able to use D-serine. PA3, PA5, PA8, PA10, PA16 and PA17 were unable to use i-erythritol. PA1, PA7, PA8, PA10 and PAO1 were able to utilize D-mannose. Both PA10 and PA12 were able to use L-alanyl-glycine. PAO1 was able to use glycyl-L-glutamic acid.

Table 3.6 Results of Biolog GN identification for *Pseudomonas aeruginosa* strains.

Carbon Source	PA 1	PA 3	PA 5	PA 7	PA 8	PA 9	PA 10	PA 11	PA 12	PA 13	PA 16	PA 17	PA O1
Water	-	-	-	-	-	-	-	-	-	-	-	-	-
α-cyclodextrin	-	-	-	-	-	-	-	+	+	-	-	-	-
Dextrin	+	-	-	-	-	+	+	+	+	+	-	-	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-D-glucosamine	+	+	-	+	+	+	+	+	+	+	-	-	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	-	+	+	-	+	-	-	+	-	-	+
D-arabitol	+	+	-	+	+	-	+	+	-	-	-	-	+
Cellobiose	-	-	-	-	-	-	-	-	+	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	+	-	-	-	-
β-methyl D-glucoside	+	-	-	+	-	-	-	+	+	-	-	-	-
D-psicose	+	-	-	+	+	-	+	+	+	+	+	-	+
D-raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	+	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	+	+	-	-	-	-	-	-	-	-
Trehalose	+	-	-	-	-	+	-	+	-	-	-	-	-
Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+
Mono-methyl succinate	+	+	+	+	+	+	+	+	+	+	+	+	+
p-hydroxyphenylacetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
itaconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-keto butyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-keto glutaric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-keto valeric acid	-	+	+	+	+	+	+	+	+	+	+	+	+
D, L-lactic acid	-	+	+	+	+	+	+	+	+	+	+	+	+
Malonic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionic acid	-	+	+	+	+	+	+	+	+	+	+	+	+
Quinic acid	-	+	+	+	+	-	+	+	+	+	+	+	+
D-saccharic acid	+	-	-	-	-	-	-	-	-	-	-	-	-
Sebacic acid	+	-	-	+	+	-	-	-	-	-	+	-	+
	-	+	+	+	+	+	+	+	+	+	+	+	+

Succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-histidine	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxy L-proline	+	+	-	+	+	-	+	+	+	-	+	-	+
L-leucine	+	+	+	+	+	+	+	+	+	+	+	-	+
L-ornithine	-	-	-	-	-	+	-	-	-	-	-	-	-
L-phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+
L-pyroglutamic acid	-	-	-	+	-	-	+	-	-	-	-	-	-
D-serine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-serine	+	+	+	+	+	+	+	+	+	+	+	-	+
L-threonine	+	+	+	+	+	-	+	+	+	+	+	+	+
D, L-carnitine	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-amino butyric acid	+	-	-	+	-	+	-	+	+	+	-	-	+
i-erythritol	+	+	-	+	+	+	+	+	+	+	-	-	+
D-fructose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-fructose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
m-inositol	-	-	-	-	-	-	-	-	-	-	-	-	-
α-D-lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	-	-	+	+	-	+	-	-	-	-	-	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
cis-aconitic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Citric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Formic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactonic acid lactone	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galacturonic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
D-gluconic acid	-	-	-	-	-	-	-	-	+	-	-	-	-
D-glucosaminic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
D-glucuronic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-hydroxybutyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
β-hydroxybutyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-
γ-hydroxybutric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Bromo-succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinamic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucuronamide	+	+	+	+	+	+	+	+	+	+	+	+	+

Alaninamide	+	+	+	+	+	+	+	+	+	+	+	+	+
D-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-alanine	-	-	-	-	-	-	+	-	+	-	-	-	-
L-alanyl-glycine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycyl-L-aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-	+
Glycyl-L-glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Urocanic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Inosine	+	+	-	-	-	-	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl ethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+
Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	+
2-amino ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+
2,3-butanediol	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-
D, L-α-glycerol phosphate	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose-1-phosphate	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose-6-phosphate													

Strain	**PA 1**	**PA 3**	**PA 5**	**PA 7**	**PA 8**	**PA 9**	**PA 10**	**PA 11**	**PA 12**	**PA 13**	**PA 16**	**PA 17**	**PAO1**
PROB (%)	100	100	100	100	100	99	100	100	100	100	100	100	100
SIM	0.60	0.68	0.81	0.60	0.66	0.89	0.69	0.66	0.64	0.52	0.99	0.87	0.67

3.1.6 Identification of isolates using 16S rRNA gene analysis

Genotypic-based identification eliminates the problem of variable phenotype and allows for more accurate identification of bacteria. The use of 16S rRNA in the classification of bacterial species has now been well established and 16S rRNA gene sequencing is now the gold standard of bacterial identification. 16S rRNA genes are highly conserved among all organisms. However, all organisms possess various unique species-specific regions that allow for bacterial identification. The 16S rRNA genes from all of the isolates were amplified and sequenced to confirm their identification and were compared to each other and other similar species using phylogenetic analysis.

3.1.6.1 Amplification of 16S rRNA gene

The 16S rRNA genes from all the isolates were amplified using the universal primers in Table 2.11. The amplified 1300 bp PCR product seen in Figure 3.1 was identical to the product produced by all of the isolates

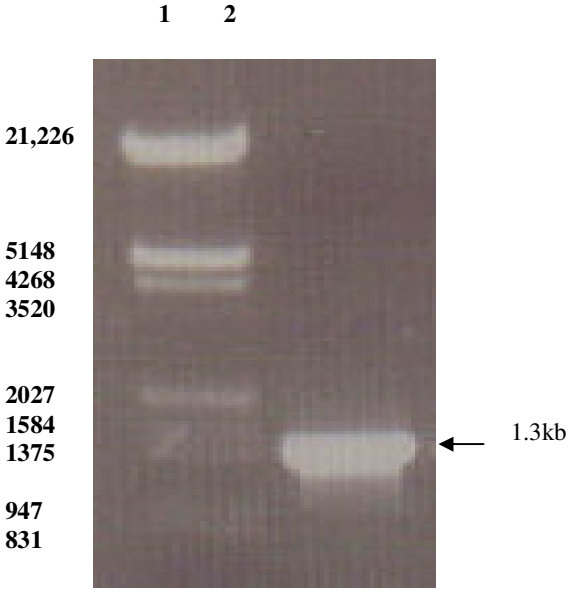


Figure 3.1 Agarose gel showing the (1.3 kbp) 16S rRNA PCR product amplified using universal 16S rRNA primers. Lane 1, DNA ladder. Lane 2, 16S rRNA PCR product.

The amplified 16S rRNA PCR products from all the isolates were sequenced by Qiagen. The 16S rRNA gene sequences of the twelve isolates and *P. aeruginosa* PAO1 were aligned and this is shown in Figure 3.2. These isolates shared 99-100% nucleotide similarity with eachother. BLASTN search analysis was used to compare the obtained 16S rRNA sequence data form the isolates with other 16S rRNA gene sequences available in the GenBank database (www.ncbi.nlm.nih.gov/BLAST). Sequences from the isolates showed 99%-100% sequence similarity to a known strain of *Pseudomonas aeruginosa*, *P. aeruginosa* S8 (Wang *et al*., 2007), as supported by Figure 3.3.

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PA3                          TGTCTGATTCACGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 59
PA10                         TGTCTGATTCACGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 59
PA16                         T-TCTGATTC-CGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 50
PA1                          TGTCTGATTC-CGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 57
PA13                         T-TCTGATTCACGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 54
PA9                          T-TCTGATTC-CGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 47
PA5                          T-TCTGATTC-CGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 47
PA11                         TGTCTGATTCACGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 49
PA12                         TGTCTGATTCACGATTACTAAGCGATTCCGACTTCACGCAGTCGAG 48
PA8                          T-TCTGATTC-CGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 47
PA17                         TTTCTGATTCACGATTACTACGCGATTCCGACTTCACGCAGTCGAG 46
PAO1                         CTTCTGATTCACGATTACTA-GCGATTCCGACTTCACGCAGTCGAG 46
PA7                          TTTCTGATTCACGATTACTACGCGATTCCGACTTCACGCAGTCGAG 46
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PA3             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 119
PA10            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 119
PA16            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 110
PA1             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 117
PA13            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 114
PA9             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 107
PA5             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 107
PA11            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 109
PA12            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 108
PA8             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 107
PA17            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 106
PAO1            TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 106
PA7             TTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG 106
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PA3             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 179
PA10            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 179
PA16            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 170
PA1             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 177
PA13            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 174
PA9             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 167
PA5             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 167
PA11            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 169
PA12            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 168
PA8             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 167
PA17            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 166
PAO1            GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 166
PA7             GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG 166
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PA3             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 239
PA10            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 239
PA16            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 230
PA1             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 237
PA13            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 234
PA9             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 227
PA5             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 227
PA11            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 229
PA12            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 228
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PA8             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 227
PA17            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 226
PAO1            ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 226
PA7             ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC 226
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PA3             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 299
PA10            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 299
PA16            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 290
PA1             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 297
PA13            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 294
PA9             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 287
PA5             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 287
PA11            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 289
PA12            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 288
PA8             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 287
PA17            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 286
PAO1            CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 286
PA7             CCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT 286
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PA3             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 359
PA10            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 359
PA16            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 350
PA1             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 357
PA13            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 354
PA9             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 347
PA5             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 347
PA11            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 349
PA12            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 348
PA8             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 347
PA17            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 346
PAO1            CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 346
PA7             CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAAT 346
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PA3             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 419
PA10            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 419
PA16            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 410
PA1             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 417
PA13            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 414
PA9             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 407
PA5             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 407
PA11            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 409
PA12            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 408
PA8             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 407
PA17            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 406
PAO1            CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 406
PA7             CCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAA 406
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PA3             TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCG-GTCAATTCATTTGAGTTTTAACCC 478
PA10            TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACCC 478
PA16            TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 468
PA1             TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCCGTCAATTCATTTGAGTTTTAACC- 476
PA13            TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 472
PA9             TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 465
PA5             TTAAACCACATGCTCCACCGCTTGTGCGGGGCCCCCGTCAATTCATTTGAGTTTTAACC- 466
PA11            TTAAACCACATGCTCCACCGCTTGTGCCGGGCCCCCGTCAATTCATTTGAGTTTTAACC- 468
PA12            TTAAACCACATGCTCCACCGCTTGTGC-GGGCCCCCGTCAATTCATTTGAGTTTTAACC- 466
PA8             TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 465
PA17            TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 464
PAO1            TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCCGTCAATTCATTTGAGTTTTAACC- 465
PA7             TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC-GTCAATTCATTTGAGTTTTAACC- 464
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PA3             TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 534
PA10            TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 534
PA16            TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 524
PA1             TTGCGG-CCGTACTCCCC-AGGCCGGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 533
PA13            TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 528
PA9             TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 521
PA5             TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 522
PA11            TTGCGG-CCGTACTCCCCCAGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 525
PA12            TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 522
PA8             TTGCGG-CCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 521
PA17            TTGCGGCCCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 521
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PAO1            TTGCGGGCCGTACTCCCCCAGGC-GGTCGACTTTATCGCGTTAGCTGCGCCACTAAGATC 524
PA7             TTGCGGCCCGTACTCCCC-AGGC-GGTCGACTT-ATCGCGTTAGCTGCGCCACTAAGATC 521
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PA3             TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 592
PA10            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 592
PA16            TC-AAGGATCCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 583
PA1             TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 591
PA13            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 586
PA9             TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 579
PA5             TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 580
PA11            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 583
PA12            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 580
PA8             TCCAAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 580
PA17            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 579
PAO1            TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 582
PA7             TC-AAGGATCCC-AACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA 579
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PA3             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 652
PA10            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 652
PA16            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 643
PA1             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 651
PA13            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 646
PA9             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 639
PA5             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 640
PA11            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 643
PA12            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 640
PA8             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 640
PA17            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 639
PAO1            TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 642
PA7             TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTT 639
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PA3             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 712
PA10            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 712
PA16            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 703
PA1             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 711
PA13            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 706
PA9             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 699
PA5             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 700
PA11            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 703
PA12            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 700
PA8             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 700
PA17            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 699
PAO1            CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 702
PA7             CGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCAC 699
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PA3             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGGAT 772
PA10            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGGAT 772
PA16            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 762
PA1             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 770
PA13            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 765
PA9             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 758
PA5             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 759
PA11            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGAAT 763
PA12            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGA-T 759
PA8             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 759
PA17            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 758
PAO1            CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 761
PA7             CCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGG-AT 758
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PA3             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 832
PA10            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 832
PA16            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 822
PA1             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 830
PA13            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 825
PA9             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 818
PA5             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 819
PA11            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 823
PA12            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 819
PA8             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 819
PA17            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 818
PAO1            TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG 821
PA7             TTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCCATTAACG 818
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PA3             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTACCCGGTGCTTATTCTGTT 891
PA10            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 891
PA16            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 881
PA1             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 889
PA13            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 884
PA9             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 877
PA5             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 878
PA11            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 882
PA12            CTTGCACCCTTCGTATTACCGCGGCTGCTGGGCACGAAGTTAGCCGGTGCTTATTCTGTT 879
PA8             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 878
PA17            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 877
PAO1            CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 880
PA7             CTTGCACCCTTCGTATTACCGCGGCTGCTGG-CACGAAGTTAGCCGGTGCTTATTCTGTT 877
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PA3             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 951
PA10            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 951
PA16            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 941
PA1             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 949
PA13            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 944
PA9             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 937
PA5             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 938
PA11            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 942
PA12            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 939
PA8             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 938
PA17            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 937
PAO1            GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 940
PA7             GGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTT 937
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PA3             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1011
PA10            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1011
PA16            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1001
PA1             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1009
PA13            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1004
PA9             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 997
PA5             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 998
PA11            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1002
PA12            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 999
PA8             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 998
PA17            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 997
PAO1            ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 1000
PA7             ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC 997
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PA3             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1071
PA10            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1071
PA16            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1061
PA1             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1069
PA13            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1064
PA9             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1057
PA5             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1058
PA11            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1062
PA12            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1059
PA8             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1058
PA17            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1057
PAO1            CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1060
PA7             CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGAC 1057
                ************************************************************

PA3             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCAACCAACT 1131
PA10            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCAACCAACT 1131
PA16            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1121
PA1             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1129
PA13            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1124
PA9             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1117
PA5             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1118
PA11            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1122
PA12            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1119
PA8             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1118
PA17            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1117
PAO1            TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1120
PA7             TGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACT 1117
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PA3             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1191
PA10            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1191
PA16            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1181
PA1             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1189
PA13            AGCTAATCCGACCTAGGCTCATC-GATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1183
PA9             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1177
PA5             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1178
PA11            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1182
PA12            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1179
PA8             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1178
PA17            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1177
PAO1            AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1180
PA7             AGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCC 1177
                ********************** ***********************************

PA3             TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1251
PA10            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1251
PA16            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1241
PA1             TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1249
PA13            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1243
PA9             TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1237
PA5             TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1238
PA11            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1242
PA12            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1239
PA8             TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1238
PA17            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1237
PAO1            TCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1240
PA7             TCAGGACGTATGCGGTATTAGCGCCCCTTTCCGGACGTTATCCCCCACTACCAGGCAGAT 1237
                ************************** ***********************************

PA3             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1296
PA10            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1296
PA16            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1285
PA1             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1293
PA13            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1288
PA9             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1283
PA5             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1284
PA11            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1293
PA12            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1281
PA8             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1289
PA17            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1281
PAO1            TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1291
PA7             TCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGC---------- 1281
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Figure 3.2: Nucleotide alignment of the 16S rRNA gene sequences from all the isolates and *P. aeruginosa* PAO1.

3.1.6.2 Phylogenetic analysis

Phylogenetic analysis of the sequences was performed following alignment of the 1,300 bp consensus sequences using ClustalW software. The phylogenetic tree was generated using the Multiphyl program (Keane *et al.*, 2007) and visualised using Treeview software package. The Multiphyl program chose a general time reversible model as the best model for comparing the strains. With this substitution model the relative frequencies of each character do not change. For a time reversible model, there is no assumption that substitutions preferentially change in certain directions

over time. For example, A→C→G is the same as G→C→A. The tree search used was the nearest neighbour interchange.

The phylogenetic tree in Figure 3.3 illustrated the relationship between the 16S rRNA sequences of the bacterial isolates (PA1-PA17) and a known strain of *P. aeruginosa*, *Pseudomonas aeruginosa* S8 (Wang *et al*., 2007). It also compared the isolates to other members of the Pseudomonads which have many phenotypic characteristics common to *P. aeruginosa*, making them difficult to differentiate using conventional identification techniques. These were *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas stutzeri*. The sequences were also compared to those of species that were previously members of the Pseudomonads: *Xanthomonas* spp. and *Burkholderia cepacia*. The phylogenetic tree revealed a very high degree of relatedness between all of the isolates and the known strain of *Pseudomonas aeruginosa*. The isolates were closely related to the other members of the Pseudomonads which were located on different neighbouring branches and less closely related to the *Xanthomonas* and *Burkholderia cepacia* strains. The percentage of nucleotide similarity between all of these strains is shown in Figure 3.3.

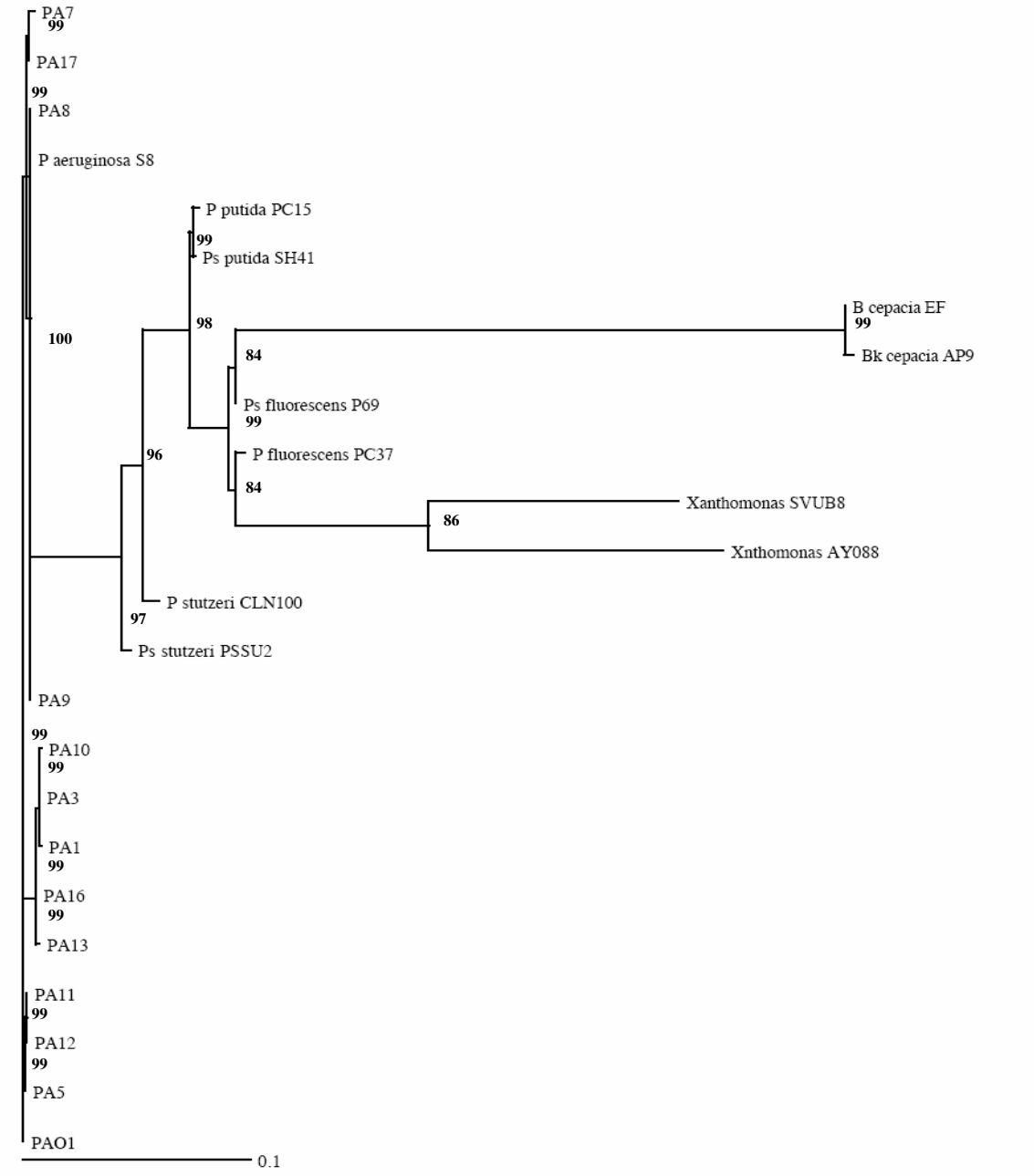

Figure 3.3: Phylogenetic tree showing the taxanomic classification of all the isolates and a comparison with other Pseudomonad strains and closely related species based on their 16S rRNA sequences. The strains used were *P. aeruginosa* S8 (EF362637) (Wang *et al*., 2007), *Xanthomonas* spp. SVUB8 (AM401582) (El-Deeb *et al*., 2006), *Xanthamonas* spp. AY088 (AF385546) (Paster *et al*., 2001), *P. fluorescens* PC37 (DQ178234)(Merimaa *et al*., 2006), *P. fluorescens* P69 (AY973267) (Heinaru *et al*., 2000), *P. putida* PC15 (AY973267) (Heinaru *et al*., 2000), *P. putida* SH41 (AJ833919) (Heiss *et al*., 2004), *P. stutzeri* CLN100 (AJ544240) (Cladera *et al*., 2004), *P. stutzeri* PSSU2 (AJ310484) (Nunez *et al*., 2001), *B. cepacia* (EF113108) (Zhao and Chen, 2006) and *B. cepacia* AB9 (AB272341) (Kobashi *et al*., 2007). 0.1 = nucleotide differences. Bold figures indicate percentage similarity.

3.2 An evaluation of the antibiotic sensitivities of the isolates

The ability of the isolates to grow in the presence of antibiotics was tested. The two approaches used were the disk diffusion method and broth microdilution method according to the CLSI guidelines. The isolates were initially screened using commercially available multidisks (Mast Diagnostics, Merseyside, U.K). They were subsequently tested for their sensitivities to a wider range of antibiotics and the minimum inhibitory concentrations (MICs) of each of these antibiotics for all of the isolates were obtained. The minimum inhibitory concentration (MIC) is the minimum concentration of the antibacterial agent below which bacterial growth is not inhibited. In all, the isolates were screened against forty-one antibiotics from eleven classes of antibiotics.

3.2.1 Preliminary screening

Preliminary susceptibility screening of the isolates was performed using Mastring-S M14 multidisks (Mast Diagnostics, Merseyside, U.K.) which included eight antibiotic agents from five antibiotic classes effective against Gram-negative bacteria. They were chosen for their diversity of antibiotic classes. The multidisks were used as a quick screening method to identify which classes of antibiotics the isolates were susceptible/resistant to. The results (Table 3.7) showed that the isolates were universally resistant to ampicillin and cephalothin (β-lactams), sulphatriad and cotrimoxazole (folate pathway inhibitors) and tetracycline. All of the strains were susceptible to the lipopeptide antibiotic, colistin sulphate. However, the most interesting finding from this screening was that all of the isolates were susceptible to the aminoglycoside antibiotics, gentamicin and streptomycin, except for *P. aeruginosa* PA13, which showed resistance to both of these antimicrobial agents.

Table 3.7: *Pseudomonas aeruginosa* strains grown on Mueller-Hinton agar with antibiotic susceptibility discs at 37°C

Strain (PA)	1	3	5	7	8	9	10	11	12	13	16	17	PA01
Disk conc. (μg)													
Ampicillin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+
Cephalothin (5)	+	+	+	+	+	+	+	+	+	+	+	+	+
Colistin Sulphate (25)	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentamicin (10)	-	-	-	-	-	-	-	-	-	+	-	-	-
Streptomycin (10)	-	-	-	-	-	-	-	-	-	+	-	-	-
Sulphatriad (200)	+	+	+	+	+	+	+	+	+	+	+	+	+
Tetracycline (25)	+	+	+	+	+	+	+	+	+	+	+	+	+
Cotrimoxazole (25)	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = resistant, - = susceptible

3.2.2 Definitive susceptibility screening

Definitive antibiotic susceptibility testing was performed using either the disk diffusion method or the broth microdilution method according to the methods of the CLSI (Section 2.7). Antibiotic susceptibility disks (Oxoid, Hampshire, U. K.) were generally used when antibiotic powders were either unavailable or expensive. The aminoglycosides were the first class of antibiotics to be tested. This was because of the result obtained during preliminary screening (Table 3.7) showing that *P. aeruginosa* PA13 was resistant to the clinically important aminoglycosides, gentamicin and streptomycin. After the aminoglycosides were tested, other antibiotic classes commonly used to treat Gram-negative bacteria were also screened to build up a comprehensive antibiotic resistance profile for all of the isolates. These included antibiotics from the β-lactam, the fluoroquinolone, folate sythesis inhibitor, tetracycline and phenicol classes.

Aminoglycosides

The MIC values of antibiotics from the aminoglycoside class for all of the isolates are shown in Table 3.8. In all, fourteen aminoglycosides were screened using the broth microdilution method. The isolates were universally susceptible to four aminoglycosides: amikacin (1-8 μg/ml), apramycin (2-4 μg/ml), butirosin A (2-4 μg/ml) and lividomycin A (0.5-2 μg/ml). However, all of the isolates except *P. aeruginosa* PA13 were susceptible to another five of the aminoglycosides: gentamicin (1-2 μg/ml), netilmicin (4-8 μg/ml), sisomicin (2 μg/ml), streptomycin (2-4 μg/ml) and tobramycin (1-2 μg/ml). All of the strains were resistant to kanamycin, hygromycin B, paramomycin and spectinomycin with low-level resistance to paramomycin (16-64 μg/ml), spectinomycin (32 μg/ml) and hygromycin B (32-64 μg/ml) and higher level of resistance to kanamycin (128 μg/ml). The MIC values of kanamycin, spectinomycin and neomycin were identical for all the isolates. *P. aeruginosa* PA13 stood out from the other isolates because in addition to having high-level resistance to the latter four antibiotics, it also had a very high level of resistance to netilmicin (1024 μg/ml), a high level of resistance to gentamicin and sisomicin (128 μg/ml) and a lower level of resistance to tobramycin (32 μg/ml), neomycin (32 μg/ml) and streptomycin (64 μg/ml).

Table 3.8: Minimum Inhibitory Concentration values of aminoglycoside antibiotics for all the isolates

Strain	**Minimum Inhibitory Concentration (μg/ml)**													
P. aeruginosa	**AMK**	**APR**	**BUT**	**GEN**	**KAN**	**HYG**	**LIV**	**NEO**	**NET**	**PAR**	**SIS**	**SPEC**	**STR**	**TOB**
PA1	2	2	4	2	128	64	1	32	8	32	2	32	2	1
PA3	2	4	2	2	128	64	0.5	32	8	16	2	32	2	1
PA5	2	4	4	2	128	64	1	32	8	16	2	32	2	2
PA7	2	4	4	1	128	64	1	32	8	16	2	32	2	2
PA8	2	2	4	2	128	64	1	32	4	16	2	32	2	2
PA9	1	2	2	2	128	64	0.5	32	4	16	2	32	2	1
PA10	2	4	4	2	128	64	1	32	4	16	2	32	2	2
PA11	2	4	4	2	128	64	1	32	4	16	2	32	2	1
PA12	2	4	4	2	128	64	1	32	4	16	2	32	4	1
PA13	8	4	4	128	128	64	2	32	1024	64	128	32	64	32
PA16	2	2	2	2	128	32	1	32	4	16	2	32	2	1
PA17	2	2	2	2	128	64	1	32	8	16	2	32	2	1
PAO1	2	4	2	2	128	32	1	32	4	16	2	32	2	1

AMK, amikacin; **APR**, apramycin; **BUT**, butirosin A; **GEN**, gentamicin; **HYG**, hygromycin B; **KAN**, kanamycin; **LIV**, lividomycin A; **NEO**, neomycin; **NET**, netilmicin; **PAR**, paromomycin; **SIS**, sisomicin; **SPEC**, spectinomycin; **STR**, streptomycin; **TOB**, tobramycin (Grey shading indicates resistant strain).

β-lactams

All the isolates were tested for their sensitivities to twenty-one β-lactams. They were screened using both the broth microdilution method and the disk diffusion method. The MIC values of ten β-lactam antibiotics for all the isolates obtained using the broth microdilution method are shown in Table 3.9. The zones of inhibition for twelve β-lactam antibiotics using the disk diffusion method are shown in Table 3.10. The β-lactam ceftazidime was tested using both methods.

The isolates were universally susceptible to six β-lactams: ceftazidime (1-2 μg/ml), piperacillin-tazobactam (≤64/4 μg/ml), cefepime (≤8 μg/ml), ceftriaxone (≤8 μg/ml), meropenem (≤4 μg/ml) and aztreonam (≤8 μg/ml). However, all of the isolates except *P. aeruginosa* PA13 were susceptible to another seven of the β-lactams: cefsulodin (4-8 μg/ml), piperacillin (2-4 μg/ml), carbenicillin (16-64 μg/ml), ticarcillin (≤64 μg/ml), ticarcillin-clavulanic acid (≤64/2 μg/ml), ceftizoxime (≤8 μg/ml) and imipenem (≤4 μg/ml).

The isolates were universally resistant to eight β-lactams, having high-level resistance to cephalothin (≥1024 μg/ml), cloxacillin (512-1024 μg/ml), oxacillin (128-1024 μg/ml), amoxicillin (≥1024 μg/ml), penicillin G (≥1024 μg/ml), ampicillin (≥1024 μg/ml) and intermediately resistant to cefotaxime (≥8 μg/ml) and moxalactam (≥8 μg/ml). In addition to the latter antibiotics, *P. aeruginosa* PA13 was also resistant to a further seven β-lactams including high-level resistance to piperacillin (256 μg/ml), carbenicillin (512 μg/ml), ticarcillin (≥128 μg/ml), ticarcillin-clavulanic acid (≥128/2 μg/ml) and intermediately resistant to imipenem (≥16 μg/ml) and cefsulodin (32 μg/ml) and ceftizoxime (≥8 μg/ml)

Although *P. aeruginosa* PA13 was resistant to piperacillin, neither it nor any of the other isolates were resistant to the β-lactam/β-lactamase inhibitor combination of piperacillin and tazobactam. *P. aeruginosa* PA13 was also the only strain with resistance to ticarcillin with an MIC of ≥128 μg/ml. Interestingly, it was also resistant to the β-lactam/β-lactamase inhibitor combination of ticarcillin and clavulanic acid, indicating that it was also resistant to the β-lactamase inhibitor, clavulanic acid.

The susceptibility testing of the isolates for carbapenems showed that all of the isolates were sensitive to meropenem. Although also sensitive to this antibiotic, *P. aeruginosa* PA13 grew at higher concentrations than the other strains, as was shown by a much smaller zone of inhibition (Table 3.10).

Table 3.9: MICs values of ß-lactams for *P. aeruginosa* strains

CEFS, cefsulodin; **CEFT**, ceftazidime; **CEPH**, cephalothin; **CLX**, cloxacillin; **PIP**, piperacillin; **OXA**, oxacillin; **CAR**, carbenicillin; **AMX**, amoxicillin; **PEN**, penicillin G; **AMP** ampicillin (Grey shading indicates resistant strain)

Strain	**Minimum Inhibitory Concentration (µg/ml)**									
P. aeruginosa	**CEFS**	**CEFT**	**CEPH**	**CLX**	**PIP**	**OXA**	**CAR**	**AMX**	**PEN**	**AMP**
PA1	4	1	1024	512	4	128	16	1024	1024	1024
PA3	4	1	1024	512	4	128	32	1024	1024	1024
PA5	4	1	1024	512	4	256	64	1024	1024	1024
PA7	4	1	1024	512	4	128	64	1024	1024	1024
PA8	4	1	1024	512	4	256	64	1024	1024	1024
PA9	4	1	1024	512	4	128	64	1024	1024	1024
PA10	4	1	1024	512	4	256	32	1024	1024	1024
PA11	4	1	1024	512	4	256	32	1024	1024	1024
PA12	8	1	1024	512	4	256	64	1024	1024	1024
PA13	32	2	>1024	1024	256	1024	512	>1024	>1024	>1024
PA16	8	1	1024	512	4	256	64	1024	1024	1024
PA17	8	1	1024	512	4	128	64	1024	1024	1024
PAO1	2	1	1024	512	2	256	16	1024	1024	1024

Table 3.10: Zone diameters and equivalent MICs (μg/ml) of β-lactam antibiotics for the *P. aeruginosa* strains

(Grey shading indicates resistant strain)

Antimicrobial Agent (Disk content)	PA1	PA3	PA5	PA7	PA8	PA9	PA10	PA11	PA12	PA13	PA16	PA17	PAO1
	Zone Diameter (mm) / equivalent MIC (μg/ml)												
Ticarcillin (75μg)	28	25	23	25	22	25	22	27	25	7 (≥128μg/ml)	23	25	25
Ticarcillin-clavulanic acid (75/10μg)	28	25	23	26	23	25	23	27	25	7 (≥128/2 μg/ml)	23	26	25
Pipercillin-taobactam (100/10μg)	31	30	30	27	26	28	30	28	29	25	30	31	30
Ceftazidime (30μg)	30	24	22	26	22	22	28	24	25	23	24	22	22
Cefepime (30μg)	31	29	28	29	27	29	23	25	27	20	29	28	28
Cefotaxime (30μg)	18 ≥8μg/ml	18 ≥8μg/ml	19 ≥8μg/ml	19 ≥8μg/ml	19 ≥8μg/ml	18 ≥8μg/ml	18 ≥8μg/ml	19 ≥8μg/ml	19 ≥8μg/ml	17 ≥8μg/ml	18 ≥8μg/ml	19 ≥8μg/ml	19 ≥8μg/ml
Ceftriaxone (30μg)	24	23	19	23	22	23	23	23	23	23	23	24	23
Ceftizoxime (30μg)	21	22	22	22	21	22	22	22	22	15 (≥8μg/ml)	21	22	22
Moxalactam (30μg)	23 ≥8μg/ml	23 ≥8μg/ml	22 ≥8μg/ml	23 ≥8μg/ml	22 ≥8μg/ml	22 ≥8μg/ml	20 ≥8μg/ml	20 ≥8μg/ml	22 ≥8μg/ml	18 ≥8μg/ml	23 ≥64μg/ml	20 ≥64μg/ml	23 ≥64μg/ml
Imipenem (10μg)	20	22	22	23	22	23	23	22	23	15 (≥16 μg/ml)	21	23	22
Meropenem (10μg)	31	32	32	34	30	32	35	31	34	17	33	30	30
Aztreonam (30μg)	28	26	26	25	24	26	25	26	24	24	24	26	25

Standard error = ± 0.5 mm in all cases

Fluoroquinolones

The isolates were tested for their sensitivities to two fluoroquinolone antibiotics: ofloxacin and ciprofloxacin. The sensitivities of the isolates to ofloxacin were tested using the microbroth dilution method and the sensitivities of the isolates to ciprofloxacin were tested using the disk diffusion method. The results obtained are shown in Tables 3.11 and 3.12 respectively.

All of the isolates were sensitive to ofloxacin (0.5-1 μg/ml) except for *P. aeruginosa* PA13 which was resistant to the antibiotic (16 μg/ml) (Table 3.11). Likewise, all of the isolates were sensitive to ciprofloxacin (≤1 μg/ml) except for *P. aeruginosa* PA13 which was resistant to the antibiotic (≥4 μg/ml) (Table 3.12).

Table 3.11: MIC values (μg/ml) of ofloxacin (fluoroquinolone) for *P. aeruginosa* strains

(Grey shading indicates resistant strain)

Strain	MIC of Ofloxacin (μg/ml)
PA1	1
PA3	0.5
PA5	1
PA7	1
PA8	1
PA9	1
PA10	1
PA11	1
PA12	1
PA13	16
PA16	1
PA17	1
PAO1	0.5

Table 3.12: Zone diameters and equivalent MIC values (μg/ml) of ciprofloxacin (fluoroquinolone) for *P. aeruginosa* strains.

(Grey shading indicates resistant strain)

Strain	MIC of ciprofloxacin Zone Diameter (mm)/equivalent MIC (μg/ml)
PA1	31
PA3	32
PA5	32
PA7	32
PA8	33
PA9	32
PA10	32
PA11	32
PA12	33
PA13	12 (≥4 μg/ml)
PA16	32
PA17	31
PAO1	32

Standard error = ± 0.5 mm in all cases

Tetracycline

The isolates were screened for their sensitivity to tetracycline, a member of the tetracycline class of antibiotics. The sensitivities of the isolates to the antibiotic were tested using the disk diffusion method. The results obtained are shown in Table 3.13. They were all found to be resistant to the antibiotic with MIC values of ≥16 μg/ml.

Table 3.13: Zone diameters and equivalent MIC value of tetracycline for *P. aeruginosa* strains
(Grey shading indicates resistant strain).

Strain	MIC of tetracycline Zone Diameter (mm)/equivalent MIC (µg/ml)
PA1	3 (≥16 µg/ml)
PA3	4 (≥16 µg/ml)
PA5	3 (≥16 µg/ml)
PA7	7 (≥16 µg/ml)
PA8	3 (≥16 µg/ml)
PA9	5 (≥16 µg/ml)
PA10	4 (≥16 µg/ml)
PA11	4 (≥16 µg/ml)
PA12	4 (≥16 µg/ml)
PA13	2 (≥16 µg/ml)
PA16	4 (≥16 µg/ml)
PA17	3 (≥16 µg/ml)
PAO1	4 (≥16 µg/ml)

Standard error = ± 0.5 mm in all cases

Folic acid synthesis inhibitor

The strains were screened for their sensitivity to co-trimoxazole, a member of the folic acid synthesis inhibitors class of antibiotics. The sensitivities of the isolates to the antibiotic were tested using the disk diffusion method. The results obtained are shown in Table 3.14. They were all found to be resistant to the antibiotic with MIC values of ≥32 µg/ml.

Table 3.14: Zone diameters of co-trimoxazole (folate synthesis inhibitor) for *P. aeruginosa*

(Grey shading indicates resistant strain).

Strain	MIC of co-trimoxazole Zone Diameter (mm)/equivalent MIC (μg/ml)
PA1	15 (≥32 μg/ml)
PA3	16 (≥32 μg/ml)
PA5	16 (≥32 μg/ml)
PA7	15 (≥32 μg/ml)
PA8	15 (≥32 μg/ml)
PA9	15 (≥32 μg/ml)
PA10	16 (≥32 μg/ml)
PA11	15 (≥32 μg/ml)
PA12	15 (≥32 μg/ml)
PA13	15 (≥32 μg/ml)
PA16	16 (≥32 μg/ml)
PA17	16 (≥32 μg/ml)
PAO1	16 (≥32 μg/ml)

Standard error = ± 0.5 mm in all cases

Phenicols

The isolates were screened for their sensitivity to chloramphenicol, a member of the phenicol class of antibiotics. The sensitivities of the isolates to the antibiotic were tested using the broth microdilution method. The results obtained are shown in Table 3.15. All of the isolates were found to be resistant to the antibiotic with MIC values of 128-256 μg/ml.

Table 3.15: MICs of chloramphenicol for *P. aeruginosa* strains
(Grey shading indicates resistant strain).

Strain	MIC of Chloramphenicol (μg/ml)
PA1	256
PA3	256
PA5	256
PA7	128
PA8	256
PA9	128
PA10	128
PA11	256
PA12	256
PA13	256
PA16	256
PA17	256
PAO1	128

3.2.3 Overall antibiotic resistance profiles (ARPs) for the clinical isolates

The overall antibiotic resistance profiles of the isolates are summarised in Table 3.16. All of the antibiotics tested fell into seven classes. There was considerable uniformity in the results, showing a similar response by all the isolates except *P. aeruginosa* PA13. This strain was distinguished by its resistance to the clinically important aminoglycosides, gentamicin, netilmicin and tobramycin and to the β-lactam antibiotics piperacillin, ticarcillin and the β-lactam-β-lactamase inhibitor combination, ticarcillin-clavulanic acid. Importantly, it also had intermediate resistance to the third generation cephalosporins: ceftizoxime and cefsulodin and to the carbapenem, imipenem. Therefore, *P. aeruginosa* PA13 was a multiresistant strain and was chosen for further study.

Table 3.16: Sensitivities of *P. aeruginosa* strains to antibiotic agents from various antibiotic classes

Antimicrobial class	Agent	PA1	PA3	PA5	PA7	PA8	PA9	PA10	PA11	PA12	PA13	PA16	PA17	PAO1
β-lactams														
Penicillins	Penicillin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Amoxicillin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Ampicillin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Pipercillin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Carbenicillin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Ticarcillin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Cloxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Oxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R
β-lactam/β-lactamase inhibitor combinations	Ticarcillin-clavulanic acid	S	S	S	S	S	S	S	S	S	R	S	S	S
	Pipercillin-tazobactum	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephems	Cephalothin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Ceftazidime	S	S	S	S	S	S	S	S	S	S	S	S	S
	Cefotaxime	I	I	I	I	I	I	I	I	I	I	I	I	I
	Ceftizoxime	S	S	S	S	S	S	S	S	S	I	S	S	S
	Cefsulodin	S	S	S	S	S	S	S	S	S	I	S	S	S
	Moxalactam	I	I	I	I	I	I	I	I	I	I	I	I	I
	Cefepime	S	S	S	S	S	S	S	S	S	S	S	S	S
	Ceftriaxone	S	S	S	S	S	S	S	S	S	S	S	S	S
Monobactams	Aztreonam	S	S	S	S	S	S	S	S	S	S	S	S	S
Penems	Imipenem	S	S	S	S	S	S	S	S	S	I	S	S	S
	Meropenem	S	S	S	S	S	S	S	S	S	S	S	S	S

Antimicrobial class	Agent	PA1	PA3	PA5	PA7	PA8	PA9	PA10	PA11	PA12	PA13	PA16	PA17	PAO1
Aminoglycosides	Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S
	Gentamicin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Kanamycin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Netilmicin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Spectinomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Streptomycin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Tobramycin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Apramycin	S	S	S	S	S	S	S	S	S	S	S	S	S
	Butirosin A	S	S	S	S	S	S	S	S	S	S	S	S	S
	Hygromycin B	R	R	R	R	R	R	R	R	R	R	R	R	R
	Lividomycin A	S	S	S	S	S	S	S	S	S	S	S	S	S
	Neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Paromomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
	Sisomicin	S	S	S	S	S	S	S	S	S	R	S	S	S
Quinolones	Ciprofloxacin	S	S	S	S	S	S	S	S	S	R	S	S	S
	Ofloxacin	S	S	S	S	S	S	S	S	S	R	S	S	S
Folate pathway inhibitors	Co-trimoxazole	R	R	R	R	R	R	R	R	R	R	R	R	R
Tetracyclines	Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R
Phenicols	Chloramphenicols	R	R	R	R	R	R	R	R	R	R	R	R	R
Lipopeptides	Colistin	S	S	S	S	S	S	S	S	S	S	S	S	S

R = Resistant, **S** = Sensitive, **I** = Intermediate resistance. Grey shading indicates resistant strain

3.3 Molecular analysis of antibiotic resistance

Aminoglycosides are commonly the first-line antibiotics used to treat *P. aeruginosa* infections. It was of interest to understand a mechanism, or mechanisms that conferred aminoglycoside resistance on *P. aeruginosa* PA13, which was able to grow in the presence of high concentrations of aminoglycoside antibiotics such as gentamicin, netilmicin and tobramycin. There are many mechanisms that confer resistance to the aminoglycosides. These include decreased permeability of the bacterial cell membrane, increased efflux of the antibiotic from the cell, modification of the antibiotic target and modification of the antibiotic by aminoglycoside modifying enzymes. However, aminoglycoside modifying enzymes are the most common mechanism for conferring resistance to aminoglycosides. There are three families of aminoglycoside modifying enzymes, classified as *N*-acetyltransferases (AACs), *O*-nucleotidyltransferases (ANTs) and *O*-phosphotransferases (APHs). Genes encoding these enzymes were investigated. Primers to amplify aminoglycoside resistance genes that conferred resistance to gentamicin were designed from sequences available in Genbank and from the literature.

3.3.1 Screening for aminoglycoside modifying enzymes

All of the isolates were screened for aminoglycoside modifying enzymes (AMEs) using the primers in Table 2.12. In all, the isolates were screened for seventeen AME genes. From all of these genes, only one product was amplified for one of the isolates. The primers for the *aac(6')*-IIa gene amplified a 2.2kb product in *P. aeruginosa* PA13 and this product is shown in Figure 3.4.

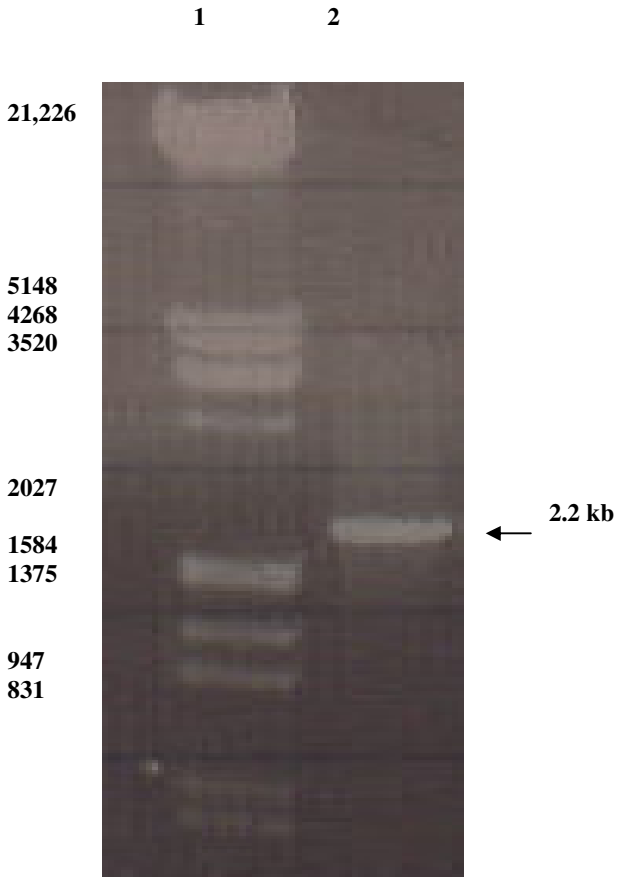

Figure 3.4: Agarose gel showing the 2.2 kb product amplified in *P. aeruginosa* PA13. Lane 1 - DNA ladder, Lane 2 – amplfied product from *P. aeruginosa* PA13

3.3.2 Cloning and sequencing of the 2.2 kbp product

The amplified 2.2 kbp product (Figure 3.4) was cloned into a Qiagen pDrive cloning vector to allow for the sequencing and analysis of the cloned PCR product. The plasmid construct was transformed into the host *E. coli* strain XL1 Blue. Positive clones were identified by blue/white colony screening, from which positive plasmid DNA was purified using the Sigma Genelute plasmid purification kit. A plasmid from a positive clone is shown in Figure 3.5a. The plasmid DNA from the positive clone was digested with the restriction enzyme *Eco*R1 (a restriction site on both ends of the multiple cloning site within the pDrive vector) to confirm the insertion of the amplified product. The agarose gel of the restricted plasmid shows a band corresponding to the 3.85 kb vector and a band corresponding to the restricted 2.2 kb insert (Figure 3.5b). The 2.2 kb insert was then fully sequenced and analysed. Three consensus sequences were used to make the consensus sequence. The complete sequence was submitted to the EMBL/GenBank database and was assigned the Accession number (DQ767903).

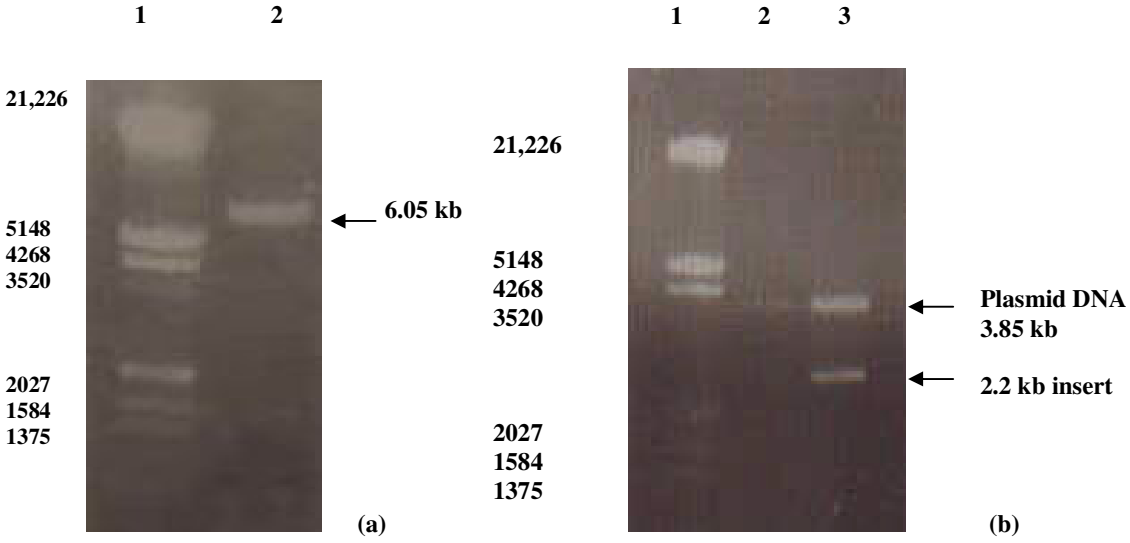

Figure 3.5: (a) An agarose gel showing purified, undigested, plasmid DNA, containing the 2.2 kbp product isolated from positive white colonies of *E. coli* XL1 Blue (b) an agarose gel showing the restriction digest of plasmid DNA from a positive clone with *Eco* R1, verifying the insertion of the 2.2 kbp PCR product. Lanes 1 (a+b) – DNA ladder, Lane 2(a) – pDrive vector containing the 2.2 kb insert, Lane 2 (b) – empty. Lane 3 (b) - the upper band corresponds to the pDrive cloning vector, while the bottom bands corresponds to the size of the inserted PCR product.

3.3.3 Sequence analysis of amplified product

The complete sequence of the amplified 2.2 kb product is shown in Figure 3.6. An open reading frame search of the sequenced 2.2 kb product using an ORF finder programme on the NCBI website (www.ncbi/nlm/nih/gov) identified four open reading frames (ORFs), which are highlighted by four colours in Figure 3.6. These were ORF1 (pink), ORF2 (green), ORF3 (blue) and ORF4 (purple).

attC **recombination site**

1201 CTTCCATCGG AGGGGGACGT CCAAGGGCTG GCGCCCTTGG CCGCCCCTCA TGTCAAAC**GT**

ORF3

1261 **TAGGC**GTCAA AGGAAACTTA **ATG**GCAATCC GATTCCTCAC CATACTGCTA TCTACTTTTT

1321 TTCTTACCTC ATTCGTGCAT GCGCAAGAAC ACGTGCTAGA GCGTTCTGAC TGGAAGAAGT

1381 TCTTCAGCGA CCTCCGGGCC GAAGGTGCAA TCGTTATTTC AGACGAACGT CAAGCGGAGC

1441 ATGCTTTATT GGTTTTTGGT CAAGAGCGAG CAGCAAAGCG TTACTCGCCT GCTTCAACCT

1501 TCAAGCTTCC ACACACACTT TTTGCACTCG ATGCAGACGC CGTTCGTGAT GAGTTCCAGG

1561 TTTTTCGATG GGACGGCGTT AAACGGAGCT TTGCGGGCCA TAATCAAGAC CAAGACTTGC

1621 GATCAGCGAT GCGAAATTCT GCGGTCTGGG TTTATGAGCT ATTTGCAAAA GAGATCGGAG

1681 AGGACAAAGC AAGACGCTAT TTAAAGCAAA TTGATTATGG CAACGCCGAC CCTTCGACAA

1741 TCAAGGGCGA TTACTGGATA GATGGCAATC TTGAAATCTC AGCGCACGAA CAGATTTCGT

1801 TTCTCAGAAA ACTCTATCGA AATCAGCTGC CATTTCAGGT GGAACATCAG CGCTTGGTCA

1861 AAGATCTCAT GATTACGGAA GCCGGGCGCA ACTGGATACT ACGCGCAAAG ACCGGCTGGG

1921 AAGGCAGGTT TGGCTGGTGG GTAGGGTGGG TGGAGTGGCC AACCGGTCCC GTATTCTTCG

1981 CACTGAATAT TGATACGCCA AACAGAACGG ATGATCTTTT CAAAAGAGAG GCAATCGCGC

2041 GGGCAATCCT TCGCTCTATC GACGCATTGC CGCCCAACTAA TCAATCCAG CGGACGCCTT

attC **recombination site** **ORF4**

2101 CGGCGCCGCT GATTTCAAC**G TTAGAT**GCAC TAAGCACATA ATTGCTCACA GCCAAACTAT

2161 CAGGTCAAGT CTGCTTTTAT TATTTTTAAG CGTGCATAAT AAGCCCTACA CAAATTGGGA

2221 GATATATCAT GAAAGGCTGG CTTTTTCTTG TTATCGCAAT AGTGGCGAAG AATC

Figure 3.6: The overall sequence of the integron from *P. aeruginosa* PA13. The four open reading frames are indicated by different colours: ORF1 (pink), ORF2 (green), ORF3 (blue) and ORF4 (purple). The integron promoters P_{ant} and P2 are indicated with horizontal arrows. The *attI* and *attC* recombination sites are underlined.

ORF1

ORF1 was found at the 5'-end of the sequence (Figure 3.6). BLASTN analysis identified that it was 99-100% similar to previously published integrase (*IntI*) genes (Figure 3.7). When translated, the open reading frame encoded a partial protein of 133 amino acids (Figure 3.8) that was 100% homologous to previously published *Intl*1 integrase enzyme sequences in *P. aeruginosa* (AAS20532) (Aubert *et al*., 2004), *P. aeruginosa* (CAA11470) (Laraki *et al*., 1999), *Salmonella typhimurium* (AAS18383) (Daly *et al*., 2005) and *Corynebacterium diphtheriae* (BX248359) (Cerdeno-Tarraga *et al*., 2003). The integrase was translated in the 3'→ 5' direction. This integrase gene was typical of class 1 integrons where integrases are found on the 5' conserved segment. Integrons are genetic elements capable of the acquisition, rearrangement and expression of genes contained in gene cassettes.

```
P.aeruginosa_AAS20532        GATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAA 538
Pseudom_CAA11470             GATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAA 538
Salmonella_AAS18383          GATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAA 643
PA13                         ----------------------------------------GATCGAA- 7
Corynebacterium_BX248359     GATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAA 190
                                                                       *******

P.aeruginosa_AAS20532        TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGT 588
Pseudom_CAA11470             TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGT 588
Salmonella_AAS18383          TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGT 693
PA13                         TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGT 57
Corynebacterium_BX248359     TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGT 240
                             **************************************************

P.aeruginosa_AAS20532        TCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGA 638
Pseudom_CAA11470             TCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGA 638
Salmonella_AAS18383          TCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGA 743
PA13                         TCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGA 107
Corynebacterium_BX248359     TCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGA 290
                             **************************************************

P.aeruginosa_AAS20532        GGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGAC 688
Pseudom_CAA11470             GGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGAC 688
Salmonella_AAS18383          GGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGAC 793
PA13                         GGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGAC 157
Corynebacterium_BX248359     GGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGAC 340
                             **************************************************

P.aeruginosa_AAS20532        GGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC 738
Pseudom_CAA11470             GGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC 738
Salmonella_AAS18383          GGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC 843
PA13                         GGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC 207
Corynebacterium_BX248359     GGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC 390
                             **************************************************

P.aeruginosa_AAS20532        CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGA 788
Pseudom_CAA11470             CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGA 788
Salmonella_AAS18383          CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGA 893
PA13                         CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGA 257
Corynebacterium_BX248359     CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGA 440
                             **************************************************

P.aeruginosa_AAS20532        CCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG 838
Pseudom_CAA11470             CCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG 838
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Salmonella_AAS18383          CCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG 943
PA13                         CCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG 307
Corynebacterium_BX248359     CCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG 490
                             **************************************************

P.aeruginosa_AAS20532        CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC 888
Pseudom_CAA11470             CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC 888
Salmonella_AAS18383          CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC 993
PA13                         CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC 357
Corynebacterium_BX248359     CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC 540
                             **************************************************

P.aeruginosa_AAS20532        ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGT 938
Pseudom_CAA11470             ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGT 938
Salmonella_AAS18383          ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGT 1043
PA13                         ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGT 407
Corynebacterium_BX248359     ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGT 590
                             ********************************** *************

P.aeruginosa_AAS20532        AGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT 988
Pseudom_CAA11470             AGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT 988
Salmonella_AAS18383          AGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT 1093
PA13                         AGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT 457
Corynebacterium_BX248359     AGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT 640
                             **************************************************

P.aeruginosa_AAS20532        GGTAACGGCGCAGTGGCGGTTTTCAT------------------------ 1014
Pseudom_CAA11470             GGTAACGGCGCAGTGGCGGTTTTCAT------------------------ 1014
Salmonella_AAS18383          GGTAACGGCGTAGTGGCGGTTTTCAT------------------------ 1119
PA13                         GGTAACGGCGCAGTGGCGGTTTTCAT------------------------ 507
Corynebacterium_BX248359     GGTAACGGCGCAGTGGCGGTTTTCAT------------------------ 666
```

Figure 3.7: Clustal W alignments of the nucleotide sequences of *IntI* genes. The compared integrase genes were from *Pseudomonas aeruginosa* (AAS20532), *Pseudomonas aeruginosa* (CAA11470), *Salmonella typhimurium* (AAS18383), *Pseudomonas aeruginosa* PA13 and *Corynebacterium diphtheriae* (BX248359). Nucleotides that are identical in all strains are underlined with an asterisk.

Figure 3.8: Clustal W alignments comparing the partial amino acid sequences of the integrase enzymes, *IntI*. The compared integrase genes were from *Pseudomonas aeruginosa* (AAS20532), *Pseudomonas aeruginosa* (CAA11470), *Salmonella typhimurium* (AAS18383), *Pseudomonas aeruginosa* PA13 and *Corynebacterium diphtheriae* (AAS18383). Residues that are identical in all strains are underlined with an asterisk.

ORF2

ORF2, which was 519 bp in length, was located downstream of the integrase gene (Figure 3.6). BLASTN analysis of the ORF showed 100% nucleotide identity with several aminoglycoside acetyltransferases (AAC(6')-Ib) encoded products from many species including *Pseudomonas aeruginosa* (AAD02244) (Mugnier *et al*., 1998), *P. aeruginosa* (CAE48335) (Mendes *et al*., 2004) and *Burkholderia cepacia* (AAK55331) (Crowley *et al*, 2002) (Figure 3.9). When translated it encoded a 172 amino acid protein (Figure 3.10).

However, the aminoglycoside resistance phenotype of *P. aeruginosa* PA13 suggested production of AAC(6')-IIa enzyme and not AAC(6')-1b enzyme, due the fact that the strain was resistant to gentamicin and not resistant to amikacin. AAC(6')-IIa confers

resistance to gentamicin, tobramycin, netilmicin and sisomicin (but not to amikacin), whereas AAC(6')-Ib confers resistance to amikacin, tobramycin, kanamycin, netilmicin, and sisomicin (but not to gentamicin). The most similar enzymes (Figure 3.10) to the AAC(6')-Ib in *P. aeruginosa* PA13 also conferred resistance to gentamicin and not to amikacin. The sequences of these *aac(6')-Ib* genes were compared with an *aac(6')-Ib* gene from a *Vibrio cholerae* strain, which was known to confer resistance to amikacin and not to gentamicin (Figure 3.9). The *Vibrio cholerae* gene differed by one nucleotide from the sequence of the other genes. *P. aeruginosa* PA13 and its most similar genes had a cytosine at position 276, whereas the *aac(6')-Ib* gene from *Vibrio cholerae* had a thymine at this position. When translated this nucleotide difference changed the amino acid at position 119 from a leucine in the AAC(6')-Ib enzyme from *Vibrio cholerae* to a serine in the AAC(6')-Ib enzyme in *P. aeruginosa* PA13 and its most similar enzymes (Figure 3.10). This suggested that *P. aeruginosa* PA13 possessed a mutant form of the AAC(6')-Ib enzyme.

```
B.cepaciaAAK55331                                                    ATGACT 6
PseudoCAE48335                                                       ATGACT 6
PA13                                                                 ATGACT 6
P.aerAAD02244                                                        ATGACT 6
VirioABC54722                                                        ATGACT 6
                                                                     ******

B.cepacia_AAK55331    GAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG 66
Pseudo_CAE48335       GAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG 66
PA13                  GAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG 66
P.aerAAD_02244        GAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG 66
Vibrio_ABC54722       GAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG 66
                      ************************************************************

B.cepacia_AAK55331    GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTT 126
Pseudo_CAE48335       GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTT 126
PA13                  GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTT 126
P.aer AAD02244         GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTT 126
Vibrio ABC54722       GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTT 126
                      ************************************************************

B.cepacia_AAK55331    TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 186
Pseudo_CAE48335       TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 186
PA13                  TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 186
P.aer_AAD02244        TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 186
Vibrio_ABC54722       TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 186
                      ************************************************************

B.cepacia_AAK55331    GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 246
Pseudo_CAE48335       GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 246
PA13                  GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 246
P.aer_AAD02244        GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 246
Vibrio_ABC54722       GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 246
                      ************************************************************
```

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B.cepacia_AAK55331    GGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 306
Pseudo_CAE48335       GGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 306
PA13                  GGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 306
P.aer_AAD02244        GGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 306
Vibrio_ABC54722       GGAGTACGCGGAATAGACCAGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 306
                      ********************** ************************************

B.cepacia_AAK55331    ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 366
Pseudo_CAE48335       ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 366
PA13                  ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 366
P.aer_AAD02244        ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 366
Vibrio_ABC54722       ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 366
                      ************************************************************

B.cepacia_AAK55331    CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 426
Pseudo_CAE48335       CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 426
PA13                  CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 426
P.aer_AAD02244        CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 426
Vibrio_ABC54722       CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 426
                      ************************************************************

B.cepacia_AAK55331    GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 486
Pseudo_CAE48335       GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 486
PA13                  GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 486
P.aer_AAD02244        GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 486
Vibrio_ABC54722       GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 486
                      ************************************************************

B.cepacia_AAK55331    CAGGCATTCGAGCGAACACGCAGTGATGCCTAA 519
Pseudo_CAE48335       CAGGCATTCGAGCGAACACGCAGTGTTGCCTAA 519
PA13                  CAGGCATTCGAGCGAACACGCAGTGATGCCTAA 519
P.aer_AAD02244        CAGGCATTCGAGCGAACACGCAGTGATGCCTAA 519
Vibrio_ABC54722       CAGGCATTCGAGCGAACACGCAGTGATGCCTAA 519
                      ************************* *******
```

Figure 3.9: Clustal W alignments comparing the nucleotide sequences of the *aac(6')-Ib*. The compared *aac(6')-Ib* were from, *Burkholderia cepacia* (AAK55331), *P. aeruginosa* (CAE48335), *P. aeruginosa* PA13, *P. aeruginosa* (AAD02244) and *Vibrio cholerae* (ABC54722). The single nucleotide mutation from the wildtype TTA (serine) in *Vibrio cholerae* (ABC54722) can be seen aligned to the mutant TCA (leucine) in *Burkholderia cepacia* (AAK55331), *P. aeruginosa* (CAE48335), *P. aeruginosa* PA13, *P. aeruginosa* (AAD02244). Residues that are identical in all strains are underlined with an asterisk.

```
Vibrio_ABC54722     MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYIAMLNGEPI 60
PA13                MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYIAMLNGEPI 60
B.cepacia_AAK55331  MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYIAMLNGEPI 60
P.aer_AAD02244      MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYIAMLNGEPI 60
Pseudo_CAE48335     MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYIAMLNGEPI 60
                    ************************************************************

Vibrio_ABC54722     GYAQSYVALGSGDGWWEEETDPGVRGIDQLLANASQLGKGLGTKLVRALVELLFNDPEVT 120
PA13                GYAQSYVALGSGDGWWEEETDPGVRGIDQSLANASQLGKGLGTKLVRALVELLFNDPEVT 120
B.cepacia_AAK55331  GYAQSYVALGSGDGWWEEETDPGVRGIDQSLANASQLGKGLGTKLVRALVELLFNDPEVT 120
P.aer_AAD02244      GYAQSYVALGSGDGWWEEETDPGVRGIDQSLANASQLGKGLGTKLVRALVELLFNDPEVT 120
Pseudo_CAE48335     GYAQSYVALGSGDGWWEEETDPGVRGIDQSLANASQLGKGLGTKLVRALVELLFNDPEVT 120
                    ************************** ******************************
```

```
Vibrio_ABC54722     KIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA 172
PA13                KIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA 172
B.cepacia_AAK55331  KIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA 172
P.aer_AAD02244      KIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA 172
Pseudo_CAE48335     KIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA 172
                    **************************************************
```

Figure 3.10: Clustal W alignments comparing the amino acid sequences of AAC(6')-Ib enzymes. The compared sequences were from *P. aeruginosa* PA13, *Vibrio cholerae* (ABC54722), *P. aeruginosa* (AAD02244), *Burkholderia cepacia* (AAK55331), and *P. aeruginosa* (CAE48335). The serine residue at position 119 in *P. aeruginosa* PA13, *Burkholderia cepacia* (AAK55331), *P. aeruginosa* (AAD02244) and *P. aeruginosa* (CAE48335) can be seen, aligned with the more typical leucine residue of the wild-type gene in *Vibrio cholerae* (ABC54722) (highlighted and underlined). Residues that are identical in all strains are underlined with an asterisk.

ORF3

ORF3 was identified 82 bp downstream from the *aac(6')*-Ib gene (Figure 3.6). ORF3 encoded a 266 amino acid polypeptide. This ORF showed 99% nucleotide sequence identity with an oxacillinase (β-lactamase) gene from an unidentified bacterium from a wastewater treatment plant in Germany (AAN41427) (Tennstedt *et al*, 2003), 99% sequence identity with an oxacillinase gene from a clinical isolate of *Burkholderia cepacia* (AAK55330) isolated in Cork (Crowley *et al*., 2002) and 92% sequence identity with OXA-46 from a *Pseudomonas aeruginosa* (AAN63499) strain isolated in Italy (Giuliani *et al*., 2005) (Figure 3.11). It also showed 82% amino acid sequence similarity to OXA-2 from *Acinetobacter baumannii* (ABN48512) (Yum *et al*., 2007), 82% similarity to OXA-53 from *Salmonella enterica* (AAP43641) (Mulvey *et al*., 2004), 82% similarity to OXA-3 from *P. aeruginosa* (PSEBLA) (Sanschagrin *et al*., 1995) and 71% similarity to OXA-20 from *Acinetobacter baumannii* (CAC85643) (Gombac *et al*., 2002) (Figure 3.11).

Analysis of β-lactamase sequences allows them to be divided into four molecular classes according to their amino acid content, classes A, B, C and D. Oxacillinase enzymes belong to the Class D β-lactamases. Following sequence alignment using the CLUSTALW algorithm (Thompson *et al*., 1994) several conserved motifs typically

found in Class D enzymes were identified (Figure 3.12). These included a serine-threonine-phenylalanine-lysine (S-T-F-K) tetrad located at position 72-75, an S-X-V motif at positions 118 to 120, Y-G-N at position 146-148, a modified W-X-E-X-X-L-X-I-S motif at positions 159-167 where aspartate, glutamine and glutamate replaced the more common residues glutamate, serine and alanine (at positions 161, 163 and 165 respectively), Q-X-X-X-L at position 171-175, K-T-G at positions 210-212 and W-W-V-G-W at positions 219-223 (Couture *et al*., 1992). The amino acids in Class D enzymes are numbered using the DBL numbering system (Couture *et al*., 1992). These numbers are shown in parenthesis below the alignment in Figure 3.12.

```
OXA2_ABN48512        ATGGCAATCCGAATCTTCGCGATACTTTTCTCCATTTTTTCTCTTGCCACTTTCGCGCAT 60
OXA3_PSEBLA          ATGGCAATCCGAATCTTTGCAATACTTTTCTCCACTTTTGTTTTTGGCACGTTCGCGCAT 60
OXA53_AAP43641       ATGGCAATCCAAATCTTCGCAATACTTTTCTCCACTTTTGTTCTTGCCACTTTTGCACAT 60
PA13                 ATGGCAATCCGATTCCTCACCATACTGCTATCTACTTTTTTTCTTACCTCATTCGTGCAT 60
Bcepacia_AAK55330    ATGGCAATCCGATTCCTCACCATACTGCTATCTACTTTTTTTCTTACCTCATTCGTGCAT 60
Uncultured_AAN41427  ATGGCAATCCGATTCCTCACCATACTGCTATCTACTTTTTTTCTTACCTCATTCGTGCAT 60
OXA46_AAN63499       ATGGCAATCCGATTCTTCACCATACTGCTATCCACCTTCTTTCTTACCTCATTCGTGTAT 60
OXA20_CAC85643       TTGATAATCCGATTTCTAGCACTGCTTTTCTCAGCTGTTGTACTTGTCTCTCTTGGTCAT 60
                     **  ***** * *  *  *  * **  * **     *     **  * *  * *   **

OXA2_ABN48512        GCGCAAGAAGGCACGCTAGAACGTTCTGACTGGAGGAAGTTTTTCAGCGAATTTCAAGCC 120
OXA3_PSEBLA          GCACAAGAAGGCATGCGCGAACGTTCTGACTGGCGGAAGTTTTTCAGCGAATTTCAAGCC 120
OXA53_AAP43641       GCGCAAGATGGCACGCTGGAACGTTCTGACTGGGGGAAATTTTTCAGCGATTTTCAGGCC 120
PA13                 GCGCAAGAACACGTGCTAGAGCGTTCTGACTGGAAGAAGTTCTTCAGCGACCTCCGGGCC 120
Bcepacia_AAK55330    GCGCAAGAACACGTGCTAGAGCGTTCTGACTGGAAGAAGTTCTTCAGCGACCTCCGGGCC 120
Uncultured_AAN41427  GCGCAAGAACACGTGCTAGAGCGTTCTGACTGGAAGAAGTTCTTCAGCGACCTCCGGGCC 120
OXA46_AAN63499       GCGCAAGAACATGTGGTAATCCGTTCGGACTGGAAAAAGTTCTTCAGCGACCTCCAGGCC 120
OXA20_CAC85643       GCACAAGAAAAAACGCATGAGAGCTCTAATTGGGGGAAATACTTTAGTGATTTCAACGCT 120
                     ** *****      *       * **  * ***   ** *  ** ** **  *    **

OXA2_ABN48512        AAAGGCACGATAGTTGTGGCAGACGAACGCCAAGCGGATCGTGCCATGTTGGTTTTTAAT 180
OXA3_PSEBLA          AAAGGCACGATAGTTGTGGCAGACGAACGCCAAACAGATCGTGTCATATTGGTTTTTGAT 180
OXA53_AAP43641       AAAGGTACGATAGTTGTGGCAGACGAACGCCAAGCGGATCATGCGATATTGGTTTTTGAT 180
PA13                 GAAGGTGCAATCGTTATTTCAGACGAACGTCAAGCGGAGCATGCTTTATTGGTTTTTGGT 180
Bcepacia_AAK55330    GAAGGTGCAATCGTTATTTCAGACGAACGTCAAGCGGAGCATGCTTTATTGGTTTTTGGT 180
Uncultured_AAN41427  GAAGGTGCAATCGTTATTTCAGACGAACGTCAAGCGGAGCATGCTTTATTGGTTTTTGGT 180
OXA46_AAN63499       GAAGGTGCAATCGTTATTGCAGACGAACGTCAAGCGAAGCATACTTTATCGGTTTTTGAT 180
OXA20_CAC85643       AAAGGTACAATAGTTGTAGTAGATGAACGCACAAACGGTAATTCCACATCGGTTTATAAT 180
                      ****  * ** *** *   *** *****   *         *      * ***** *  *

OXA2_ABN48512        CCTGTGCGATCGAAGAAACGCTACTCGCCTGCATCGACATTCAAGATACCTCATACACTT 240
OXA3_PSEBLA          CAGGTGCGGTCAGAGAAACGCTACTCGCCGGCCTCGACATTCAAGATTCCACATACACTT 240
OXA53_AAP43641       CAAGCACGGTCAATGAAACGCTACTCGCCTGCGTCGACATTCAAGATTCCACATACACTT 240
PA13                 CAAGAGCGAGCAGCAAAGCGTTACTCGCCTGCTTCAACCTTCAAGCTTCCACACACACTT 240
Bcepacia_AAK55330    CAAGAGCGAGCAGCAAAGCGTTACTCGCCTGCTTCAACCTTCAAGCTTCCACACACACTT 240
Uncultured_AAN41427  CAAGAGCGAGCAGCAAAGCGTTACTCGCCTGCTTCAACCTTCAAGCTTCCACACACACTT 240
OXA46_AAN63499       CAAGAGCGAGCGGCAAAGCGTTACTCGCCAGCTTCAACCTTCAAGATACCCCACACACTT 240
OXA20_CAC85643       GAATCCCGGGCTCAGCAGCGCTATTCGCCTGCGTCCACATTCAAGATTCCGCATACCCTT 240
                           **  *     * ** ** ***** ** ** ** ****** * ** ** ** ***

OXA2_ABN48512        TTTGCACTTGATGCAGGCGCTGTTCGTGATGAGTTCCAGATTTTTCGATGGGACGGCGTT 300
OXA3_PSEBLA          TTTGCACTTGACGCAGGCGCTGCACGTGATGAGTTTCAAGTTTTCCGATGGGACGGCATC 300
OXA53_AAP43641       TTTGCACTTGATGCAGGCGCCGTTCGCGATGAGTTTCAGATTTTCCGCTGGGACGGCGTC 300
PA13                 TTTGCACTCGATGCAGACGCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTT 300
Bcepacia_AAK55330    TTTGCACTCGATGCAGACGCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTT 300
Uncultured_AAN41427  TTTGCACTCGATGCAGACGCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTT 300
OXA46_AAN63499       TTTGCACTTGATGCAGACGCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTT 300
OXA20_CAC85643       TTTGCGCTGGATGCAGGGGCGGTTCGCGATGAGTTTCATGTTTTTCGATGGGACGGCGCT 300
                     ***** ** ** ****  ** *  ** ******** **  **** ** *********
```

```
OXA2_ABN48512        AACAGGGGCTTTGCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATTCT 360
OXA3_PSEBLA          AAAAGAAGCTTTGCAGCTCACAACCAAGACCAAGACTTGCGATCAGCAATGCGGAATTCT 360
OXA53_AAP43641       AAAAGGAGCTTTGCAGGTCACAATAAAGACCAAGATTTGCGATCAGCAATGCGAAATTCT 360
PA13                 AAACGGAGCTTTGCGGGGCCATAATCAAGACCAAGACTTGCGATCAGCGATGCGAAATTCT 360
Bcepacia_AAK55330    AAACGGAGCTTTGCGGGGCCATAATCAAGACCAAGACTTGCGATCAGCGATGCGAAATTCT 360
Uncultured_AAN41427  AAACGGAGCTTTGCGGGGCCATAATCAAGACCAAGACTTGCGATCAGCGATGCGAAATTCT 360
OXA46_AAN63499       AACCGAAGCTTTGCAGGTCACAATCAAGACCAAGATTTGCGATCAGCGATGCGAAATTCT 360
OXA20_CAC85643       AAAAGAAGCTTTGCAGGTCACAATCAAGACCAAAACCTACGATCGGCAATGCGCAATTCT 360
                     **  *  ******* *  ** **  ******** *  * ***** ** ***** ******

OXA2_ABN48512        ACTGTTTGGGTGTATGAGCTATTTGCAAAGGAAATTGGTGATGACAAAGCTCGGCGCTAT 420
OXA3_PSEBLA          ACTGTCTGGATTTATGAGCTATTTGCAAAAGAGATCGGTGAAGACAAGGCTCGACGCTAT 420
OXA53_AAP43641       ACTGTCTGGGTTTATGAGCTATTTGCAAAGGAAATCGGTGATGGCAAGGCTCGACGCTAT 420
PA13                 GCGGTCTGGGTTTATGAGCTATTTGCAAAAGAGATCGGAGAGGACAAAGCAAGACGCTAT 420
Bcepacia_AAK55330    GCGGTCTGGGTTTATGAGCTATTTGCAAAAGAGATCGGAAAGGACAAAGCAAGACACTAT 420
Uncultured_AAN41427  GCGGTCTGGGTTTATGAGCTATTTGCAAAAGAGATCGGAGAGGACAAAGCAAGACGCTAT 420
OXA46_AAN63499       ACGGTTTGGGTTTATGAGCTGTTTGCAAAAGATATCGGAGAGGACAAAGCAAGACGTTAT 420
OXA20_CAC85643       ACCGTTTGGGTCTATCAACTATTCGCAAAAGAAATAGGCGAAAACAAAGCACGAAGCTAC 420
                           * ** *** * *** * ** ** ***** ** ** **  *   *** **  *    **

OXA2_ABN48512        TTGAAGAAAATCGACTATGGCAACGCCGATCCTTCGACAAGTAATGGCGATTACTGGATA 480
OXA3_PSEBLA          TTGAAGCAAATCGACTATGGCAACGCCGATCCTTCGACAAGTAATGGCGATTACTGGATA 480
OXA53_AAP43641       TTGAAGCAAATCGGCTATGGCAACGCCGATCCTTCGACAAGTCATGGCGATTACTGGATA 480
PA13                 TTAAAGCAAATTGATTATGGCAACGCCGACCCTTCGACAATCAAGGGCGATTACTGGATA 480
Bcepacia_AAK55330    TTAAAGCAAATTGATTATGGCAACGCCGACCCTTCGACAATCAAGGGCGATTACTGGATA 480
Uncultured_AAN41427  TTAAAGCAAATTGATTATGGCAACGCCGACCCTTCGACAATCAAGGGCGATTACTGGATA 480
OXA46_AAN63499       TTAAAGCAAATTGATTATGGCAACGTCGATCCTTCGACAATCAAGGGCGATTACTGGATA 480
OXA20_CAC85643       CTAGAAAAAATTAAACTACGGCAATGCAGACCCCTCGACCAAGAGCGGTGACTACTGGATA 480
                      *  *  ** *    ** ***** *  ** ** ***** *     ** ** **********

OXA2_ABN48512        GAAGGCAGCCTTGCAATCTCGGCGCAGGAGCAAATTGCATTTCTCAGGAAGCTCTATCGT 540
OXA3_PSEBLA          GATGGCAATCTTGCTATCGCGGCACAAGAACAGATTGCATTTCTCAGGAAGCTCTATCAT 540
OXA53_AAP43641       GAAGGCAGCCTTGCAATCTCAGCACAGGAACAGATCGCGTTTCTCAGAAAGCTCTATCAA 540
PA13                 GATGGCAATCTTGAAATCTCAGCGCACGAACAGATTTCGTTTCTCAGAAAACTCTATCGA 540
Bcepacia_AAK55330    GATGGCAATCTTGAAATCTCAGCGCACGAACAGATTTCGTTTCTCAGAAAACTCTATCGA 540
Uncultured_AAN41427  GATGGCAATCTTGAAATCTCAGCGCACGAACAGATTTCGTTTCTCAGAAAACTCTATCGA 540
OXA46_AAN63499       GATGGAAATCTTAAAATCTCAGCGCACGAACAGATTTTGTTTCTCAGAAAACTCTATCGA 540
OXA20_CAC85643       GATGGAAATCTTGCAATTTCAGCAAATGAACAAATTTCCATCCTAAAGAAGCTTTATCGA 540
                     ** ** *  ***   **  * **  * ** ** **     * ** *  ** ** ****

OXA2_ABN48512        AACGAGCTGCCCTTTCGGGTAGAACATCAGCGCTTGGTCAAGGATCTCATGATTGTGGAA 600
OXA3_PSEBLA          AACGAGTTGCCCTTTCGGGTAGAACATCAGCGCTTGGTCAAGGACCTCATGATTGTGGAA 600
OXA53_AAP43641       AACGATCTGCCCTTTAGGGTGGAACATCAGCGCTTGGTCAAGGATCTGATGATTGTGGAA 600
PA13                 AATCAGCTGCCATTTCAGGTGGAACATCAGCGCTTGGTCAAAGATCTCATGATTACGGAA 600
Bcepacia_AAK55330    AATCAGCTGCCATTTCAGGTGGAACATCAGCGCTTGGTCAAAGATCTCATGATTACGGAA 600
Uncultured_AAN41427  AATCAGCTGCCATTTCAGGTGGAACATCAGCGCTTGGTCAAAGATCTCATGATTACGGAA 600
OXA46_AAN63499       AATCAGTTACCATTTAAGGTGGAGCACCAGCGCTTGGTGAAAGATCTCATGATTACGGAA 600
OXA20_CAC85643       AATGAGCTTCCTTTTAGGGTAGAGCACCAACGCTTGGTTAAAGACTTGATGATTGTCGAA 600
                     **  *  * ** ***  *** ** ** ** ******** ** **  * ******   ***

OXA2_ABN48512        GCCGGTCGCAACTGGATACTGCGTGCAAAGACGGGCTGGGAAGGCCGTATGGGTTGGTGG 660
OXA3_PSEBLA          GCCGGTCGCAACTGGATACTGCGCGCAAAGACGGGCTGGGAAGGCCGCATTGGTTGGTGG 660
OXA53_AAP43641       GCGGGACGCAACTGGATTCTGCGCGCGAAGACGGGCTGGGAAGGCAGCATGGGTTGGTGG 660
PA13                 GCCGGGCGCAACTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGTGG 660
Bcepacia_AAK55330    GCCGGGCGCAACTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGTGG 660
Uncultured_AAN41427  GCCGGGCGCAATTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGTGG 660
OXA46_AAN63499       GCCGGGCGCAGTTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGTGG 660
OXA20_CAC85643       GCCAAACGTGATTGGATACTACGTGCCAAAACAGGCTGGGATGGTCAAATGGGTTGGTGG 660
                     **    **    ***** ** ** ** ** ** ******** **     * ** ******

OXA2_ABN48512        GTAGGATGGGTTGAGTGGCCGACTGGCTCCGTATTCTTCGCACTGAATATTGATACGCCA 720
OXA3_PSEBLA          GTAGGATGGGTTGAGTGGCCGACTGGCCCCGTATTCTTCGCACTGAATATTGATACGCCA 720
OXA53_AAP43641       GTGGGGTGGGTTGAATGGCCAACCGGTCCCGTATTCTTTGCCTTGAATATCGATACGCCA 720
PA13                 GTAGGGTGGGTGGAGTGGCCAACCGGTCCCGTATTCTTCGCACTGAATATTGATACGCCA 720
Bcepacia_AAK55330    GTAGGGTGGGTGGAGTGGCCAACCGGTCCCGTATTCTTCGCGCTGAATATTGATACGCCA 720
Uncultured_AAN41427  GTAGGGTGGGTGGAGTGGCCAACCGGTCCCGTATTCTTCGCGCTGAATATTGATACGCCA 720
OXA46_AAN63499       GTAGGGTGGATTGAATGGCCAACAGGCCCCGTATTCTTTGCGCTGAATATTGATACGCCA 720
OXA20_CAC85643       GTCGGTTGGGTAGAGTGGCCTACAGGCCCAGTATTTTTTGCGTTAAATATCGACACGCCA 720
                     ** ** *** * ** ***** ** **  * ***** ** **  * ***** ** ******

OXA2_ABN48512        AACAGAATGGATGATCTTTTCAAGAGGGAGGCAATCGTGCGGGCAATCCTTCGCTCTATT 780
OXA3_PSEBLA          AACAGGATGGATGACCTTTTCAAAAGGGAGGCAATAGTGCGGGCAATCCTTCGCTCTATC 780
OXA53_AAP43641       AACAGAATGGACGATCTTTTCAAGAGGGAAGCAATAGCGCGAGCGATACTTCTCTCTATC 780
PA13                 AACAGAACGGATGATCTTTTCAAAAGAGAGGCAATCGCGCGGGCAATCCTTCGCTCTATC 780
Bcepacia_AAK55330    AACAGAACGGATGATCTTTTCAAAAGAGAGGCAATCGCGCGGGCAATCCTTCGCTCTATC 780
```

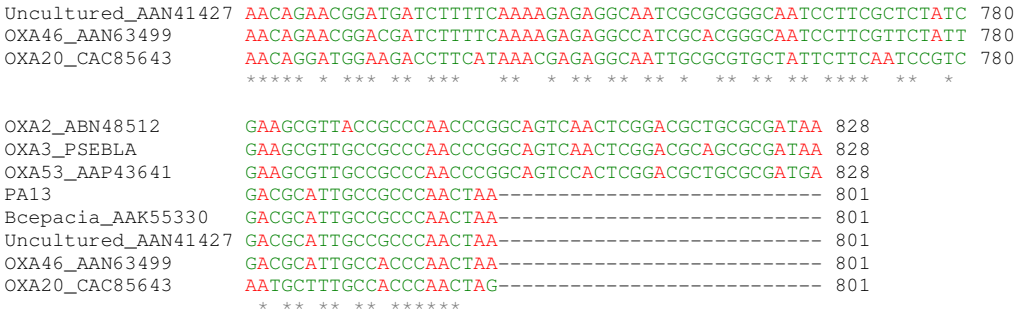
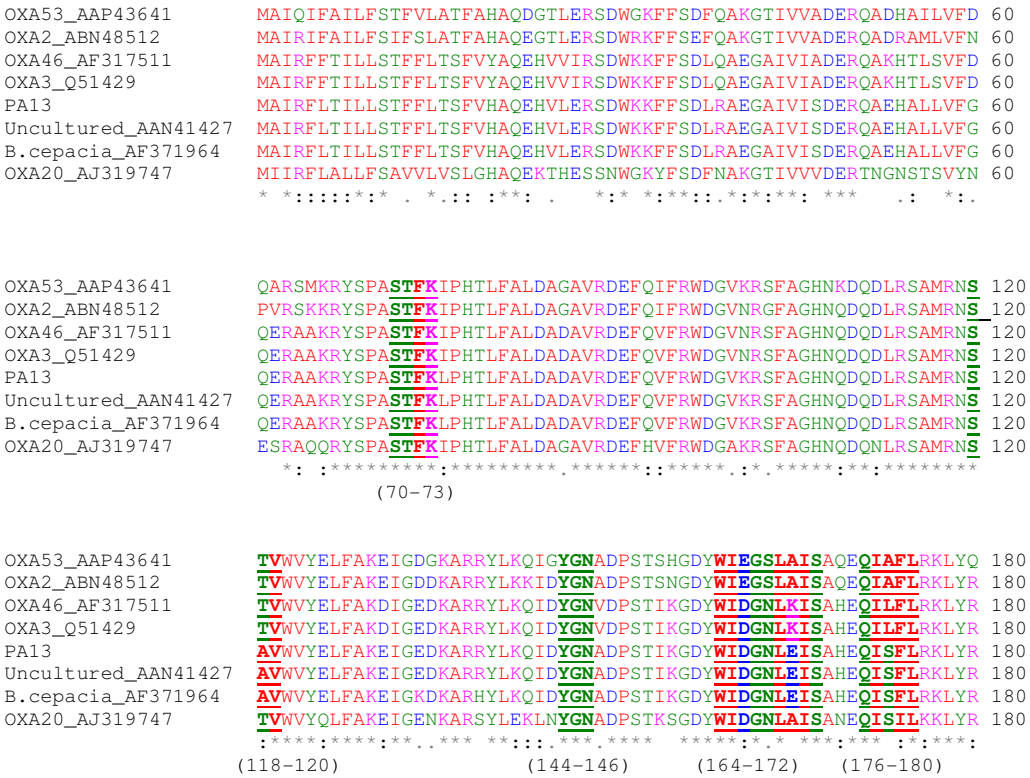


Figure 3.11: Clustal W alignments comparing the nucleotide sequences of oxacillinase enzymes. The compared genes are from *P. aeruginosa* PA13 with OXA genes from *A. baumannii* (ABN48512), *P. aeruginosa* (PSEBLA), *S. enterica* (AAP43641), *Burkholderia cepacia* (AAK55330), an unidentified bacterium from a wastewater treatment plant in Germany (AAN41427), *P. aeruginosa* (AAN63499) and *A. baumannii* (CAC85643). Nucleotides that are identical in all strains are underlined with an asterisk.



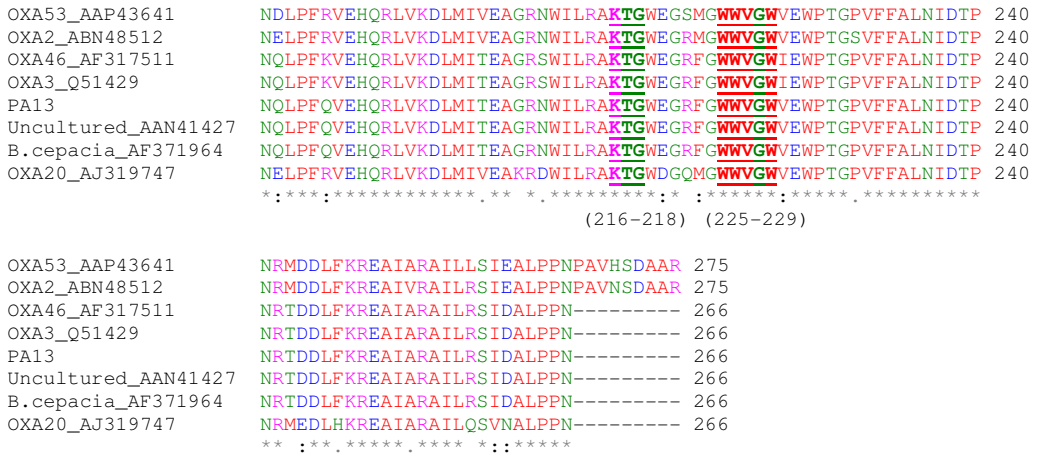


Figure 3.12: Clustal W alignments comparing the amino acid sequences of oxacillinase enzymes. The compared amino acid sequences are from *P. aeruginosa* PA13 with OXA-53 (AAP43641), OXA-2 (ABN48512), OXA-46 (AF317511), OXA-3 (Q51429), an OXA-type enzyme from unidentified bacterium from a wastewater treatment plant in Germany (AAN41427), an OXA-type enzyme from *Burkholderia cepacia* (AF371964) and OXA-20 (AJ319747). All these enzymes are representative of the OXA-2 lineage. The conserved motifs of OXA-type enzymes conserved structural regions are highlighted and underlined. DBL numbering is in parenthesis below the alignments. Residues that are identical in all strains are underlined with an asterisk.

ORF4

Downstream from the oxacillinase gene cassette, ORF4 was identified (Figure 3.6). ORF4 encoded a partial sequence that shared 100% nucleotide similarity to previously published *qacE*Δ1 genes located on integrons from many strains including *E. coli* (U12441) (Sundstrum *et al*., 1988) and *P. aeruginosa* (U63835) (Danel *et al*., 1997) and *P. aeruginosa* (AJ620678) (Castanheira *et al*., 2004) which confer resistance to quaternary ammonium compounds (Figure 3.13). This quaternary ammonium compound resistance gene is typical of class 1 integrons where they are found in the 3' conserved segment (Daly and Fanning, 2000).

```
Paer_U63835        ------------ATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCT 119
PA_AJ620678        ------------ATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCT 118
E.coli_U12441      ------------ATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCT 119
PA13               ------------ATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCT 91
                               *********************************************

Paer_U63835        GCTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 179
PA_AJ620678        GCTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 178
E.coli_U12441      GCTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 179
PA13               GCTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 151
                   ************************************************************

Paer_U63835        ACTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 239
PA_AJ620678        ACTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 238
E.coli_U12441      ACTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGA 239
PA13               AAGGCTGGCTTTTTCTTGTTATCGCAATAGTGGCGAAG--AATC---------------- 193
                   *    *    ****   *    *  ****    * *    ***
```

Figure 3.13: Clustal W alignments comparing the partial nucleotide sequences of quaternary ammonium compound resistance genes, *qacEΔ1*. The compared *qacEΔ1* genes are from *Pseudomonas aeruginosa* (U63835), *Pseudomonas aeruginosa* (AJ620678), *E. coli* (U12441) and *Pseudomonas aeruginosa* PA13. Residues that are identical in all strains are underlined with an asterisk.

3.3.4 The Integron

The presence of both the integrase gene (*intI*) and the quaternary ammonium compound resistance gene (*qacΔE1*) (Figure 3.6) indicated that the resistance genes in *P. aeruginosa* PA13 were on an integron. The complete nucleotide sequence of the integron amplified from *P. aeruginosa* PA13 was compared to organisms containing integrons with the most similar sequences using the GenBank database (Figure 3.14). Analysis of the sequence showed that the integron was 95% similar to an integron found in a *Pseudomonas aeruginosa* strain in Italy (Giuliani *et al*, 2005), 92% similar to an integron found in a *Vibrio cholerae* strain in Argentina (Soler Bistué *et al*., 2006), 92% similar to an integron described in a *Morganella morganii* strain in Argentina (Power *et al*., 2005), and 88% similar to an integron found in a *Pseudomonas aeruginosa* strain in France (Naas *et al*., 1998).

```
Morganii_AJ621187    -AGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATC 491
Paeruginosa_AF024602 -AGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATC 491
Vibrio_DQ310703      -AGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATC 5719
PA13                 ----------------------------------GATCGAA-TCCAGATC 15
Paeruginosa_AF317511 TAGCAGATGCGGCATAACAAATCGTTGGAGCGGGACTTTTGCTACGCAGG 1035
                                                       *     * *  *
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Morganii_AJ621187    CTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCC---CGTTCCA- 537
Paeruginosa_AF024602 CTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCC---CGTTCCA- 537
Vibrio_DQ310703      CTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCC---CGTTCCA- 5765
PA13                 CTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCC---CGTTCCA- 61
Paeruginosa_AF317511 CTGCGCCTACT-CCGCAAAAGCCCCTCAACTCAGGCGTTAGGCATCACAA 1084
                     **   **  *    ***** * *    *  *    *     * *  **

Morganii_AJ621187    --TACAGAAGCTGGGCGAACA--AACGATGC--TCGCCTT---CCAGAAA 578
Paeruginosa_AF024602 --TACAGAAGCTGGGCGAACA--AACGATGC--TCGCCTT---CCAGAAA 578
Vibrio_DQ310703      --TACAGAAGCTGGGCGAACA--AACGATGC--TCGCCTT---CCAGAAA 5806
PA13                 --TACAGAAGCTGGGCGAACA--AACGATGC--TCGCCTT---CCAGAAA 102
Paeruginosa_AF317511 AGTACAGCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATG 1134
                       ***** * *  * * ****  ****** *  ** *  *   **  *

Morganii_AJ621187    ACCGAGGATG----CGAACCACTTCATCCGGGGTCAGCAC-------CAC 617
Paeruginosa_AF024602 ACCGAGGATG----CGAACCACTTCATCCGGGGTCAGCAC-------CAC 617
Vibrio_DQ310703      ACCGAGGATG----CGAACCACTTCATCCGGGGTCAGCAC-------CAC 5845
PA13                 ACCGAGGATG----CGAACCACTTCATCCGGGGTCAGCAC-------CAC 141
Paeruginosa_AF317511 ACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATAT 1184
                     ** *** ***     *     **  **  * **  *   *        *

Morganii_AJ621187    CGGCAAGCGCCGCGACGGCCGAGGT---CTTCCGAT--CTCCTGAAGC-C 661
Paeruginosa_AF024602 CGGCAAGCGCCGCGACGGCCGAGGT---CTTCCGAT--CTCCTGAAGC-C 661
Vibrio_DQ310703      CGGCAAGCGCCGCGACGGCCGAGGT---CTTCCGAT--CTCCTGAAGC-C 5889
PA13                 CGGCAAGCGCCGCGACGGCCGAGGT---CTTCCGAT--CTCCTGAAGC-C 185
Paeruginosa_AF317511 CGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTAC 1234
                     ** * ** *  * * ***   **     *  ****    * **** *  *

Morganii_AJ621187    AGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCC 711
Paeruginosa_AF024602 AGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCC 711
Vibrio_DQ310703      AGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCC 5939
PA13                 AGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCC 235
Paeruginosa_AF317511 AGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATAC 1284
                     ***     * * ***  ***   **  ** *  **  *  **   *   *

Morganii_AJ621187    AATGCCTGACGATGCGTGGAGA-CCGA------------AACCTTGCGCT 748
Paeruginosa_AF024602 AATGCCTGACGATGCGTGGAGA-CCGA------------AACCTTGCGCT 748
Vibrio_DQ310703      AATGCCTGACGATGCGTGGAGA-CCGA------------AACCTTGCGCT 5976
PA13                 AATGCCTGACGATGCGTGGAGA-CCGA------------AACCTTGCGCT 272
Paeruginosa_AF317511 ATTGC--AATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTT 1332
                     * ***   * * **  ****** ****           *  * * ** *

Morganii_AJ621187    -CGTTCGCCAGCCAGGACAGA---------AATGCCTCGACTTCGCTGCT 788
Paeruginosa_AF024602 -CGTTCGCCAGCCAGGACAGA---------AATGCCTCGACTTCGCTGCT 788
Vibrio_DQ310703      -CGTTCGCCAGCCAGGACAGA---------AATGCCTCGACTTCGCTGCT 6016
PA13                 -CGTTCGCCAGCCAGGACAGA---------AATGCCTCGACTTCGCTGCT 312
Paeruginosa_AF317511 GCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGT 1382
                      *  * *  ***  **** **          * *   *   * *     *

Morganii_AJ621187    GCCCAAGGTTGCCGGGT----GACGCACACCGTGGAAACGGATGAAGGCA 834
Paeruginosa_AF024602 GCCCAAGGTTGCCGGGT----GACGCACACCGTGGAAACGGATGAAGGCA 834
Vibrio_DQ310703      GCCCAAGGTTGCCGGGT----GACGCACACCGTGGAAACGGATGAAGGCA 6062
PA13                 GCCCAAGGTTGCCGGGT----GACGCACACCGTGGAAACGGATGAAGGCA 358
Paeruginosa_AF317511 ACGCGGAATAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCT 1432
                      * *    * * *  **    * ** *  *     **  **   *****

Morganii_AJ621187    CG--AACCCAGTTGACATAAGCCT--GTTCGGTTCGTGAACTGTAATGC- 879
Paeruginosa_AF024602 CG--AACCCAGTTGACATAAGCCT--GTTCGGTTCGTGAACTGTAATGC- 879
Vibrio_DQ310703      CG--AACCCAGTTGACATAAGCCT--GTTCGGTTCGTAAACTGTAATGC- 6107
PA13                 CG--AACCCAGTGGACATAAGCCT--GTTCGGTTCGTAAGCTGTAATGC- 403
Paeruginosa_AF317511 TGGGAACCAAGCTGGTTCGAGCACTGGTTGAGTTGCTGTTCAATGATCCC 1482
                      *  **** **  *     ***    ***  ***  *   *  * ** *

Morganii_AJ621187    -AAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCA 928
Paeruginosa_AF024602 -AAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCA 928
Vibrio_DQ310703      -AAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCA 6156
PA13                 -AAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCA 452
Paeruginosa_AF317511 GAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGAT 1532
                      * **  *  *    *  *** * * *   * ** *      *** **

Morganii_AJ621187    GCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA---TGACTGT 975
Paeruginosa_AF024602 GCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA---TGACTGT 975
Vibrio_DQ310703      GCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA---TGACTGT 6203
PA13                 GCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA---TGACTGT 499
Paeruginosa_AF317511 CCGATGCTA-CGAGAAAGCGG-GGTTTGAGAGGCAAGGTACCGTAACCAC 1580
                      ** ** ** **    ** ** *****    ***  * **   * **
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```
Morganii_AJ621187    TTTT--TTGTACAGTC-----TATGCCTCGGGCAT-CCAAGCAG-CAAGC 1016
Paeruginosa_AF024602 TTTT--TTGTACAGTC-----TATGCCTCGGGCAT-CCAAGCAG-CAAGC 1016
Vibrio_DQ310703      TTTT--TTGTACAGTC-----TATGCCTCGGGCAT-CCAAGCAG-CAAGC 6244
PA13                 TTTG--TTGTACAGTC-----TATGCCTCGGGCAT-CCAAGCAG-CAAGC 540
Paeruginosa_AF317511 CCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGC 1630
                           * ** *** *      ***  **   **  *** ***  * ***

Morganii_AJ621187    GCGTTACGCCGTGG-GTCGATGTTTGATGTTATGGAGCAACAACGATGTT 1065
Paeruginosa_AF024602 GCGTTACGCCGTGG-GTCGATGTTTGATGTTATGGAGCAACAACGATGTT 1065
Vibrio_DQ310703      GCGTTACGCCGTGG-GTCGATGTTTGATGTTATGGAGCAGCAACGATGTT 6293
PA13                 GCGTTACGCCGTGG-GTCGATGTTTGATGTTATGGAGCAGCAACGATGTT 589
Paeruginosa_AF317511 GAAC-ACGCAGTGATGCCTAACCCTTCCATCGAGGGGGACGTCCAAGGGC 1679
                     *    **** ***  * * *    *    *   ** * *    * * *

Morganii_AJ621187    ACGCAGCAG-----------TAAAACAAA-GTTAGGCATCACAAAGTACA 1103
Paeruginosa_AF024602 ACGCAGCAG-----------TAAAACAAA-GTTAGGCATCACAAAGTACA 1103
Vibrio_DQ310703      ACGCAGCAG-----------TAAAACAAA-GTTAGGCATCACAAAGTACA 6331
PA13                 ACGCAGCAGGGCAGTCGCCCTAAAACAAA-GTTAGGCATCACAAAGTACA 638
Paeruginosa_AF317511 TGGCGCCCTTGGCCGCCCCTCATGTCAAACGTTAGGCATCACAAAGTACA 1729
                       **  *              *   **** ********************

Morganii_AJ621187    GCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG 1153
Paeruginosa_AF024602 GCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG 1153
Vibrio_DQ310703      GCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG 6381
PA13                 GCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG 688
Paeruginosa_AF317511 GCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG 1779
                     **************************************************

Morganii_AJ621187    CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGA 1203
Paeruginosa_AF024602 CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGA 1203
Vibrio_DQ310703      CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGA 6431
PA13                 CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGA 738
Paeruginosa_AF317511 CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGA 1829
                     **************************************************

Morganii_AJ621187    GTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC 1253
Paeruginosa_AF024602 GTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC 1253
Vibrio_DQ310703      GTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC 6481
PA13                 GTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC 788
Paeruginosa_AF317511 GTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC 1879
                     **************************************************

Morganii_AJ621187    AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA 1303
Paeruginosa_AF024602 AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA 1303
Vibrio_DQ310703      AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA 6531
PA13                 AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA 838
Paeruginosa_AF317511 AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA 1929
                     **************************************************

Morganii_AJ621187    ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGG 1353
Paeruginosa_AF024602 ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGG 1353
Vibrio_DQ310703      ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGG 6581
PA13                 ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGG 888
Paeruginosa_AF317511 ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGG 1979
                     **************************************************

Morganii_AJ621187    AAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAA 1403
Paeruginosa_AF024602 AAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAA 1403
Vibrio_DQ310703      AAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAA 6631
PA13                 AAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAA 938
Paeruginosa_AF317511 AAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAA 2029
                     **************************************************

Morganii_AJ621187    TAGACCAGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACC 1453
Paeruginosa_AF024602 TAGACCAGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACC 1453
Vibrio_DQ310703      TAGACCAGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACC 6681
PA13                 TAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACC 988
Paeruginosa_AF317511 TAGACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACC 2079
                     ********* ****************************************

Morganii_AJ621187    AAGCTGGTCCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAC 1503
Paeruginosa_AF024602 AAGCTGGTCCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAC 1503
Vibrio_DQ310703      AAGCTGGTCCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAC 6731
PA13                 AAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAC 1038
Paeruginosa_AF317511 AAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCAC 2129
                     ******** ****************************************
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Morganii_AJ621187    CAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCT 1553
Paeruginosa_AF024602 CAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCT 1553
Vibrio_DQ310703      CAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCT 6781
PA13                 CAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCT 1088
Paeruginosa_AF317511 CAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCT 2179
                     **************************************************

Morganii_AJ621187    ACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT 1603
Paeruginosa_AF024602 ACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT 1603
Vibrio_DQ310703      ACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT 6831
PA13                 ACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT 1138
Paeruginosa_AF317511 ACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT 2229
                     **************************************************

Morganii_AJ621187    CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAG 1653
Paeruginosa_AF024602 CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAG 1653
Vibrio_DQ310703      CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAG 6881
PA13                 CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAG 1188
Paeruginosa_AF317511 CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAG 2279
                     **************************************************

Morganii_AJ621187    TGATGCCTAACCCTTCCATCG-AGGGGGACGTCCAAGGGCTGGCGCCCTT 1702
Paeruginosa_AF024602 TGATGCCTAACCCTTCCATCG-AGGGGGACGTCCAAGGGCTGGCGCCCTT 1702
Vibrio_DQ310703      TGATGCCTAACCCTTCCATCG-AGGGGGACGTCCAAGGGCTGGCGCCCTT 6930
PA13                 TGATGCCTAACCCTTCCATCGGAGGGGGACGTCCAAGGGCTGGCGCCCTT 1238
Paeruginosa_AF317511 TGATGCCTAACCCTTCCATCG-AGGGGGACGTCCAAGGGCTGGCGCCCTT 2328
                     ********************* ****************************

Morganii_AJ621187    GGCCGCCCCTCATGTCAAACGTTGGGCATTAAGGAAAAGTTAATGGCAAT 1752
Paeruginosa_AF024602 GGCCGCCCCTCATGTCAAACGTTGGGCATTAAGGAAAAGTTAATGGCAAT 1752
Vibrio_DQ310703      GGCCGCCCCTCATGTCAAACGTTGGGCATTAAGGAAAAGTTAATGGCAAT 6980
PA13                 GGCCGCCCCTCATGTCAAACGTTAGGCGTCAAAGGAAACTTAATGGCAAT 1288
Paeruginosa_AF317511 GGCCGCCCCTCATGTCAAACGTTGGGCATCAAAGGAAATTTAATGGCAAT 2378
                     ********************** *** * ** * *** ************

Morganii_AJ621187    CCGAATCTTCGCGATACTTTTCTCCATTTTTTCTCTTGCCACTTTCGCGC 1802
Paeruginosa_AF024602 CCGAATCTTCGCGATACTTTTCTCCATTTTTTCTCTTGCCACTTTCGCGC 1802
Vibrio_DQ310703      CCGAATCTTCGCGATACTTTTCTCCATTTTTTCTCTTGCCACTTTCGCGC 7030
PA13                 CCGATTCCTCACCATACTGCTATCTACTTTTTTTCTTACCTCATTCGTGC 1338
Paeruginosa_AF317511 CCGATTCTTCACCATACTGCTATCCACCTTCTTTCTTACCTCATTCGTGT 2428
                     **** ** ** * *****  * ** *  ** * **** ** * **** *

Morganii_AJ621187    ATGCGCAAGAAGGCACGCTAGAACGTTCTGACTGGAGGAAGTTTTTCAGC 1852
Paeruginosa_AF024602 ATGCGCAAGAAGGCACGCTAGAACGTTCTGACTGGAGGAAGTTTTTCAGC 1852
Vibrio_DQ310703      ATGCGCAAGAAGGCACGCTAGAACGTTCTGACTGGAGGAAGTTTTTCAGC 7080
PA13                 ATGCGCAAGAACACGTGCTAGAGCGTTCTGACTGGAAGAAGTTCTTCAGC 1388
Paeruginosa_AF317511 ATGCGCAAGAACATGTGGTAATCCGTTCGGACTGGAAAAAGTTCTTCAGC 2478
                     ***********     * **   ***** *******  ***** ******

Morganii_AJ621187    GAATTTCAAGCCAAAGGCACGATAGTTGTGGCAGACGAACGCCAAGCGGA 1902
Paeruginosa_AF024602 GAATTTCAAGCCAAAGGCACGATAGTTGTGGCAGACGAACGCCAAGCGGA 1902
Vibrio_DQ310703      GAATTTCAAGCCAAAGGCACGATAGTTGTGGCAGACGAACGCCAAGCGGA 7130
PA13                 GACCTCCGGGCCGAAGGTGCAATCGTTATTTCAGACGAACGTCAAGCGGA 1438
Paeruginosa_AF317511 GACCTCCAGGCCGAAGGTGCAATCGTTATTGCAGACGAACGTCAAGCGAA 2528
                     **  * *  *** ****  * ** *** *  ********** ****** *

Morganii_AJ621187    TCGTGCCATGTTGGTTTTTGATCCTGTGCGATCGAAGAAACGCTACTCGC 1952
Paeruginosa_AF024602 TCGTGCCATGTTGGTTTTTGATCCTGTGCGATCGAAGAAACGCTACTCGC 1952
Vibrio_DQ310703      TCGTGCCATGTTGGTTTTTGATCCTGTGCGATCGAAGAAACGCTACTCGC 7180
PA13                 GCATGCTTTATTGGTTTTTGGTCAAGAGCGAGCAGCAAAGCGTTACTCGC 1488
Paeruginosa_AF317511 GCATACTTTATCGGTTTTTGATCAAGAGCGAGCGGCAAAGCGTTACTCGC 2578
                      * * *  * * ******** **  * **** *    ** ** *******

Morganii_AJ621187    CTGCATCGACATTCAAGATACCTCATACACTTTTTGCACTTGATGCAGGC 2002
Paeruginosa_AF024602 CTGCATCGACATTCAAGATACCTCATACACTTTTTGCACTTGATGCAGGC 2002
Vibrio_DQ310703      CTGCATCGACATTCAAGATACCTCATACACTTTTTGCACTTGATGCAGGC 7230
PA13                 CTGCTTCAACCTTCAAGCTTCCACACACACTTTTTGCACTCGATGCAGAC 1538
Paeruginosa_AF317511 CAGCTTCAACCTTCAAGATACCCCACACACTTTTTGCACTTGATGCAGAC 2628
                     * ** ** ** ****** * ** ** ************** ******* *

Morganii_AJ621187    GCTGTTCGTGATGAGTTCCAGATTTTTCGATGGGACGGCGTTAACAGGGG 2052
Paeruginosa_AF024602 GCTGTTCGTGATGAGTTCCAGATTTTTCGATGGGACGGCGTTAACAGGGG 2052
Vibrio_DQ310703      GCTGTTCGTGATGAGTTCCAGATTTTTCGATGGGACGGCGTTAACAGGGG 7280
PA13                 GCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTTAAACGGAG 1588
Paeruginosa_AF317511 GCCGTTCGTGATGAGTTCCAGGTTTTTCGATGGGACGGCGTTAACCGAAG 2678
                     ** ****************** **********************  *  *
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Morganii_AJ621187    CTTTGCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATT 2102
Paeruginosa_AF024602 CTTTGCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATT 2102
Vibrio_DQ310703      CTTTGCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATT 7330
PA13                 CTTTGCGGGCCATAATCAAGACCAAGACTTGCGATCAGCGATGCGAAATT 1638
Paeruginosa_AF317511 CTTTGCAGGTCACAATCAAGACCAAGATTTGCGATCAGCGATGCGAAATT 2728
                     ****** ** ** ************** *********** ***** ****

Morganii_AJ621187    CTACTGTTTGGGTGTATGAGCTATTTGCAAAGGAAATTGGTGATGACAAA 2152
Paeruginosa_AF024602 CTACTGTTTGGGTGTATGAGCTATTTGCAAAGGAAATTGGTGATGACAAA 2152
Vibrio_DQ310703      CTACTGTTTGGGTGTATGAGCTATTTGCAAAGGAAATTGGTGATGACAAA 7380
PA13                 CTGCGGTCTGGGTTTATGAGCTATTTGCAAAAGAGATCGGAGAGGACAAA 1688
Paeruginosa_AF317511 CTACGGTTTGGGTTTATGAGCTGTTTGCAAAAGATATCGGAGAGGACAAA 2778
                     ** * ** ***** ******** ******** ** ** ** ** ******

Morganii_AJ621187    GCTCGGCGCTATTTGAAGAAAATCGACTATGGCAACGCCGATCCTTCGAC 2202
Paeruginosa_AF024602 GCTCGGCGCTATTTGAAGAAAATCGACTATGGCAACGCCGATCCTTCGAC 2202
Vibrio_DQ310703      GCTCGGCGCTATTTGAAGAAAATCGACTATGGCAACGCCGATCCTTCGAC 7430
PA13                 GCAAGACGCTATTTAAAGCAAATTGATTATGGCAACGCCGACCCTTCGAC 1738
Paeruginosa_AF317511 GCAAGACGTTATTTAAAGCAAATTGATTATGGCAACGTCGATCCTTCGAC 2828
                     **  * ** ***** *** **** ** ********** *** ********

Morganii_AJ621187    AAGTAATGGCGATTACTGGATAGAAGGCAGCCTTGCAATCTCGGCGCAGG 2252
Paeruginosa_AF024602 AAGTAATGGCGATTACTGGATAGAAGGCAGCCTTGCAATCTCGGCGCAGG 2252
Vibrio_DQ310703      AAGTAATGGCGATTACTGGATAGAAGGCAGCCTTGCAATCTCGGCGCAGG 7480
PA13                 AATCAAGGGCGATTACTGGATAGATGGCAATCTTGAAATCTCAGCGCACG 1788
Paeruginosa_AF317511 AATCAAGGGCGATTACTGGATAGATGGAAATCTTAAAATCTCAGCGCACG 2878
                     **  ** ***************** ** *  ***  ****** ***** *

Morganii_AJ621187    AGCAAATTGCATTTCTCAGGAAGCTCTATCGTAACGAGCTGCCCTTTCGG 2302
Paeruginosa_AF024602 AGCAAATTGCATTTCTCAGGAAGCTCTATCGTAACGAGCTGCCCTTTCGG 2302
Vibrio_DQ310703      AGCAAATTGCATTTCTCAGGAAGCTCTATCGTAACGAGCTGCCCTTTCGG 7530
PA13                 AACAGATTTCGTTTCTCAGAAAACTCTATCGAAATCAGCTGCCATTTCAG 1838
Paeruginosa_AF317511 AACAGATTTTGTTTCTCAGAAAACTCTATCGAAATCAGTTACCATTTAAG 2928
                     * ** ***   ******** ** ******** **  ** * ** ***  *

Morganii_AJ621187    GTAGAACATCAGCGCTTGGTCAAGGATCTCATGATTGTGGAAGCCGGTCG 2352
Paeruginosa_AF024602 GTAGAACATCAGCGCTTGGTCAAGGATCTCATGATTGTGGAAGCCGGTCG 2352
Vibrio_DQ310703      GTAGAACATCAGCGCTTGGTCAAGGATCTCATGATTGTGGAAGCCGGTCG 7580
PA13                 GTGGAACATCAGCGCTTGGTCAAAGATCTCATGATTACGGAAGCCGGGCG 1888
Paeruginosa_AF317511 GTGGAGCACCAGCGCTTGGTGAAAGATCTCATGATTACGGAAGCCGGGCG 2978
                     ** ** ** ************ ** *************  ********* **

Morganii_AJ621187    CAACTGGATACTGCGTGCAAAGACGGGCTGGGAAGGCCGTATGGGTTGGT 2402
Paeruginosa_AF024602 CAACTGGATACTGCGTGCAAAGACGGGCTGGGAAGGCCGTATGGGTTGGT 2402
Vibrio_DQ310703      CAACTGGATACTGCGTGCAAAGACGGGCTGGGAAGGCCGTATGGGTTGGT 7630
PA13                 CAACTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGT 1938
Paeruginosa_AF317511 CAGTTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGT 3028
                     **  ******** ** ******** ************ *  * ** ****

Morganii_AJ621187    GGGTAGGATGGGTTGAGTGGCCGACTGGCTCCGTATTCTTCGCACTGAAT 2452
Paeruginosa_AF024602 GGGTAGGATGGGTTGAGTGGCCGACTGGCTCCGTATTCTTCGCACTGAAT 2452
Vibrio_DQ310703      GGGTAGGATGGGTTGAGTGGCCGACTGGCTCCGTATTCTTCGCACTGAAT 7680
PA13                 GGGTAGGGTGGGTGGAGTGGCCAACCGGTCCCGTATTCTTCGCACTGAAT 1988
Paeruginosa_AF317511 GGGTAGGGTGGATTGAATGGCCAACAGGCCCCGTATTCTTTGCGCTGAAT 3078
                     ******* *** * ** ***** ** **  ********** ** ******

Morganii_AJ621187    ATTGATACGCCAAACAGAATGGATGATCTTTTCAAGAGGGAGGCAATCGT 2502
Paeruginosa_AF024602 ATTGATACGCCAAACAGAATGGATGATCTTTTCAAGAGGGAGGCAATCGT 2502
Vibrio_DQ310703      ATTGATACGCCAAACAGAATGGATGATCTTTTCAAGAGGGAGGCAATCGT 7730
PA13                 ATTGATACGCCAAACAGAACGGATGATCTTTTCAAAAGAGAGGCAATCGC 2038
Paeruginosa_AF317511 ATTGATACGCCAAACAGAACGGACGATCTTTTCAAAAGAGAGGCCATCGC 3128
                     ****************** *** *********** ** ***** ****

Morganii_AJ621187    GCGGGCAATCCTTCGCTCTATTGAAGCGTTACCGCCCAACCCGGCAGTCA 2552
Paeruginosa_AF024602 GCGGGCAATCCTTCGCTCTATTGAAGCGTTACCGCCCAACCCGGCAGTCA 2552
Vibrio_DQ310703      GCGGGCAATCCTTCGCTCTATTGAAGCGTTACCGCCCAACCCGGCAGTCA 7780
PA13                 GCGGGCAATCCTTCGCTCTATCGACGCATTGCCGCCCAACTAATCAATCC 2088
Paeruginosa_AF317511 ACGGGCAATCCTTCGTTCTATTGACGCATTGCCACCCAACTAACCAATCC 3178
                      ************** ***** ** ** ** ** ******    ** **

Morganii_AJ621187    ACTCGGACGCTGCGCGATAAAACCGCGCAGCGCCGGTTACTTCAA-CGTT 2601
Paeruginosa_AF024602 ACTCGGACGCTGCGCGATAAAACCGCGCAGCGCCGGTTACTTCAA-CGTT 2601
Vibrio_DQ310703      ACTCGGACGCTGCGCGATAAAACCGCGCAGCGCCGGTTACTTCAA-CGTT 7829
PA13                 AG-CGGACGCCTT--------------CGGCGCCGCTGATTTCAA-CGTT 2122
Paeruginosa_AF317511 AGCCG-ACGCCTT--------------CGACGCGGCTGATTTCAAACGTT 3213
                     *  ** ****                 *  *** * * * ***** ****
```

```
Morganii_AJ621187    AGTACCACTGAAACCCTCCTTTATTTCGCCCATGTTTATTCAAACGGCAT 2651
Paeruginosa_AF024602 AGTACCACTGAAACCCTCCTTTATTTCGCCCATGTTTATTCAAACGGCAT 2651
Vibrio_DQ310703      AGTACCACTGAAACCCTCCTTTATTTCGCCCATGTTTATTCAAACGGCAT 7879
PA13                 AGATGCACTAAGCACATAATTGCTCACAGCCAAACTA--TCAGGTCAAGT 2170
Paeruginosa_AF317511 AGATGCACT-AAGCACATAATTGCT------------------------- 3237
                     **   **** *         *

Morganii_AJ621187    TCAGTTTCTCAAACGCTGTGCAGCGCTGGGTTTGCCGTTTCTCTGGGCTT 2701
Paeruginosa_AF024602 TCAGTTTCTCAAACGCTGTGCAGCGCTGGGTTTGCCGTTTCTCTGGGCTT 2701
Vibrio_DQ310703      TCAGTTTCTCAAACGCTGTGCAGCGCTGGGTTTGCCGTTTCTCTGGGCTT 7929
PA13                 CTGCTTTTATTATTTTTAAGC-GTGCATAATAAGCC----CTACACAAAT 2215
Paeruginosa_AF317511 --------------------------------------------------

Morganii_AJ621187    CGCCTGGTGGCGTTACGCTGGTTTGTGGTCTTTTTGGCCTCTGGCCCTTG 2751
Paeruginosa_AF024602 CGCCTGGTGGCGTTACGCTGGTTTGTGGTCTTTTTGGCCTCTGGCCCTTG 2751
Vibrio_DQ310703      CGCCTGGTGGCGTTACGCTGGTTTGTGGTCTTTTTGGCCTCTGGCCCTTG 7979
PA13                 TGGGAGATA-TATCATGAAAGGCTGGCTTTTTCTTG--TTATCGCAATAG 2262
Paeruginosa_AF317511 --------------------------------------------------

Morganii_AJ621187    TGTAGCAAGCGCGAGCAGCTATTTTTTTCGTAGTGCTGTGCCGCCTCGGT 2801
Paeruginosa_AF024602 TGTAGCAAGCGCGAGCAGCTATTTTTTTCGTAGTGCTGTGCCGCCTCGGT 2801
Vibrio_DQ310703      TGTAGCAAGCGCGAGCAGCTATTTTTTTCGTAGTGCTGTGCCGCCTCGGT 8029
PA13                 TGGCGAAGAATC------------------------------------- 2274
Paeruginosa_AF317511 --------------------------------------------------
```

Figure 3.14: A comparison of the integron nucleotide sequence from *P. aeruginosa* PA13 with similar integron sequences from *Morganella morganii* (AJ621187), *P. aeruginosa* (AF024602), *Vibrio cholerae* (DQ310703) and *P. aeruginosa* (AF317511). Residues that are identical in all strains are underlined with an asterisk.

Integrons possess two essential elements, located at the 5' conserved segment (CS), which are able to mobilize and insert gene cassettes. These are an *intI* gene encoding a site-specific recombinase belonging to the integrase family and its associated primary recombination site, *attI* (Collis and Hall, 1995). Captured genes (usually antibiotic resistance genes) are part of discrete mobile cassettes that contain the protein-coding region and a 3'-associated integrase-specific recombination site known as *attC*, belonging to the family of sites known as 59-base elements (Recchia and Hall, 1995). The outer boundaries of the 59-base element contain the conserved seven base pair core site GTTRRRY at the recombinant cross-over point, and an inverse core site RYYYAAC at the 3' end of the inserted gene cassette (where Y = pyrimidine [T or C] and R = purine [A or G]).

Located downstream of the *aac(6')-Ib* (ORF2) gene cassette and upstream of *bla-$_{OXA}$* gene (ORF3) was a short imperfect repeat element which matched the consensus 59-base element (*attC*) which is associated with fused ORFs within gene cassettes (GTTAGGC [Figure 3.5] located between nucleotide position 1259 and 1265). The

attC recombination site (59-base element) of the the *aac(6')-Ib* gene cassette was 73 base pairs long and includes all the elements typical of the *attC* sites (Figure 3.15). The sequence of this *attC* site was compared with the most similar *attC* site sequences from other integrons containing *aac(6')*-Ib genes using information in the Genbank database (Figure 3.15. It shared to be 99% similarity with *attC* sites found in two *Pseudomonas aeruginos*a isolates (Mugnier *et al*., 1998) (Mendes *et al*., 2004) and 96% similar to *attC* sites from a *Burkholderia cepacia* strain and a *Pseudomonas aeruginosa* strain (Crowley *et al*., 2002) (Danel *et al*., 1997).

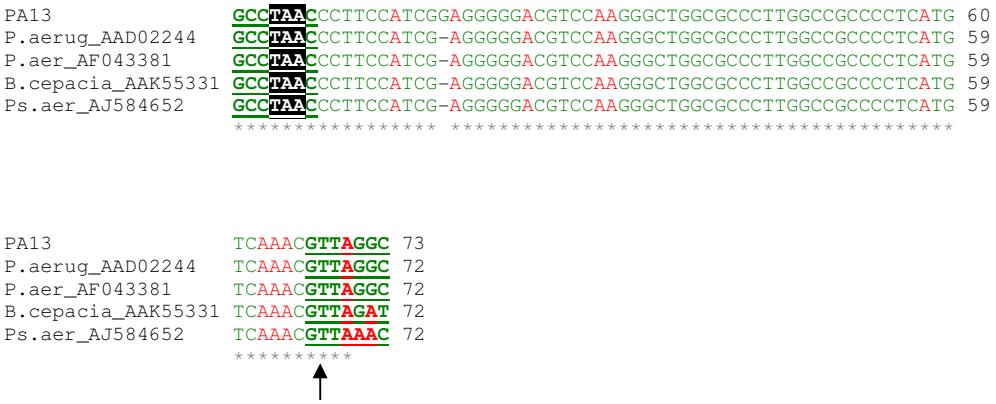

Figure 3.15: Comparison of the *attC* recombination sites (59-base elements) from *aac(6')-Ib* gene cassettes.The compared *attC* sites include the *aac(6')-Ib* gene from *P. aeruginosa* PA13, *P. aeruginosa* (AAD02244), *P. aeruginosa* (AF043381) and *Burkholderia cepacia* (AAK55331) and *P.aeruginosa* (CAE48335). The sequences are shown from the inverse core site to the core site, as they would appear on a circular cassette. The core and inverse core sites are highlighted and underlined. The *aac(6')-Ib* stop codons are in white letters on a black background. The vertical arrow represents the recombination point. Residues that are identical in all strains are underlined with an asterisk.

The *attC* recombination site (59-be) of the *bla-$_{OXA}$* gene cassette in *P. aeruginosa* PA13 was 55 base pairs long and comprises all the elements typical of the *attC* sites (Figure 3.16). The sequence of this *attC* site was compared with the most similar *attC*

site sequences from other integrons containing oxacillinase genes using information in the Genbank database (Figure 3.16). It appears to be the same size and closely related to those of gene cassettes encoding the most similar oxacillinase enzymes (OXA-type enzyme from an unidentified wastewater bacterium [Tennstedt *et al*, 2003], OXA-type enzyme from *B. cepacia* [Crowley *et al*, 2002] and OXA-46 from *P. aeruginosa* [Giuliani *et al*., 2005]) with a sequence identity of 100%, 96% and 90% respectively. They were also consistently divergent in size and sequence from those of the cassettes encoding less similar oxacillinase enzymes.

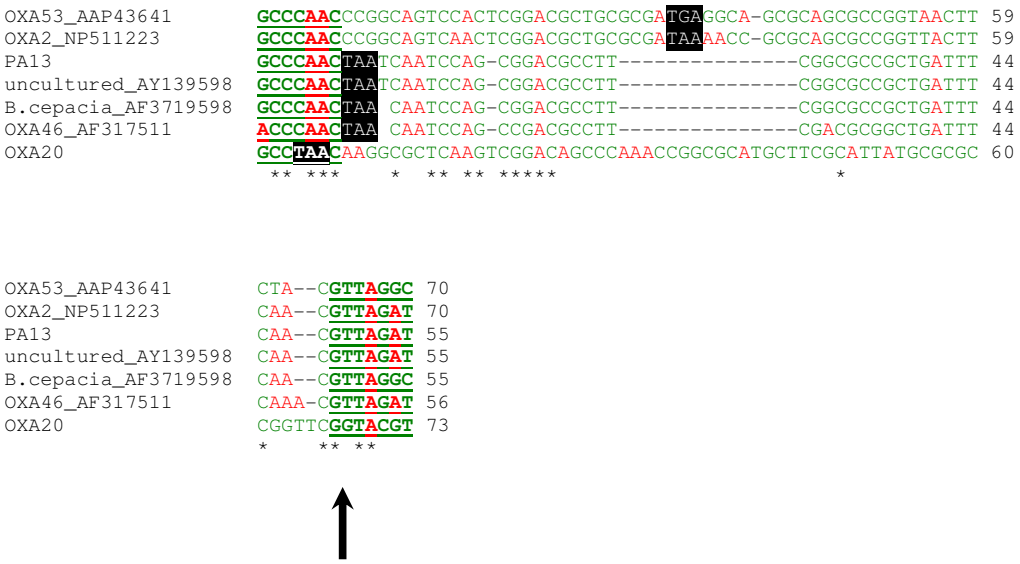

Figure 3.16: Comparison of the *attC* recombination sites (59-base elements) from *bla-$_{OXA}$* gene cassettes. The *attC* sites are from the oxacillinase gene from *P. aeruginosa* PA13 compared to its most similar genes including OXA-53 (AAP43641), OXA-2 (AJ295229), an OXA-type enzyme from unidentified bacterium from a wastewater treatment plant in Germany (AY139598), an OXA-type enzyme from *Burkholderia cepacia* clinical isolate (AF371964), OXA-46 (AF317511) and OXA-20 (AJ319747). The sequences are shown from the inverse core site to the core site, as they would appear on a circular cassette. The core and inverse core sites are highlighted and underlined. The stop codons are in white letters on a black background. The vertical arrow represents the recombination point. Residues that are identical in all strains are underlined with an asterisk.

Promoters

Gene cassettes are generally promoterless. Therefore, most gene cassettes are expressed from a common promoter located in the 5'-CS region of integrons. Two promoters were identified on the integron and are highlighted in Figure 3.6. The first, known as P_{ant} (also known as P_C and P1), was found in the 5' conserved segment 194 bp downstream of the *attI* recombination site and within the integrase gene (Figure 3.6). It had the sequence TTGTTA N_{17} TAAGCT. The second was P2 (TTGTTA/TACAGT). This promoter was found 75 bp downstream of the recombination site (*attI*).

A schematic representation of the Class 1 integron structure from *P. aeruginosa* PA13 is shown in Figure 3.17.

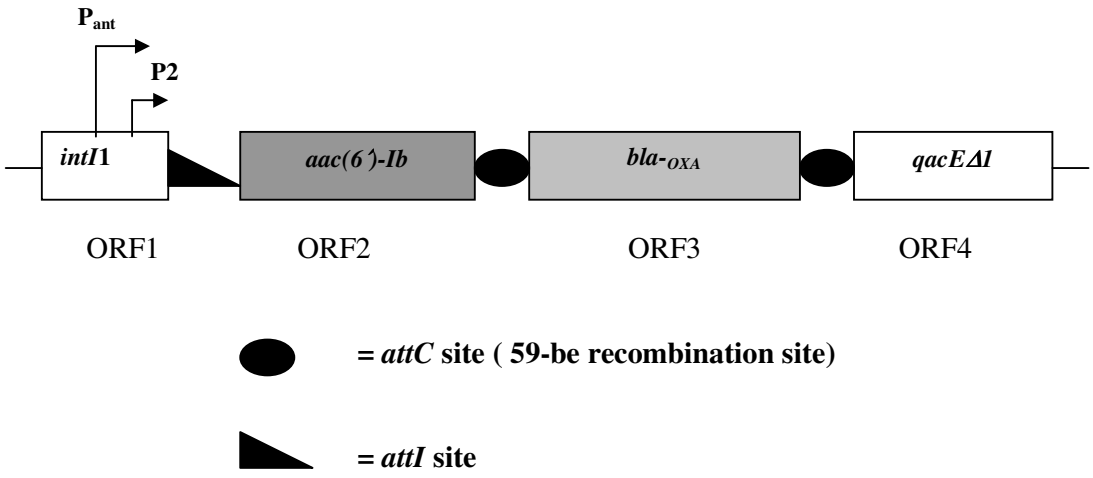

Figure 3.17: A schematic representation of the class 1 integron structure *from P. aeruginosa* PA13. The *intI1* integrase gene, which encodes the integrase, is contained in the 5'-conserved segment. The promoters P_{ant} and P2 are located within the integrase gene. The 3'-conserved segment is found downstream of the integrated gene cassette contains the disinfectant resistance determinant *qacEΔ1*. The inserted gene cassettes, *aac(6')*-Ib and *bla-*$_{OXA}$ are indicated by shaded boxes and the arrows indicate their transcriptional orientation. The *attI* and *attC* recombination sites are represented by the black triangle and circles respectively.

3.4 Investigation of the oxacillinase gene, *bla-$_{OXA}$*, from *P. aeruginosa* PA13

In addition to aminoglycoside resistance, *P. aeruginosa* PA13 was also resistant to many β-lactam antibiotics that are commonly used to treat *P. aeruginosa* infections including penicillins, cephalosporins and penems (Section 3.2.2). The most common mechanism of resistance to β-lactam antibiotics is the production of β-lactamase enzymes (Dajani, 2002). It was of interest to study the oxacillinase (β-lactamase) gene located on the integron from *P. aeruginosa* PA13, in order to characterise it further because unlike the *aac(6')*-Ib gene also found on the integron, this oxacillinase gene had not been previously reported in a *P. aeruginosa* strain. Other similar enzymes have been previously expressed and characterised by cloning the genes encoding them into an expression vector (Girlich *et al*., 2004), (Héritier *et al*., 2005), (Voha *et al*., 2006). In pursuing a method to express the enzyme from *P. aeruginosa* PA13, a number of vectors were available. Two expression vectors were chosen to investigate the oxacillinase gene. One was a commercially available vector, pET-28a (Novagen) and the other was an in-house vector, pPC, from the Laboratory of Dr. Michael O' Connell, DCU.

3.4.1 Studies of the oxacillinase gene using the pET-28a vector

The first vector chosen for the expression of the *bla-$_{OXA}$* gene was the commercially available vector, pET-28a. This vector was chosen because of its strong promoter and because its expression could be tightly controlled. The strategy used for cloning the *bla-$_{OXA}$* gene into the pET-28a expression vector is illustrated in Figure 3.18. The cloning strategy involved: (1) the amplification (by PCR) of the gene using specific primers designed to add restiction sites, *Nco* I and *Xho* I, to the ends of the gene, (2) cloning of the gene into a TA cloning vector and transformation of this construct into *E. coli* XL10 Gold for blue white screening, (3) restriction of the gene at the added restriction sites from the TA vector and excision and purification of the restricted gene from the agarose gel, (4) restriction of the pET-28a vector and treatment of the restricted vector with Antarctic phosphatase, (5) ligation of the restricted gene into the pET-28a vector, (6) transformation of this construct into *E. coli* XL10 Gold for

analysis. The correct orientation of the gene was then checked and finally (7) the transformation of the construct into *E. coli* BL21 (DE3) for induction was performed.

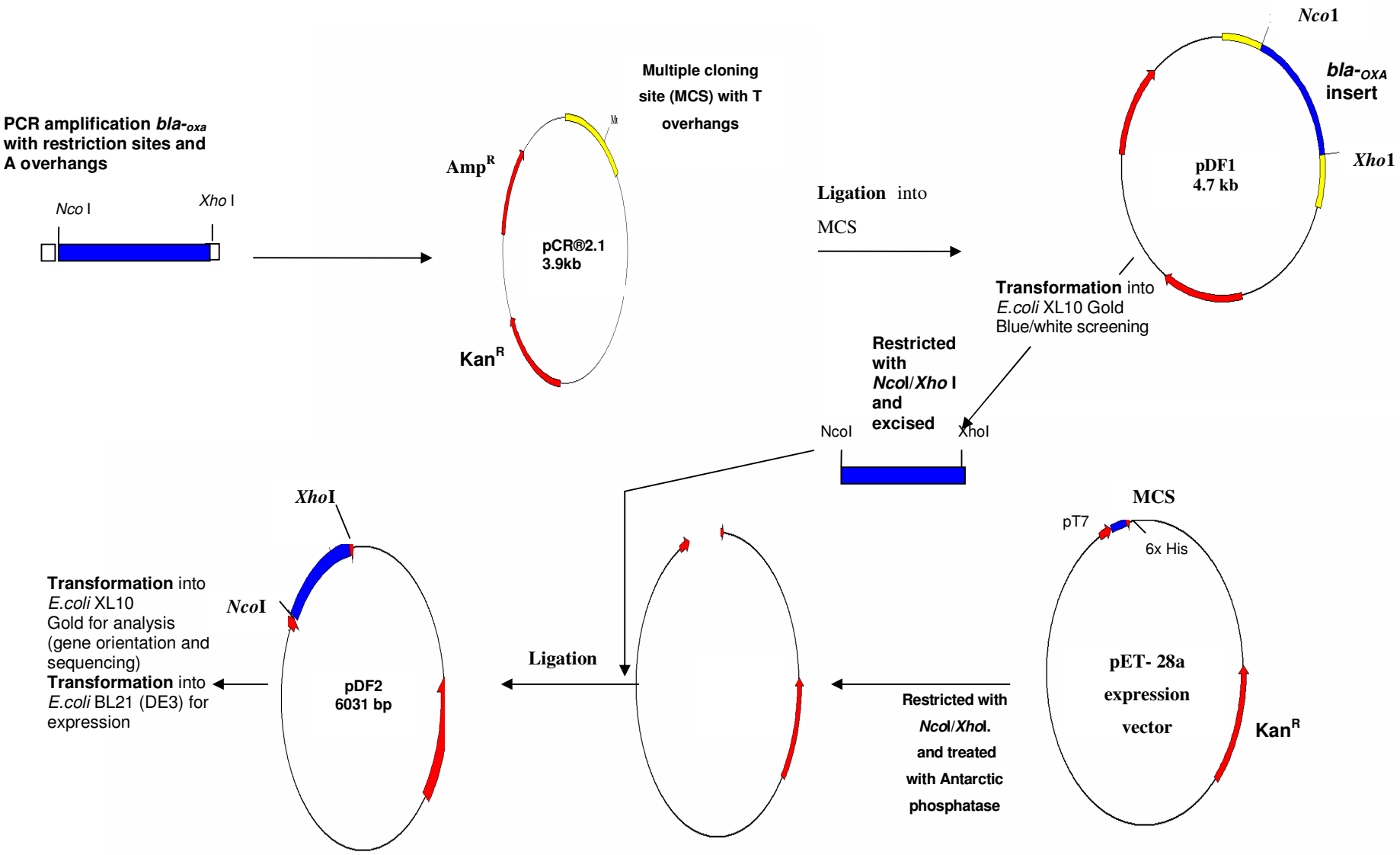

Figure 3.18: Diagram illustrating the steps used for cloning the *bla-$_{OXA}$* gene into the expression vector, pET-28a

3.4.1.1 Amplification of the *bla-$_{OXA}$* using specific primers with *Nco*1 and *Xho*1 restriction sites

The *bla-$_{OXA}$* gene for expression was amplified using the primers in Table 2.13. pETOXA F (forward primer) was designed to include the *Nco*I restriction site at the 5' end of *bla-$_{OXA}$* and pETOXA R (reverse primer) to add an *Xho*I restriction site at the 3' end, replacing the stop codon. An ~800 bp product was amplified and can be seen in Figure 3.19.

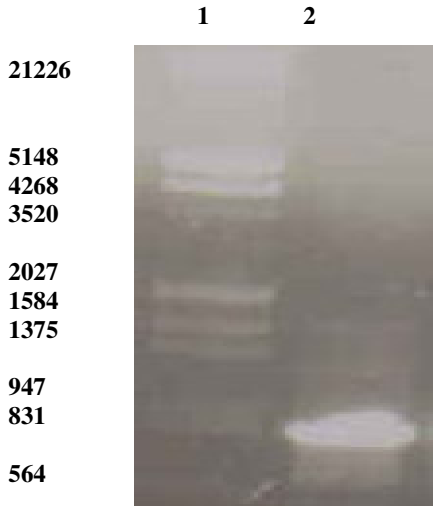

Figure 3.19: Agarose gel showing the ~800bp PCR product amplified from *P. aeruginosa* PA13, used for expression in the pET-28a expression vector. Lane 1 – DNA ladder, Lane 2 - PCR product.

3.4.1.2 Cloning of *bla-oxa* gene into the pCR®2.1 TA cloning vector

TA cloning is one of the most popular methods of cloning an amplified PCR product. When amplified using Taq polymerase, this enzyme adds a single 3'-A overhang to each end of the PCR product. This PCR product can be directly cloned into a linearized cloning vector containing single base 3'-T overhangs on each end. pCR®2.1 (Novagen) is a TA cloning vector. The amplified *bla-*$_{OXA}$ product (Figure 3.19) was first ligated into a pCR®2.1 TA vector. The resulting construct was called pDF1. pDF1 was transformed into *E. coli* XL10 Gold cells. Positive clones were selected by blue/white screening. Plasmids were purified using the Gen Elute Plasmid DNA extraction kit (Sigma). The vector containing the inserted gene (pDF1) can be seen in Figure 3.20.

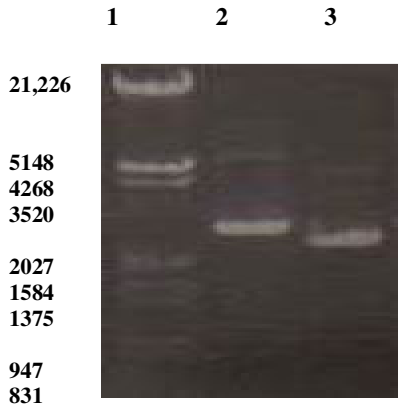

Figure 3.20: Agarose gel showing the pCR®2.1 TA vector containing the inserted *bla-*$_{OXA}$ gene (pDF1). Lane 1 – DNA ladder, Lane 2 - the TA vector containing the inserted gene, Lane 3 - the TA vector without the *bla-*$_{OXA}$ gene.

3.4.1.3 Restriction and excision of the cloned *bla-oxa* gene from pDF1

pDF1 (Figure 3.20) was restricted using the restriction enzymes *Nco* I and *Xho* I, which cut the cloned gene at the resitriction sites added to its 5' and 3' ends respectively. The restriction reaction was run on an agarose gel (Figure 3.21) and the fragment containing the restricted *bla-$_{OXA}$* gene was excised using the protocol outlined in Section 2.16.1.

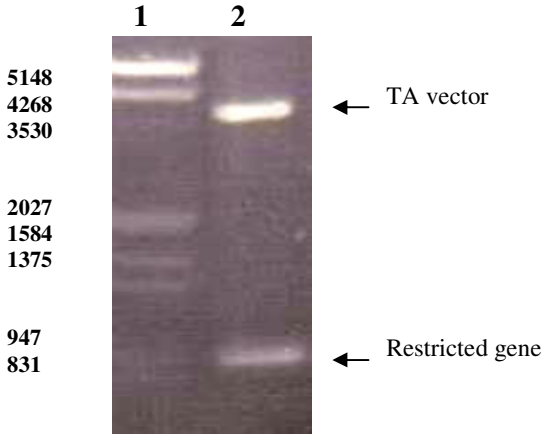

Figure 3.21: Agarose gel showing the excision of the *bla-$_{OXA}$* gene from the pDF1 using *Nco* I and *Xho* I. Lane 1 - DNA ladder, Lane 2 – restricted *bla-$_{OXA}$* gene (lower band) and TA vector (upper band).

3.4.1.4 Cloning of the *bla-$_{OXA}$* gene into the pET-28a expression vector

The pET-28a expression vector was restricted with *Nco* I and *Xho* I in preparation for ligation with the excised *bla-$_{OXA}$* gene. It was then treated with Antarctic phosphatase. Antarctic Phosphatase (New England Biolabs, UK) catalyzes the removal of 5' phosphate groups from DNA. Since phosphatase-treated fragments lack the 5'-phosphoryl termini required by ligases, they cannot self-ligate. This property was used to decrease the vector background when cloning.

The excised *bla-$_{OXA}$* gene (Figure 3.21) was cloned into the pET-28a expression vector using the ligation protocol in Section 2.13.3. The resulting product was named pDF2.

pDF2 was transformed into *E. coli* XL10 Gold cells in order to analyse the construct. Clones were selected for on kanamycin LB plates. Positive clones were screened using the Birnboim and Doly plasmid preparation method described in Section 2.9.2.2. The plasmid from a positive clone can be seen in Figure 3.22. Single and double restriction analysis was performed using the restriction enzymes *Nco* I and *Xho* I to confirm the presence of the gene in the vector. This can be seen in Figure 3.23.

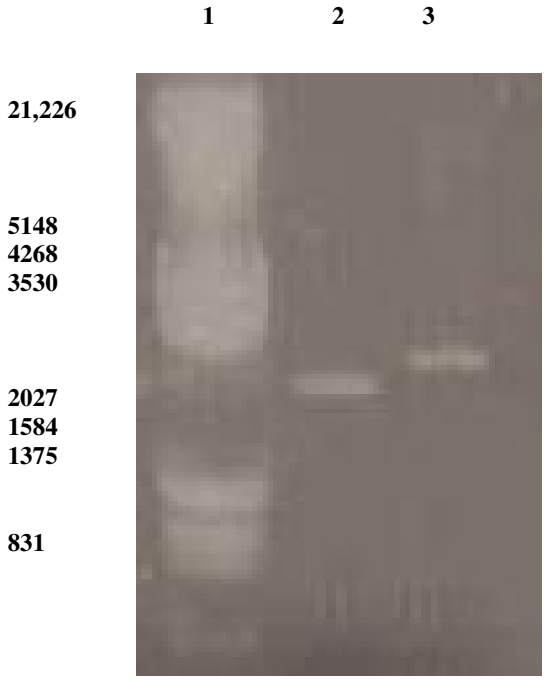

Figure 3.22: Agarose gel showing the plasmid DNA from a positive pDF2 clone. Lane 1 – DNA ladder, Lane 2 - the pET-28a vector without an insert, Lane 3 - the pET-28a with the *bla-$_{OXA}$* gene inserted (pDF2).

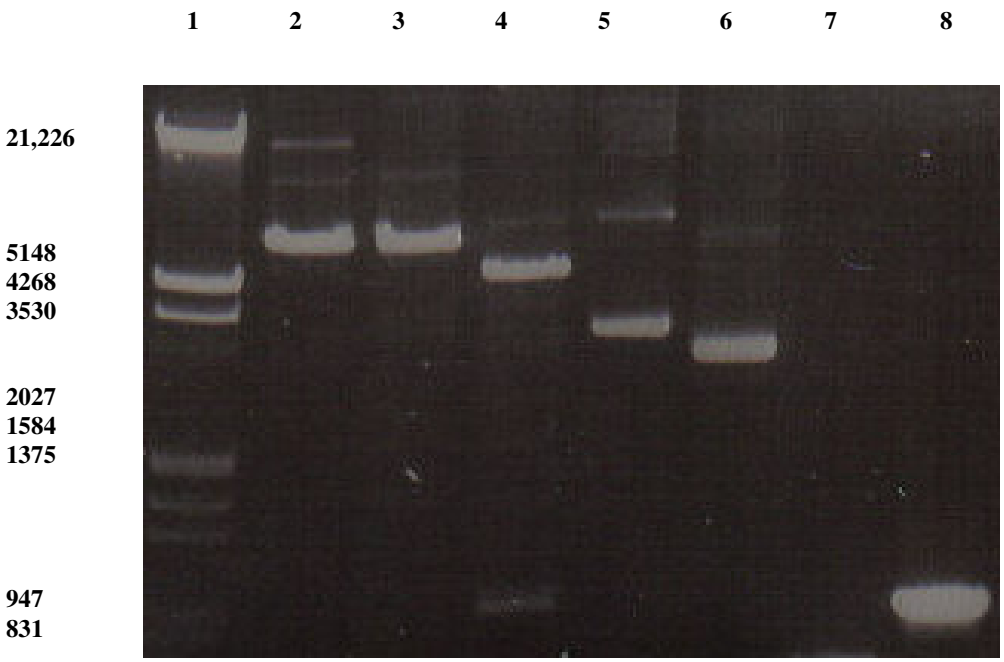

Figure 3.23: Agarose gel showing double and single restriction digest of pDF2. Lane 1 - DNA ladder, Lane 2 - the construct digested with *Nco* I, Lane 3 - the construct digested with *Xho* I, Lane 4 - a double digestion of the construct with *Nco* I and *Xho* I, Lane 5 - the uncut DNA, Lane 6 - the vector with no insert, Lane 7 – empty, Lane 8 - the amplified *bla-*$_{OXA}$ gene.

3.4.1.5 Orientation of oxacillinase gene in pDF2

The correct orientation of the oxacillinase gene in pDF2 was confirmed by restricting the construct with *Bgl* II. There was a *Bgl* II restriction site 107 bp upstream of the *bla-*$_{OXA}$ start codon. There was also a *Bgl* II restriction site 582 bp into the oxacillinase gene. When the gene was in the correct orientation, the restriction reaction produced a 689 bp fragment and ~5.4 kbp fragment. If the gene was in an incorrect orientation the restriction reaction produced a 329 bp fragment and a ~5 kbp fragment. The restriction reaction of the vector with the inserted gene in the correct orientation can be seen in Figure 3.24.

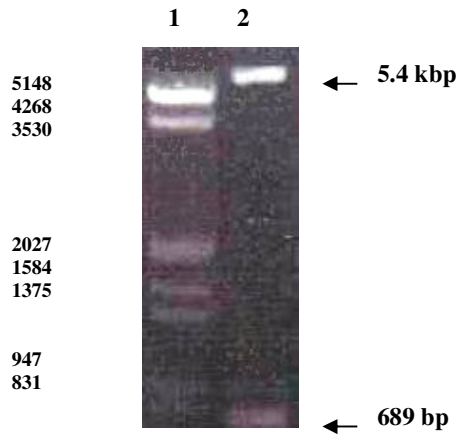

Figure 3.24: Agarose gel of restriction digest of pDF2 with *Bgl* II confirming the correct orientation of the *bla-$_{OXA}$* gene. Lane 1 - DNA ladder, Lane 2 – pDF2 restricted with *Bgl* II.

3.4.1.6 Sequencing of pDF2

Following the analysis of the pDF2 for correct orientation, the construct was sequenced by MWG Biotech using specifically designed primers (Table 2.16). The sequence can be seen in Figure 3.25. The sequence showed that the *bla-$_{OXA}$* gene inserted correctly into the pET-28a expression vector without any mutations. The promoter, ribosome binding site, restriction sites, start codon, His tags, stop codon and resistance gene were all in their correct locations. The positions of the insert gene, restriction sites etc. are illustrated in Figure 3.26.

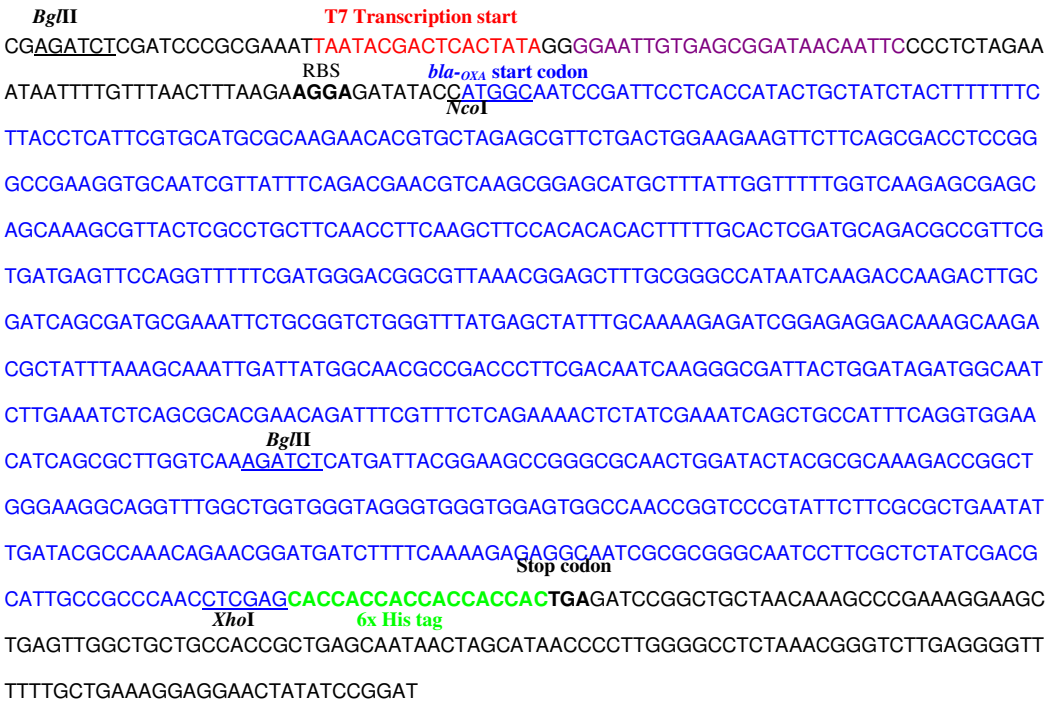

Figure 3.25: The sequence of pDF2. Start and stop codons, restriction sites, RBS, transcription start site and His tag are either highlighted or underlined. The inserted gene is highlighted in blue.

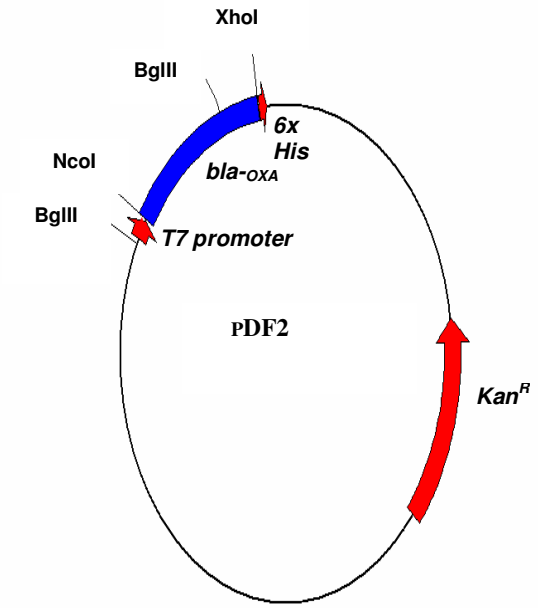

Figure 3.26: pDF2 Plasmid Map. The *bla-$_{OXA}$* gene fragment shown in blue which is under the control of the T7 promoter. His tag fusion and the kanamycin resistance gene (*Kan^R*) are also shown. See Figure 2.4 for map of parent vector pET-28a. Generated using pDRAW32.

3.4.1.7 Induction of pDF2

The sequenced pDF2 was transformed into *E. coli* BL21 (DE3) cells as described in Section 2.14. Target genes cloned in pET plasmids are under the control of a strong bacteriophage T7 transcription. Expression is induced by providing a source of T7 RNA polymerase in the host cell. *E. coli* BL21 (DE3) cells were used to provide a source of T7 RNA polymerase. These cells were induced and their protein was extracted using the protocol in Section 2.21. The extracted proteins (soluble fraction) were separated on a SDS-PAGE gel (Figure 3.27). The predicted 31 kDa (30.97 kDa) oxacillinase protein was not detected. The molecular weight of the putative enzyme was calculated using an online molecular weight calculator

(http://www.sciencegateway.org/tools/proteinmw.htm). The same bands that were identified for the induced cells were also identified in the non-induced cells.

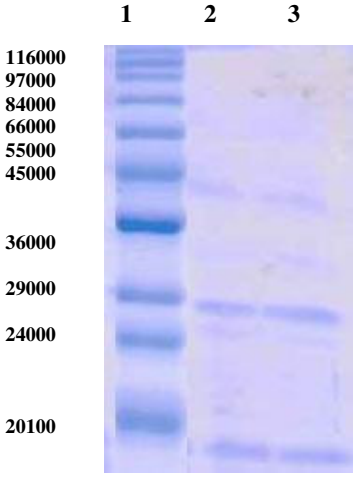

Figure 3.27: SDS-PAGE gel showing protein (soluble fraction) extracted from induced and non-induced *E. coli* BL21 (DE3) cells transformed with pDF2. Lane 1 – wide-range protein marker, Lanes 2 and 3 - proteins from induced and non-induced cells are respectively.

When the protein of interest was not found in the soluble fraction of the gel, the insoluble fraction (pellet) from the induced and non-induced cells was extracted using the protocol in Section 2.21. This fraction would contain the protein if it had been expressed in insoluble inclusion bodies, which sometimes occurs with the overexpression of proteins using T7 promoters. The extracted proteins were separated on a SDS-PAGE gel and can be seen in Figure 3.28. The putative 31 kDa protein was not seen on the gel.

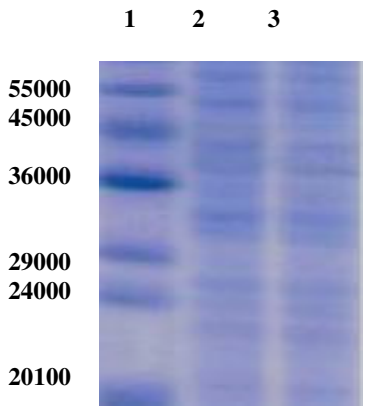

Figure 3.28: SDS-PAGE gel showing the protein (insoluble fraction) extracted from induced and non-induced *E. coli* BL21 (DE3) cells transformed with pDF2. Lane 1 – wide-range protein marker, Lanes 2 and 3 - the proteins from the induced and non-induced cells respectively.

3.4.2 Studies of the oxacillinase gene using the pPC vector

The second expression vector used was an in-house vector called pPC. This is a pQE-60-derived expression vector. It was chosen for the strength of its *tac* promoter and unlike pET vectors it was compatibile with a wide range of host cells.

The strategy used for cloning the *bla-*$_{OXA}$ gene into the pPC expression vector is illustrated in Figure 3.29. The cloning strategy involved: (1) the amplification of the gene using specific primers designed to add restriction sites to the ends of the gene, (2) cloning of the gene into a UA cloning vector (pDrive) and transformation of this construct into *E. coli* XL10 Gold cells, (3) restriction of the gene from the UA vector at the added restriction sites and excision and purification of the restricted gene from an agarose gel, (4) restriction of the pPC vector to remove the 635 bp control insert, excision and purification of the restricted pPC vector from an agarose gel and treatment of the pPC vector with Antarctic phosphatase, (5) insertion of the restricted gene into the pPC expression vector, (6) transformation of this construct into *E. coli* XL10 Gold for analysis checking for the correct orientation of the gene and sequencing and induction.

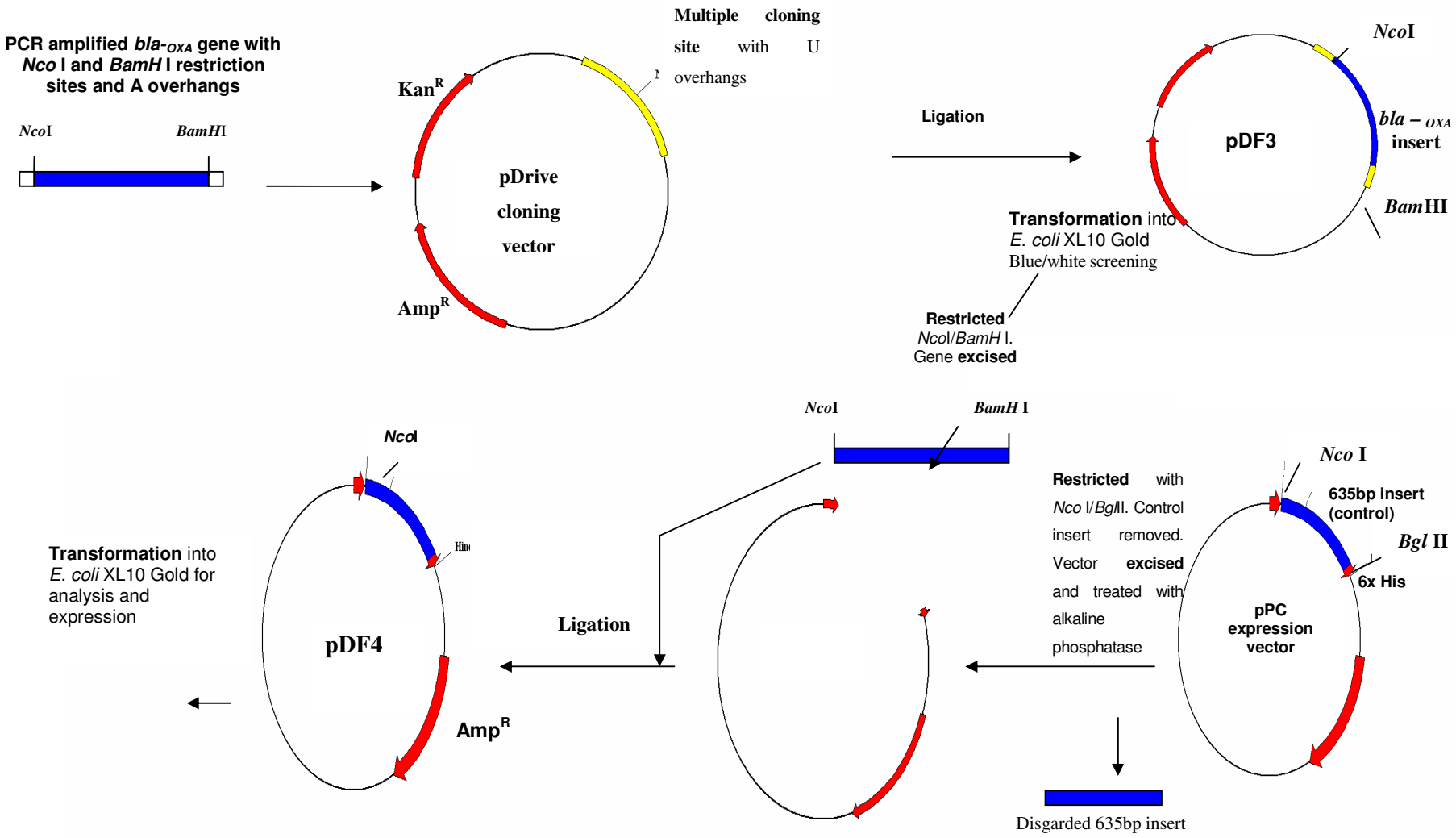

Figure 3.29: Diagram illustrating the cloning strategy for expression in pPC expression vector

3.4.2.1 Amplification of the *bla-$_{OXA}$* gene using specific primers with *Nco*1 and *BamH*1 restriction sites

The *bla-$_{OXA}$* gene for expression was amplified using the primers in Table 2.13. pPCOXA F (forward primer) was designed to include the *Nco* I restriction site at the 5' end of *bla-$_{OXA}$* and pPCOXA R (reverse primer) to add a *BamH* I restriction site at the 3' end, replacing the stop codon. The expected ~800bp product was amplified and can be seen in Figure 3.30.

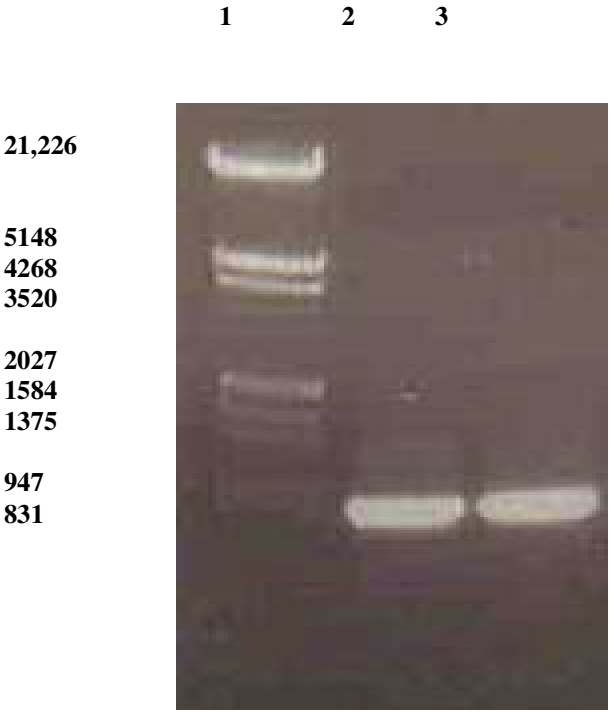

Figure 3.30: Agarose gel showing the ~800bp PCR product amplified from *P. aeruginosa* PA13, used for expression in the pPC expression vector. Lane 1 - DNA ladder, Lanes 2 and 3 – PCR product.

3.4.2.2 Cloning of the *bla-$_{OXA}$* gene into the pDrive cloning vector

The pDrive cloning vector (Qiagen) is a cloning vector containing U-overhangs. As in TA cloning, the PCR product can be directly cloned into a linearized cloning vector containing single base 3'-U overhangs on each end. The amplified *bla-$_{OXA}$* product (Figure 3.30) was first ligated into the pDrive cloning vector. The resulting construct was called pDF3. pDF3 was transformed into *E. coli* XL10 Gold cells. Positive clones were selected by blue/white screening. Plasmids were purified using the Gen Elute Plasmid DNA extraction kit (Sigma) (Section 2.9.2.1). The vector containing the inserted gene can be seen in Figure 3.31.

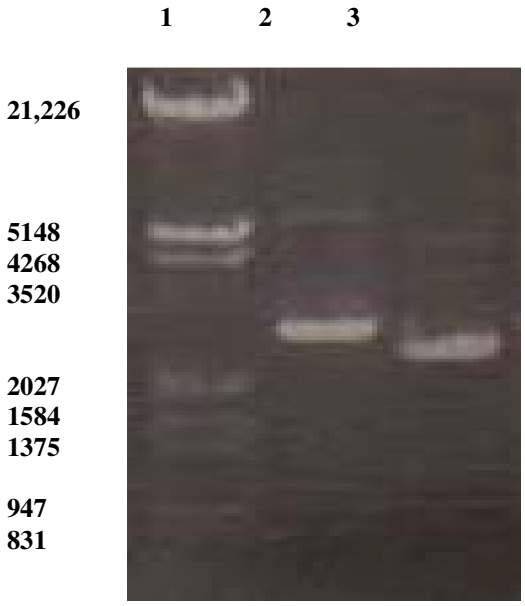

Figure 3.31: An agarose gel showing the pDrive cloning vector with the cloned *bla-$_{OXA}$* gene (pDF3). Lane 1 – DNA ladder, Lane 2 - the cloning vector with the *bla-$_{OXA}$* gene, Lane 3 - vector without the *bla-$_{OXA}$* gene.

3.4.2.3 Restriction and excision of cloned gene from pDF3

The cloned *bla-$_{OXA}$* gene was restricted from the pDF3 using the restriction enzymes *Nco* I and *BamH* I which cut the cloned gene at its 5' and 3' ends respectively. The restriction reaction was run on an agarose gel (Figure 3.32) and the fragment containing the restricted *bla-$_{OXA}$* gene was excised.

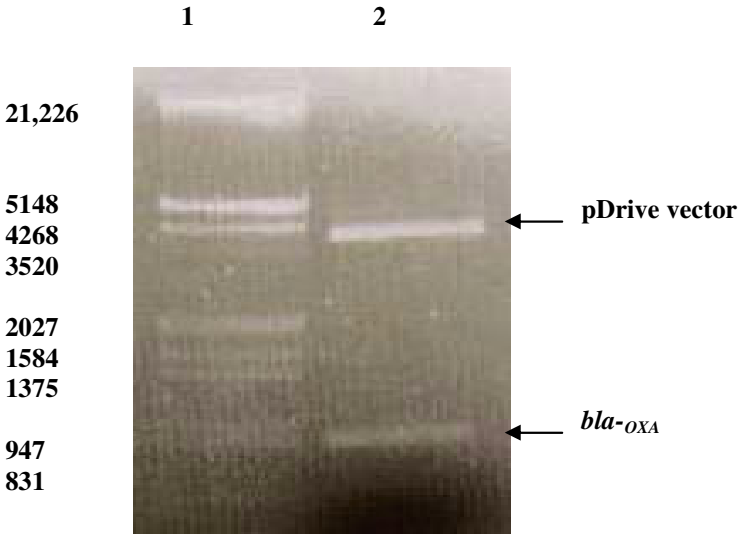

Figure 3.32: Agarose gel showing the restriction of the *bla-$_{OXA}$* gene from the pDF3 vector using *Nco* I and *BamH* I. Lane 1 – DNA ladder, Lane 2 – pDF3 restricted with *Nco* I and *BamH* I.

3.4.2.4 Removal of the 635 bp control insert from the pPC expression vector

The pPC expression vector used contained a 635 bp insert. This vector was used as a control in expression studies. However, in order to clone the *bla-$_{OXA}$* gene into pPC expression vector, it was neccesary to remove the 635 bp insert from the pPC vector. The vector was restricted with *Nco* I and *Bgl* II to remove the 635 bp insert (Figure 3.33). The restricted vector was excised and purified using the protocol in Section 2.16.2. The restricted vector was treated with Antarctic phosphatase to remove the 5' phosphate groups from the DNA and reduce the vector background during cloning.

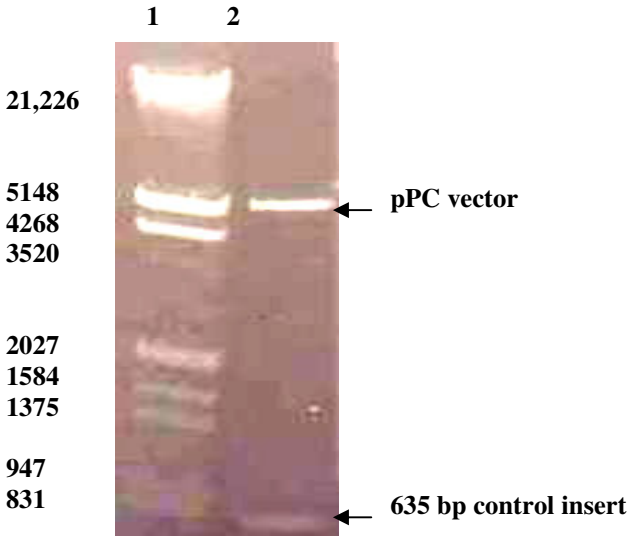

Figure 3.33: An agarose gel of the restriction digest of the pPC vector showing the 635 bp insert restricted with *Nco* I and *Bgl* II. Lane 1 – DNA ladder, Lane 2 – pPC vector restricted with *Nco* I and *Bgl* II.

3.4.2.5 Ligation of the *bla-$_{OXA}$* gene into the pPC expression vector

The excised *bla-$_{OXA}$* gene was ligated into the pPC expression vector. The resulting construct was named pDF4. The ligation reaction was transformed into *E. coli* XL10

Gold cells. Clones were selected for on LB plates containing ampicillin. Positive clones were screened using the Birnboim and Doly plasmid preparation method described in Section 2.9.2.2. The plasmid from a positive clone can be seen in Figure 3.34. Even though the vector was restricted with *Nco*I and *Bgl*II and the *bla-$_{OXA}$* gene was restricted with *Nco*I and *BamH*I, the *BamH*I and *Bgl*II restrictions were compatible with each other. The *Bgl*II enzyme could not have been used as a restriction site at the 3' end of *bla-$_{OXA}$* gene because it contains a *Bgl*II restriction site within it.

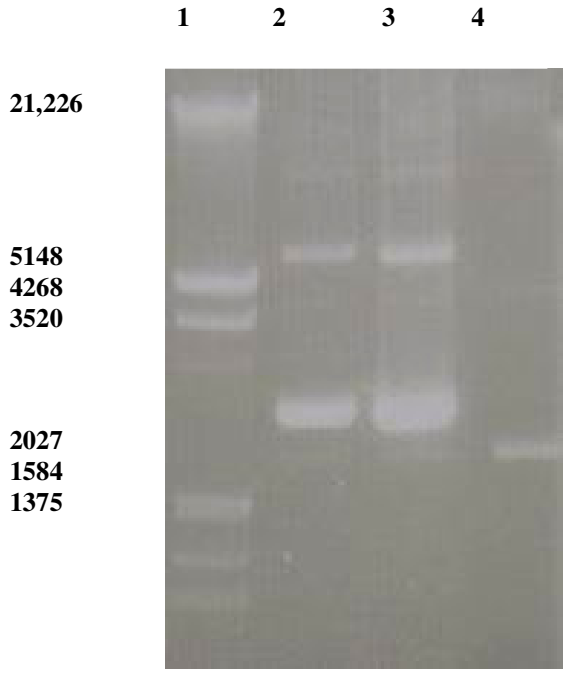

Figure 3.34: Agarose gel showing the plasmids from pPC with the *bla-$_{OXA}$* gene insert (pDF4). Lane 1 – DNA ladder, Lanes 2 and 3 - The pPC vector containing the insert, Lane 4 - the pPC vector without the *bla-$_{OXA}$* gene.

3.3.2.6 Orientation of the *bla-$_{OXA}$* gene in pDF4

The correct orientation of the *bla-$_{OXA}$* gene in pDF4 was confirmed by restricting the construct with *Hind* III. There was a *Hind* III restriction site 333 bp downstream of the *bla-$_{OXA}$* start codon. There was also a *Hind* III restriction site at the stop codon just downstream of the 6X His tag. When the gene was in the correct orientation, the restriction reaction produced a 599 bp fragment and ~4.8 kbp fragment. If the gene was in an incorrect orientation the restriction digest produced a 250 bp fragment and a ~5.2 Kbp fragment. The restriction reaction produced two fragments, 4.8kbp and 599bp (Figure 3.35). Therefore the gene was in the correct orientation in the vector.

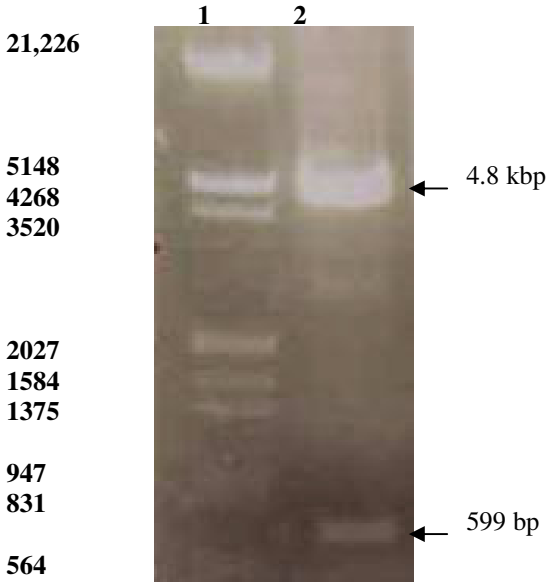

Figure 3.35: Agarose gel showing the restriction digest of pDF4 with *Hind* III to confirm correct orientation of the *bla-$_{OXA}$* gene. Lane 1 – DNA ladder, Lane 2 – pDF4 restricted with *Hind* III.

3.3.2.7 Sequencing of pDF4

Following the analysis of the pDF4 for correct orientation, the construct was sequenced by MWG Biotech using specifically designed primers (Table 2.15). The sequence can be seen in Figure 3.36. The sequence showed that the *bla-$_{OXA}$* gene inserted correctly into the pPC expression vector without any mutations. The promoter, ribosome binding site, restriction sites, start codon, His tags, stop codon and resistance gene were all in their correct locations. The positions of the insert gene, restriction sites etc. are illustrated in Figure 3.37.

Promoter region

-35

-10

transcription start

TCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG

RBS

RBS

Start codon for *bla-$_{OXA}$*

AAACAGAATTCATTAAAGAGGAGAAATTAACCATGGCAATCCGATTCCTCACCATACTGCTATCTACTTTTTTTCTTACC

*Eco*R I

Nco I

TCATTCGTGCATGCGCAAGAACACGTGCTAGAGCGTTCTGACTGGAAGAAGTTCTTCAGCGACCTCCGGGCCGAAGGT

GCAATCGTTATTTCAGACGAACGTCAAGCGGAGCATGCTTTATTGGTTTTTGGTCAAGAGCGAGCAGCAAAGCGTTACT

CGCCTGCTTCAACCTTCAAGCTTCCACACACACTTTTTGCACTCGATGCAGACGCCGTTCGTGATGAGTTCCAGGTTTTT

Hind III

CGATGGGACGGCGTTAAACGGAGCTTTGCGGGCCATAATCAAGACCAAGACTTGCGATCAGCGATGCGAAATTCTGCG

GTCTGGGTTTATGAGCTATTTGCAAAAGAGATCGGAGAGGACAAAGCAAGACGCTATTTAAAGCAAATTGATTATGGC

AACGCCGACCCTTCGACAATCAAGGGCGATTACTGGATAGATGGCAATCTTGAAATCTCAGCGCACGAACAGATTTCG

TTTCTCAGAAAACTCTATCGAAATCAGCTGCCATTTCAGGTGGAACATCAGCGCTTGGTCAAAGATCTCATGATTACGG

AAGCCGGGCGCAACTGGATACTACGCGCAAAGACCGGCTGGGAAGGCAGGTTTGGCTGGTGGGTAGGGTGGGTGGAG

TGGCCAACCGGTCCCGTATTCTTCGCACTGAATATTGATACGCCAAACAGAACGGATGATCTTTTCAAAAGAGAGGCAA

TCGCGCGGGCAATCCTTCGCTCTATCGACGCATTGCCGCCCAGGATCTCATCACCATCACCATCACTAAGCTTCTGTTT

Gly-Ser

6xHis tag

Stop codon

TGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAA

Hind III

Figure 3.36: The sequence of the pDF4. Start and stop codons, restriction sites, RBS, transcription start site and His tag are either highlighted or underlined. The inserted gene is highlighted in blue.

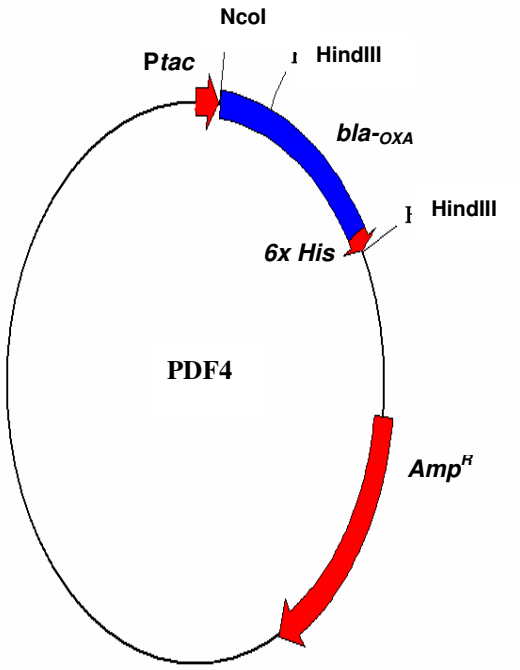

Figure 3.37: pDF4 Plasmid Map. The *bla-*$_{OXA}$ gene fragment insert is shown in blue which is under the control of the P*tac* promoter. His tag fusion and ampicillin resistance gene (*amp*^{R}) are also shown. See Figure 2.5 for map of parent vector. Generated using pDraw32.

3.3.2.8 Induction of pDF4 in *E. coli* XL10-Gold

After sequencing, pDF4 transformed *E. coli* XL-10 Gold cells were induced and their proteins were extracted using the protocol in Section 2.21. The extracted proteins were separated on a SDS-PAGE gel (Figure 3.38). Although some bands were identified, none of them corresponded to the putative 31 kDa *bla-*$_{OXA}$ protein. The same bands that were identified for the induced cells were also identified in the non-induced cells. Successful induction was seen with the control vector, pPC, which expressed a 23 kDa protein.

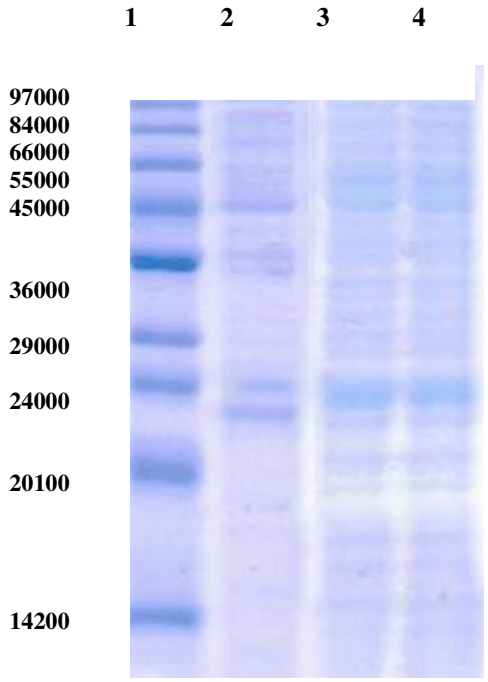

Figure 3.38: SDS-PAGE gel showing protein extracted from induced and non-induced *E. coli* XL10 Gold cells transformed with pDF4. Lane 1 – wide-range protein marker, Lane 2 - the 23 kDa protein induced in the control strain, Lanes 3 and 4 - the proteins from induced and non-induced respectively.

3.3.2.9 Induction of pDF4 in *E. coli* XL10-Gold at 28°C

When genes from one organism are expressed in another inclusion bodies are sometimes formed. Inclusion bodies are major protein aggregates, commonly occurring in recombinant bacteria upon targeted gene overexpression (Carrio *et al*., 2000). To reduce the expression of proteins in inclusion bodies the *E. coli* XL-10 Gold cells were induced using gentler culture conditions. The cells were grown at 28°C and shaken at 150 rpm. The extracted proteins from both the soluble and insoluble fractions of the induced and non-induced cultures were separated on SDS-PAGE gels (Figure 3.39 and 3.40 respectively). Even with the gentler culture conditions, the predicted 31 kDa protein was not expressed by the *E. coli* XL-10 Gold cells transformed with pDF4. The 23 kDa protein of the contol vector was expressed.

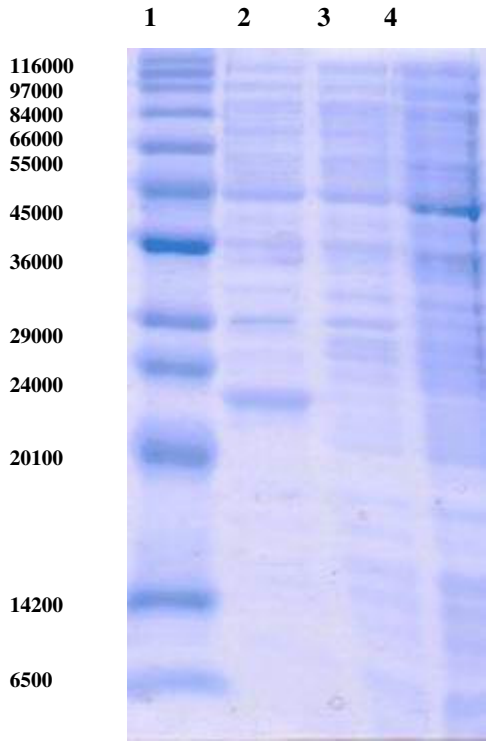

Figure 3.39: SDS-PAGE gel showing protein extracted from induced and non-induced *E. coli* XL10 Gold cells transformed with pDF4 cultivated at 28°C and 150 rpm. Lane 1 – wide-range protein marker, Lane 2 - the 23 kDa protein induced in the control strain, Lane 3 and 4 - the proteins from induced and non-induced cells respectively.

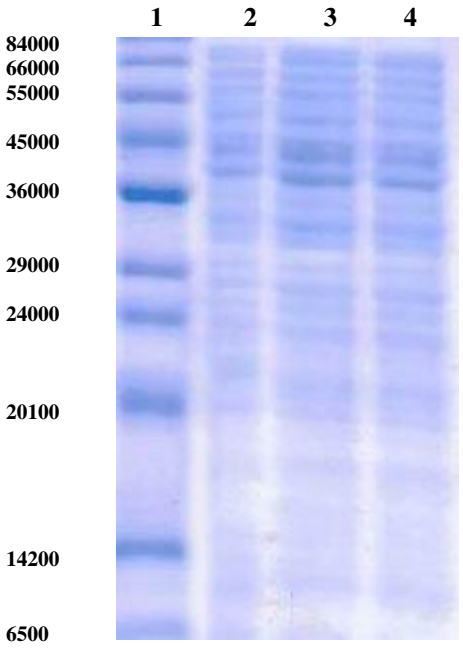

Figure 3.40: SDS-PAGE gel showing protein (insoluble fraction) extracted from induced and non-induced *E. coli* XL10 Gold cells transformed with pDF4 which were grown at 28°C and 150 rpm. Lane 1 – wide-range protein marker, Lane 2 - the proteins from the insoluble fraction of the control strain, Lanes 3 and 4 - the proteins from the induced and non-induced cells respectively.

3.3.2.10 Induction of pDF4 in *E. coli* RosettaBlue™

A subset of codons, namely AGG/AGA, CGA/CGG, AUA, CUA, GGA and CCC are rarely expressed in *E. coli* and appear to cause problems from a translational point of view. If the recombinant protein being expressed contains several of these rare codons the protein may not be expressed due to this translational limitation. The *E. coli* RosettaBlue™ strain (Novagen) has been engineered to provide the tRNAs for these rarely expressed codons on a chloramphenicol resistant plasmid, pRARE.

Thirteen rare codons were identified in the *bla-$_{OXA}$* gene. pDF4 was transformed into *E. coli* RosettaBlue™ cells. These transformants were induced and their proteins were

extracted. The extracted proteins were separated on a SDS-PAGE gel (Figure 3.41). None of the proteins on the SDS-PAGE gel corresponded to the predicted 31 kDa *bla-$_{OXA}$* protein. The same bands that were identified for the induced cells were also identified in the non-induced cells. Successful induction was seen with the control vector, pPC, which expressed a 23 kDa protein.

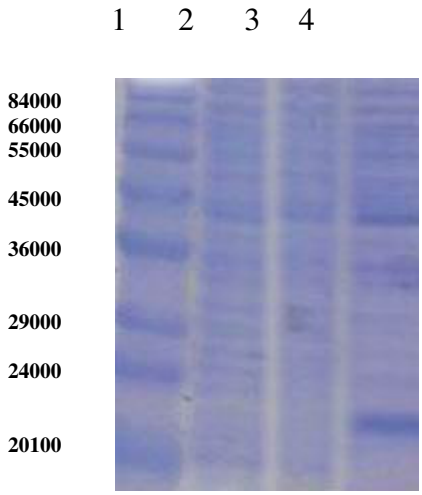

Figure 3.41: An SDS-PAGE gel showing proteins extracted from the soluble fraction from the induced and non-induced *E. coli* RosettaBlue^{TM} cells transformed with pDF4. Lane 1- wide-range DNA marker, Lanes 2 and 3 - the proteins from induced and non-induced cells respectively, Lane 4 - the 23 kDa protein induced in the control strain

When the protein of interest was not found in the soluble fraction of the gel, the insoluble fraction (pellet) from the induced and non-induced cells was extracted using the protocol in Section 2.21. This fraction would contain the protein if it had been expressed in insoluble inclusion bodies. The predicted 31 kDa *bla-$_{OXA}$* protein was not expressed in the cell (Figure 3.42).

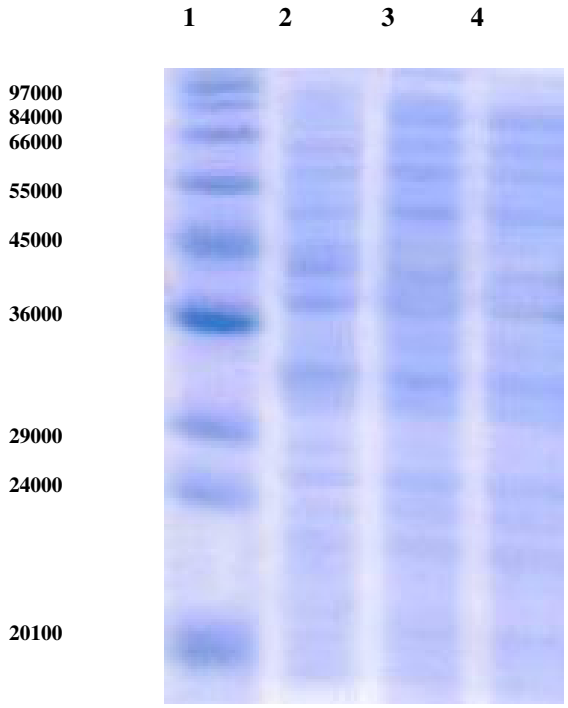

Figure 3.42: An SDS-PAGE gel showing proteins extracted from insoluble fraction from both the induced and non-induced *E. coli* RosettaBlue^{TM} cells transformed with pDF4. Lane 1 - the wide-range protein marker, Lane 2 – the proteins produced in the control strain, Lanes 3 and 4 - the proteins from induced and non-induced cells respectively.

4.0 Discussion

One of the most fundamental aspects of microbiology is the identification of bacterial species. Differential biochemical profiling and molecular methods such as the analysis of 16S rRNA genes are among the best available techniques, which can be utilised to both detect and provide epidemiological analysis of bacteria (Boettger, 1996), (Drancourt *et al*., 2000), (Woo *et al*., 2000), (Clarridge III, 2004).

Analytical techniques for identification of microorganisms have evolved due to the advancement of molecular diagnostics. These techniques particularly combined with conventional analyses have enabled rapid and definitive identification of unknown isolates (Wiedmann *et al*., 2000). These techniques were employed to identify the clinical isolates obtained from both hospital environments.

The identification of bacteria in the clinical microbiology laboratory was traditionally performed by isolating the organism and studying it phenotypically by means of Gram staining, culture, and biochemical methods, which were once the gold standard of bacterial identification (Woo *et al*., 2000). The phenotypic and morphological characteristics of the isolates were consistent with the description of typical Pseudomonads according to Cowan and Steel's manual for the identification of medical bacteria (1993) and Bergey's Manual for Systematic Bacteriology (2001) which define the genus as being Gram-negative, non spore-forming, motile, unicellular rods, with the long axis straight or slightly curved. All of the isolates had these characteristics. The cell characteristics of the isolates were also consistent with those of Pseudomonads. All of the colonies produced large non-mucoid colonies, with the exception of *P. aeruginosa* PA12, which produced large mucoid colonies. This colony type, which is often obtained from respiratory and urinary tract infections, has a mucoid appearance that is attributed to the production of alginate slime (Todar, 2004). The mucoid colonies are presumed to play a role in colonisation and virulence (Bergey's Manual for Systematic Bacteriology, 2001).

One classic result shared by all the isolates including the reference strain *P. aeruginosa* PAO1 is the positive reaction for oxidase. This is due to the presence of cytochrome c oxidase in the electron transport chain. Cytochrome oxidase is an enzyme found in some bacteria that transfers electrons to oxygen, the final acceptor in some electron transport chains (Michel *et al*., 1989). This is one of the distinguishing characteristics that differentiate the pseudomonads from enteric bacteria (Hampton and Wasilauskas, 1979). All of the isolates were also catalase positive, which is typical of *Pseudomonas* strains (Bergey's Manual for Systematic Bacteriology, 2001). There are other physiological properties that are common to all species of the genus *Pseudomonas*. These include aerobic metabolism, chemoorganotrophic nutrition, the absence of fermentation, and the capacity to grow using a large variety of organic substrates (Bergey's Manual for Systematic Bacteriology, 2001). The division of bacteria into fermenters and oxidisers is one of the most heavily weighted primary tests used for bacterial identification. All the isolates were aerobic and oxidizers i.e., they attacked carbohydrates by oxidation, which is consistent with results expected for *P. aeruginosa* strains according to Bergey's Manual for Systematic Bacteriology (2001).

Pseudomonas aeruginosa is the type species of the genus *Pseudomonas* (Bergey's Manual for Systematic Bacteriology, 2001). It can be preliminarily identified from its grape-like odour (aminoacetophenone) *in vitro* (Samer, 2005). Definitive clinical identification includes identifying the production of the pigments pyocyanin and fluorescein, β-haemolysis on blood agar and also its ability to grow at 42°C (Figure 3.3) (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993). All of the isolates were tested for these characteristics and the results obtained were consistent with those for *Pseudomonas aeruginosa*.

Pigment production is a contributory phenotypic characteristic in the classification of *P. aeruginosa* (Bergey's Manual for Systematic Bacteriology, 2001). *Pseudomonas aeruginosa* has an innate ability to produce specific fluorescent phenazine pigments, genetically encoded by two operons for the production of metabolites such as pyocyanin (blue-green) (Kanner *et al*., 1978), (Mavrodi *et al*., 2001), pyoverdin or fluorescein

(greenish-yellow), pyomelanin (red-brown) (Yabuuchi and Ohyama, 1972) and pyorubin (red) (Kandela *et al*., 1997).

Growth medium has the biggest effect on the development of pigment. Various media are required to encourage different pigment production by pseudomonads. On chemically defined media, the pigments may exhibit a wide variety of colours depending on the carbon source used for growth (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

Nine of the twelve isolates studied were found to produce the phenazine pigment, pyocyanin, when grown on *Pseudomonas* isolation agar P, a medium that stimulates the production of this pigment (Table 3.4). Pyocyanin is a low molecular weight, water- and chloroform-soluble, non-fluorescent, blue pigment characteristically produced by more than half of all clinical isolates of *P. aeruginosa* (Samer, 2005). This redox-active secondary metabolite is synthesized from chromate due to the action of the *phzABCDEFG* operon and is regulated by quorum sensing (Mavrodi *et al*., 2001), (Fuqua *et al*., 2001). Production of pyocyanin enhances the virulence of *P. aeruginosa* (O'Malley *et al*., 2004). The pigment exerts a proinflammatory effect on phagocytes, impairs the normal function of the human nasal cilia and inhibits the proliferation of human epidermis and lymphocytes. This is probably due to inhibition of electron transport (Smeal *et al*., 1987). Pyocyanins have been reported to have antifungal properties that give *P. aeruginosa* an obvious selective advantage to these organisms in their natural environment (Kaleli *et al*., 2006), (Kerr *et al*., 1999). It also induces rapid apoptosis of human neutrophils (Allen *et al*., 2005), directly oxidises glutathione and decreases its levels in airway epithelial cells (O'Malley *et al*., 2004). Human cells possess several key mechanisms to limit their exposure to reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide. The thiol compound glutathione is one of the major components of cellular antioxidant defences (Dickenson and Forman, 2002). When cells are exposed to reactive oxygen species, reduced glutathione is oxidised to a dimer by the action of glutathione peroxidase. This dimer is then reduced back to reduced glutathione by glutathione reductase. This cycling of glutathione is thought to be an important means of

limiting cellular exposure to and cytotoxicity from hydrogen peroxide (Griffith, 1999). Pyocyanin directly accepts electrons from either NADH or NADPH. Under aerobic conditions it passes those electrons to O_2, leading to the generation of reactive oxygen species. Therefore, the addition of pyocyanin to cellular systems places them under increased oxidative stress (Hassan and Fridovich, 1980).

Significantly, pyocyanin has been identified in sputum samples from patients with chronic pulmonary infections, especially CF patients (Lau *et al*., 2004), (Lee *et al*., 2005), and thus is considered to be an infection-associated virulence factor. *Pseudomonas aeruginosa* is the only known organism of the pseudomonads and other glucose fermenting Gram-negative bacteria capable of producing pyocyanin (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993). Although some *Streptomyces* species can produce cyanomycin, which is said to be identical to pyocyanin, the colonial and Gram stain morphologies of the *Streptomyces* is radically different to that of *P. aeruginosa* (Alberto Pichardo Reyes, 1981).

All thirteen clinical isolates produced pyoverdin, also known as fluorescein, when grown on *Pseudomonas* isolation agar F, a medium that stimulates the production of this pigment (Table 3.4). Pyoverdin is a yellow-green pigment that fluoresces when exposed to ultra-violet light (254 nm). This characteristic can be used to detect *P. aeruginosa* infection in burn patients. Pyoverdin, which is encoded by the *pvd* genes (Lamont and Martin, 2003), acts as a siderophore, involved in a complex iron acquisition system tightly binding and transporting soluble iron (Fe III) from the environment under iron-deficient conditions. Although iron is an essential nutrient for most bacteria, the low solubility and bioavailability of this element in nature complicates bacterial iron acquisition. Many bacteria deal with this by producing high affinity iron-chelating molecules known as siderophores, which transport iron into the cell (Stintzi *et al*., 1999). No role in virulence is known for pyoverdin (Thi-Dao *et al*., 1999). Although it is characteristic of *P. aeruginosa*, pyoverdin also is produced by three other pseudomonads, *P. putida*, *P. fluorescens* and *P. chlororaphis*, so it cannot be used as a definitive test for *P. aeruginosa* (Bergey's Manual of Systematic Bacteriology, 2001).

Strain *P. aeruginosa* PA9 was the only strain that produced a brown pigment thought to be pyomelanin (Table 3.4). Pyomelanin is another phenazine pigment. This pigment, in common with other melanins, is produced from aromatic amino acids such as tyrosine or phenylalanine (Sanchez-Amat *et al*., 1998). This pigment is not commonly produced, but where it is, it is usually associated with isolates from urinary tract infections (Yabuuchi and Ohyama, 1972) (Ogunnariwo and Hamilton-Miller, 1975) and patients in burn units (De Vos *et al*., 1997), (Masahisa *et al*., 2005). Pyomelanin production is due to extracellular accumulation and polymerisation of homogentisate (Hegedus, 2000). The function of pyomelanin is unknown but it is thought to confer benefits including protection against oxidative stress (Nosanchuk and Casadevall, 2003). A potential side effect of this pigment on human hosts is tissue inflammation (Hegedus, 2000).

A red pigment was produced in *P. aeruginosa* PA3, PA11 and PA12 (Table 3.4). This pigment is thought to be pyorubin. Pyorubin has been reported to have antimicrobial activity, which would give the strain producing it a selective advantage over other microbes in its natural environment (Kandela *et al*., 1997). Like other phenazines, pyorubin is also believed to be involved in protection of the organism against oxidative stress (Nosanchuk and Casadevall, 2003).

Pseudomonas aeruginosa strains are typically β-haemolytic on blood agar plates (Alberto Pichardo Reyes, 1981). There are three possible outcomes from the test for haemolysis on blood agar. The first is known as α-haemolysis. This can be identified from a green envelope that surrounds intact cells. The second is known as β-haemolysis. This can be identified from the clear colourless zones that surround the cells where the blood cells have been completely lysed. Finally, there is γ-haemolysis. This describes a negative result for haemolysis where there has been no action on the red blood cells (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993). All of the *P. aeruginosa* isolates were β-haemolytic on blood agar (Table 3.3).

Haemolysins are just one of the many virulence factors with which *P. aeruginosa* is equipped (Wilson and Dowling, 1998). Haemolysins contribute to host invasion through their cytotoxic effects on eukaryotic cells. Their normal function is to lyse red blood cells (Beecher and Wong, 2000). *P. aeruginosa* produces two main haemolysins that are involved in cell invasion. One is a phospholipase and the other is a lecithinase. They appear to act synergistically to break down lipids and lecithin (Liu, 1976). Several other types of haemolysins have been described. A heat-stable haemolytic glycopeptide consisting of two molecules each of L-rhamnose and 1-b-hydroxydecenoic acid has been identified that is not toxic to human cells but which is toxic to alveolar macrophages. It has also been discovered that *P. aeruginosa* strains isolated from respiratory tract infections produce more haemolysin than other strains, which suggests that haemolysin may play an important role in *P. aeruginosa* pulmonary infections (Iglewski, 1996). A heat-labile haemolysin has been described that is very similar to another *P. aeruginosa* virulence factor, Phospholipase C, which hydrolyses lecithin (Iglewski, 1996).

The temperature range for growth and the optimal temperature for growth are characteristic of different bacteria (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993). The optimal temperature for medically important bacteria is usually between 35-40°C. All of the isolates grew abundantly at 42°C but could not grow at 5°C (Table 3.3). The ability to grow at 42°C is one of the defining characteristics of *P. aeruginosa*. Other strains of fluorescent pseudomonads such as *P. putida* and *P. fluorescens* are incapable of growth at this temperature. However, *P. fluorescens* and *P. putida* are able to grow at 5°C, also differentiating them from *P. aeruginosa* (Bergey's Manual of Systematic Bacteriology, 2001).

Cetrimide (cetyltrimethylammonium bromide) agar is a selective medium for *Pseudomonas aeruginosa*. It is a modification of Tech agar, which was developed by King *et al*. (1954) that improved pyocyanin production by *Pseudomonas* species. The selectivity of the medium is due to the presence of cetrimide. Cetrimide acts as a quaternary ammonium cationic detergent causing nitrogen and phosphorus to be released from bacterial cells other than *Pseudomonas aeruginosa*. According to Lowbury and

Collins (1955), a cetrimide concentration of 0.3 g/litre inhibits the accompanying organisms satisfactorily and minimizes interference with the growth of *Pseudomonas aeruginosa*. However, *P. putida* and *P. fluorescens* can show a small amount of growth in the presence of cetrimide (Brown and Lowbury, 1965). So, to guarantee that only *Pseudomonas aeruginosa* strains grew on cetrimide agar, 15 µg/ml of the antibiotic nalidixic acid was added to the medium as was recommended by Goto and Enomoto (1970). The medium also contains magnesium chloride and potassium sulphate, which are cationic salts that act as activators and co-activators to intensify the luminescence of pyocyanin and fluorescein. All the strains were found to grow on cetrimide agar with nalidixic acid and produced fluorescein (Table 3.3).

Tween 80 is the oleic acid ester of a polyoxyalkylene derivative of sorbitan. It was added to nutrient medium. Opaque haloes were identified around the growth of each strain. This opacity is due to crystal formation, which indicates lipolytic activity. Lipase production is a useful characteristic in identifying *Pseudomonas aeruginosa* (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993). The isolates were positive for lipase production (Table 3.3). This characteristic differentiates *Pseudomonas aeruginosa* from similar species such as *P. fluorescens* and *P. putida*, which are negative for lipase production. (Bergey's Manual of Systematic Bacteriology, 2001).

The utilization of the amino acid arginine by the pseudomonads has attracted the interest of microbiologists for many years. The degradative pathway, the arginine dihydrolysed pathway system, has been used for differentiating species (Bergey's Manual of Systematic Bacteriology, 2001). The reactions catalysed by this system are the conversion of arginine to citrulline and of citrulline to ornithine with liberation of ammonia (Slade, 1954). A rise in the pH of the medium with arginine degradation, because of ammonia liberation, indicates the presence of the dihydrolase system (Thornley, 1960). Not all species of *Pseudomonas* possess arginine dihydrolyase (Bergey's Manual of Systematic Bacteriology, 2001). All of the isolates tested were positive for this enzyme (Table 3.3). Although *P. aeruginosa* is positive for arginine dihydrolase, this characteristic cannot be used as a definitive characteristic of the species

because other species such as *P. fluorescens* and *P. putida* possess this enzyme. However, it does differentiate the species from other pseudomonads such as *P. stutzeri* and *P. cichorii*.

Molecular methods offer the most precise source identification, but are limited by expense, detailed and time-consuming procedures, and are not yet suitable for assaying large numbers of samples in a reasonable time frame. Biochemical methods, for example, nutritional patterning (BIOLOG, API, Miniteck) and fatty acid methyl ester analysis (MIDI) are simpler, quicker, less costly and allow numbers of samples to be assayed in a short period of time (Mandaville, 2002). The commercial identification systems API 20NE and Biolog were used to confirm the identification of the strains. The Biolog system in particular was also useful in identifying the substrates on which the isolated strains could grow. The API 20NE system consists of twenty-one enzymatic and carbon compound assimilation tests which were performed in cupules on a plastic strip where desiccated contents were reconstituted with a suspension of the test organism. Some tests had to be overlaid with mineral oil to obtain the correct gaseous conditions. Results were available in 24-48 hours and were represented as a seven-digit profile number, which could be read from the Analytical Profile Index. API 20NE provides a quick and simple identification system that is capable of correctly identifying the majority of *Pseudomonas* species. The isolates were identified as *Pseudomonas aeruginosa* strains by the API20NE kit, with identifications ranging from good to excellent and % i.d. ranging from 91.4% to 99.99% (Table 3.5). Studies carried out by Costas *et al.* (1992) showed the correct identification of 90.4 % of 146 *Pseudomonas* strains used. 5.5 % were not identified, while 4.1 % were incorrectly identified.

The Biolog identification system establishes identification based on the exchange of electrons generated during respiration, leading to tetrazolium-based colour changes. It tests the ability of organisms to oxidize a panel of 95 different carbon sources. The Biolog GN system identified all the isolates with 99-100% probability of being *Pseudomonas aeruginosa* (Figure 3.6). Studies carried out by Costas *et al*. (1992) showed the correct identification of between 74 % and 79 % of 114 *Pseudomonas* or

Pseudomonas-like species depending on whether results were read using an automated plate reader or read manually (manual reading was more accurate). The application of API 20NE and Biolog GN identification systems in combination with a range of biochemical tests provided good to excellent identification of all the strains as being *Pseudomonas aeruginosa* and also showed that the strains differed in their biochemical characteristics. However, the high rate of incorrect identifications with both the API 20NE and the Biolog systems GN (Costas *et al*., 1992) showed that there was a need for a more precise method of identification.

Pseudomonads are nutritionally versatile, and can use a wide range of simple organic compounds as sole sources of carbon and energy (Palleroni, 1986). *Pseudomonas aeruginosa* can utilize over 80 organic compounds for growth, giving it an important role in nature (Wick *et al*., 1990). This nutrional versatility was borne out by the ability of all the *Pseudomonas aeruginosa* strains to use a large number of substrates tested for growth. The Biolog results showed that ≥ 90% of the isolated strains were able to use 49 of the 95 substrates. The utilisation of the other compounds was strain specific. The central core of universal substrates included several amino acids notably alanine, aspartic acid, asparagine, serine, histidine, threonine and proline. They also included several carboxylic acids such as gluconic acid, itaconic acid, valeric acid, acetic acid, formic acid and several fatty acids including propionic acid and α-keto butyric acid. Of the thirty-two acids available on the Biolog plate twenty-four of them were practically universal substrates for all the strains. In a taxonomic study of pseudomonads Stanier *et al*. (1966) also found that the central core of universal substrates utilised by the *Pseudomonas aeruginosa* strains in their taxonomic study were amino acids, acids and fatty acids.

In general, *P. aeruginosa* strains cannot use an extensive range of carbohydrates and polyalcohols (Prieto *et al*., 2004). Most of the isolates were able to use common monosaccharides such as glucose, fructose, galactose and L-arabinose. *P. aeruginosa* degrades most hexoses by the Entner-Doudoroff pathway (Bergey's Manual for Systematic Bacteriology, 2001). However, the strains in general were unable to use more complex carbohydrates such as disaccharides and trisaccharides. This was also the case

for the twenty-nine *P. aeruginosa* strains used by Stanier *et al*. (1966). The inability of *P. aeruginosa* to use most sugars was partly explained by Stover *et al.* (2000), who completed the sequencing of the *P. aeruginosa* genome. The complete genome sequence showed that it has nearly 300 cytoplasmic membrane transport systems. About one third of these are involved in the transport of nutrients and other molecules. It has a large amount of transporters for mono-, di- and tri-carboxylates but appears to be deficient in sugar transporters. It only possesses just two sugar transporters whereas *E. coli* has more than twenty (Stover *et al*., 2000).

In general, carbon sources utilised by the isolates in this study are comparable to the carbon sources used by *Pseudomonas aeruginosa* strains in the taxonomic study of pseudomonads by Stanier *et al.* (1966), the expected results published in Cowan and Steel's manual for the identification of medical bacteria (1993) and in Bergey's Manual for Systematic Bacteriology (2001). No two of the thirteen strains were able to utilise exactly the same range of carbon sources indicating that no two of the strains are the same strain.

To complement the conventional phenotypic analysis, 16S rRNA gene sequences were analysed for all the "*Pseudomonas aeruginosa*" isolates for definitive identification. Phylogenetic analysis based on 16S rRNA has been proven to be the one of the most powerful tools for the identification and classification of organisms (Pace, 1997), (Kolbert and Persing, 1999), (Drancourt *et al*., 2000). Sequencing the 16S rRNA gene after amplification by PCR is now universally used as the basis for assignment of species to the genus *Pseudomonas* (Bergey's Manual for Systematic Bacteriology, 2001). 16S rRNA genes are conserved among all organisms. However, all organisms possess various unique species-specific regions that allow for bacterial identification (Gobel *et al*., 1987). Advances in molecular analysis and DNA manipulation have facilitated the development of rapid identification systems in clinical analyses. Broad-range primers that recognize 16S rRNA gene sequences conserved among a wide variety of bacteria are used to amplify species-specific variable regions of interest (Marchesi *et al*., 1998). Specific oligonucleotide probes have enabled the development of efficient diagnostic

methodologies, especially where pathogenic organisms are difficult to type and culture. Cystic fibrosis is one such area where bacterial identification systems were previously difficult. Some strains of *Pseudomonas aeruginosa*, particularly isolates from cystic fibrosis patients, can present difficulties in identification with commercial tests and other phenotypic-based identification methods due to exopolymeric polysaccharide production and loss of pigment production (Lyczak *et al*., 2002). However, the advancement in 16S rRNA gene analysis has resulted in the development of rapid diagnostic techniques for the identification of *P. aeruginosa* (Spikler *et al*., 2004), (Li Puma *et al*., 1999), (O'Callaghan *et al*., 1994).

Sometimes to distinguish between particular taxa or strains it is necessary to sequence the entire 1550 bp of the 16S rRNA gene. However, sequencing of the whole gene is not always required for identifying most clinical isolates. In many cases the first 500 bp sequence provides satisfactory differentiation for the identification of these strains and can actually show greater percentage difference between strains because the region shows slightly more diversity per kilobase sequenced (Clarridge III, 2004).

The close relationship between the isolates was indicated by the results from classical identification techniques and biochemical profiling results. This close relationship was also illustrated by the close proximity of the branching on the phylogenetic tree (Figure 3.3) and the lack of nucleotide differences between the strains. The isolates had 99-100% similar 16S rRNA nucleotide sequence with the known *Pseudomonas aeruginosa* strain, *Pseudomonas aeruginosa* S8 (Wang *et al*., 2007). For a more comprehensive analysis, the isolates were genetically compared with other validated strains in the Genbank database, including those species that are very closely related to *Pseudomonas aeruginosa*: *Pseudomonas stutzeri*, *Pseudomonas putida* and *Pseudomonas fluorescens*. These species have very similar phenotypic characteristics to *P. aeruginosa* and can therefore be difficult to differentiate using classical identification techniques (Bergey's Manual for Systematic Bacteriology, 2001). These strains were located on separate branches close to the *Pseudomonas aeruginosa* strains indicating the close genetic relationship between the species. The strains were also compared to species which were

previously members of the Pseudomonads: *Xanthomonas* and *Burkholderia* (Figure 3.3) (Bergey's Manual for Systematic Bacteriology, 2001). The latter strains were located on the farthest branches from the *Pseudomonas aeruginosa* strains indicating the evolutionary distance of the species.

16S rRNA gene sequencing was useful for identifying the isolates at the species level. Sequencing of the 16S/23S spacer region, sequencing of housekeeper genes (e.g. DNA gyrase gene), whole cell protein fingerprinting, plasmid profiling or outer membrane protein profile could be used to further differentiate the isolates (Bergey's Manual for Systematic Bacteriology, 2001).

A key point in laboratory tests for *Pseudomonas aeruginosa* involves determining its susceptibility to antibiotics and identification of its resistance mechanisms (Todar, 2004). Rolinson (1971) described two criteria for judging an organism to be resistant to an antibiotic. The first is that the concentration of the drug required to inhibit growth is at a level that cannot readily be achieved at the site of infection. For example, if a strain of *P. aeruginosa* requires a concentration of gentamicin of 10 μg/ml for inhibition it is regarded as being resistant. However, if a strain requires a concentration of 50 μg/ml of carbenicillin for inhibition it is regarded as sensitive. The reason for this is toxicity. 10 μg/ml of gentamicin cannot be safely maintained in the body, whereas 50 μg/ml of carbenicillin can. The second is that the minimum inhibitory concentration is significantly higher than that of most strains of that particular species. The minimum inhibitory concentration is the minimum concentration of the antibacterial agent below which bacterial growth is not inhibited.

It is important to know whether an organism is likely to respond to a particular antibiotic treatment. Infections caused by resistant strains are a matter of great concern in hospitals worldwide because they are associated with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteraemia, a two-fold increase in the length of hospital stay and therefore an increase in healthcare costs (Giamarellou, 2002). For this reason it is essential to establish the breakpoint between a susceptible and resistant population of

bacteria. Minimum inhibitory concentration (MIC) breakpoint values are determined by reviewing each drug's pharmacokinetics, pharmacodynamics, population distributions, and clinical efficacy at different MICs (Clinical and Laboratory Standards Institute M100-S16, 2006). There are many committees around the world involved in establishing breakpoints. The Clinical and Laboratory Standards Institute (CLSI) publishes such guidance in the USA. In Europe there are six different groups: the Comite de l'Antibiogramme de la Societe Francaise de Microbiologie (CA-SFM) (France), the Norwegian Working Group on Antibiotics (NWGA) (Norway), the German Institute for Standardisation (DIN) (Germany), Swedish Reference Group for Antibiotics (SRGA) (Sweden), the Commissie Richtlijenen Gevoeligneids Depalingen (CRG) (Holland) and the British Society for Antimicrobial Chemotherapy (BSAC) Working Group (UK) (MacGowan and Wise, 2001). There is ambiguity when it comes to MIC breakpoints in the literature. Cockerill (1999) stated that when the MIC for a particular antibiotic reaches or exceeds 8 μg/ml, the organism might be classified as moderately resistant to the antibiotic. Organisms for which MICs are above 32 μg/ml are generally viewed as clinically resistant to the antibiotic. However, different antibiotics have different MICs. For example, ticarcillin typically has a MIC of ≥ 128 μg/ml whereas the MIC for tobramycin is ≥ 16μg/ml (Clinical and Laboratory Standards Institute M100-S16, 2006). Also, the MIC of a particular antibiotic may vary for different microorganisms. For example, the MIC of ampicillin for *E. coli* is ≥ 32 μg/ml whereas the MIC for *Enterococcus* spp. is ≥16 μg/ml (Clinical and Laboratory Standards Institute M100-S16, 2006). There seems to be a lack of standardisation between both the methodologies and the MIC values from these different standards agencies (Mesaros *et al*., 2007). Recently, the CLSI has approved a MIC breakpoint value of ≥16 μg/ml of gentamicin for *Pseudomonas aeruginosa* (Clinical and Laboratory Standards Institute M100-S16, 2006). The British Society for Antimicrobial Chemotherapy (BSAC) has set a much lower breakpoint of 4 μg/ml of gentamicin for *Pseudomonas aeruginosa* (BSAC, 2006). In general, the CLSI breakpoints tend to be higher than those of the BSAC. However, the Irish Health Protection Surveillence Centre recommends that all surveillence laboratories use the Clinical and Laboratory Standards Institute (CLSI) methodologies when performing antimicrobial susceptibility (Health Protection Surveillence Centre, 2006).

Complete sequencing of its genome revealed that the *P. aeruginosa* possesses ten genes encoding described and predicted drug efflux systems (Stover *et al*., 2000). This compares to only four in *E. coli*. Furthermore, given its capacity to metabolize a wide variety of organic substrates, it is possible that *P. aeruginosa* possesses greater potential for enzymatic modification and degradative drug resistance mechanisms (Stover *et al*., 2000).

Pseudomonas aeruginosa strains are generally less susceptible to a number of antibiotics than other Gram-negative bacteria. It is naturally resistant to many antibiotics. This phenomenon is known as intrinsic resistance (Hancock and Brinkman, 2002). The intrinsic resistance of a microorganism to antimicrobials varies according to the nature of the antimicrobial, the microbial species and the prevailing growth environment (Russell and Chopra, 1990). *Pseudomonas aeruginosa* is generally resistant to hydrophobic antibiotics for the same reason as other Gram-negative bacteria i.e. the presence of LPS (lipopolysaccharide) in the outer leaflet of the outer membrane (Todar, 2004). It shows intrinsic resistance to many β-lactam antibiotics and also to lipophilic antibiotics which are able to cross the outer membranes of other Gram-negative bacteria to reach their targets, such as tetracycline, chloramphenicols and some fluoroquinolones (Li *et al*., 1994[a]).

Intrinsic resistance was long attributed to the low outer membrane permeability of *P. aeruginosa*. For example, the permeability of the outer membrane of *P. aeruginosa* to small hydrophilic antibiotics is reduced compared to *E. coli* (Yoshimura and Nikaido, 1982). *Pseudomonas aeruginosa* outer membranes show about 100-fold lower permeability to cephalosporins such as cephaloridine than other Gram-negative bacteria, in part because of porins with small pores to reduce inward passage of the antibiotics into the periplasmic space (Nikaido, 1998). Although the low outer membrane permeability is an important factor, it cannot be the entire explanation for intrinsic resistance. The influx of these antibiotics into *P. aeruginosa* across its membrane is still quite rapid even with the low permeability of the outer membrane (Li *et al*., 1994[b]). It has been shown that

even relatively susceptible *P. aeruginosa* strains pump out tetracycline, chloramphenicol, fluoroquinolones and some β-lactams using the efflux pump MexC-MexD-OprM in synergy with the low permeability of its outer membrane barrier (Li *et al*., 1994[a]), (Li *et al*., 1994[b]). The combination of these mechanisms is the most likely explanation for the universal resistance of the isolates in this study to tetracycline and chloramphenicol, as *P. aeruginosa* has not been reported to modify these antibiotics. The combination of these mechanisms is also the most likely explanation for the universal resistance of the isolates to the narrow spectrum β-lactams such as penicillin G and cephalothin. Intrinsic resistance of *P. aeruginosa* to the folic acid synthesis inhbitor, co-trimoxazole has been attributed to its impermeability and the expression of the efflux pump MexAB-OprM (Köhler *et al*., 1996). *P. aeruginosa* also has an inducible chromosomal AmpC β-lactamase and is inherently resistant to those β-lactams that induce this enzyme and are hydrolysed by it such as the aminopenicillins, ampicillin and amoxicillin and cephalothin (Livermore, 2002). These mechanisms of intrinsic resistance also confer resistance to some third-generation cephalosporins such as cefotaxime and moxalactam (Vedel, 2005). This could explain the universal intermediate resistance of the isolates to these two antibiotics

All of the isolates were resistant to the aminoglycoside antibiotics: kanamycin, neomycin, paramomycin, spectinomycin and hygromycin B. The well-known chromosomally encoded aminoglycoside 3'-phosphotransferase II (APH(3')-II), occurs universally in *P. aeruginosa* and naturally provides it with resistance to kanamycin, neomycin and parmomycin (Okii *et al*., 1983). In fact, it is this enzyme that has effectively removed these aminoglycosides from clinical use. The MexXY-OprM has been reported to confer spectinomycin resistance in *P. aeruginosa* in addition to tetracycline resistance (Jeannot *et al*., 2005). This could explain the resistance of all the isolates in this study to spectinomycin. Resistance to hygromycin B is generally attributed to the production of the aminoglycoside 4'-phosphotransferase I (APH (4')-I). This gene has been cloned and used as a useful genetic marker in molecular microbiology (Wright and Thompson, 1999). All the isolates tested were resistant to this antibiotic.

P. aeruginosa PA13 was considered to be a multidrug resistant strain. Multidrug resistance is defined as showing resistance to at least three main classes of antipseudomonal agents i.e., β-lactams, carbapenems, aminoglycosides and fluoroquinolones (Obritsch *et al*., 2005). *P. aeruginosa* PA13 was resistant to all of these classes. In addition to the antibiotics that all of the isolates were intrinsically resistant to, *P. aeruginosa* PA13 was also resistant to the β-lactams: carbenicillin, piperacillin and ticarcillin, the β-lactam/β-lactamase combination ticarcillin-clavulanic acid, the cephems: ceftizoxime and cefsulodin (intermediate resistance), the carbapenem, imipenem (intermediate resistance), the aminoglycosides: gentamicin, netilmicin, tobramycin, sisomicin, streptomycin and the fluoroquinolones: ciprofloxacin and ofloxacin.

Permeability mutations are widely blamed for increased resistance to fluoroquinolones and β-lactams (Livermore, 2002). These mutations have been reported to be important in resistance to the carbapenems. This is due to the loss of OprD, a porin which forms narrow transmembrane channels that are accessible to carbapenems but not to other β-lactams (Studemeister and Quinn, 1988). Loss of OprD is associated with resistance to imipenem and reduced susceptibility to meropenem. OprD is coregulated with MexEF-OprN. The MexEF-OprN confers resistance to fluoroquinolones. Thus, the *nfxc* (MexT) mutants that are selected for by fluoroquinolones have up-regulated MexEF-OprN and reduced OprD and consequently have resistance to both fluoroquinolones and imipenem. There is also a reduced susceptibility to meropenem (Livermore, 2002). Although not investigated in this study, this combination of mechanisms could explain *P. aeruginosa* PA13's resistance to the fluoroquinolones and carbapenems. *P. aeruginosa* PA13 is resistant to ciprofloxacin and ofloxacin (fluoroquinolones) and to imipenem (carbapenem). Although it is not resistant to meropenem, it has reduced susceptibility to this antibiotic. The difference between the MIC values of imipenem and meropenem for the imipenem-resistant strain, *P. aeruginosa* PA13, has been previously attributed to the poorer β-lactamase-inducing ability of meropenem, improved β-lactamase stability of meropenem and the possibility that meropenem diffuses through the outer membrane by non-specific pathways (Bonfiglio, 1998).

Streptomycin resistance in *Pseudomonas aeruginosa* is conferred either by the aminoglycoside adenyltransferase, ANT(3'') or by a 16S rRNA methylase gene (Poole, 2005). Although this 16S rRNA methylase gene has recently been discovered in *P. aeruginosa* isolates (Yokoyama *et al*., 2003), it is rarely seen in clinical isolates and is generally found in aminoglycoside-producing actinomycetes conferring high-level resistance to streptomycin. It is therefore more likely that it was the ANT(3'') adenyltransferase enzyme that conferred low-level streptomycin resistance on *P. aeruginosa* PA13, although the gene for this enzyme was not screened for in this study.

Preliminary antibiotic susceptibility tests showed that all of the isolates had similar susceptibilities to the eight antibiotics with the exception of *P. aeruginosa* PA13, which was resistant to the aminoglycosides, gentamicin and streptomycin. Following this discovery, susceptibilty tests were performed using a range of other antibiotics from the aminoglycoside class. *P. aeruginosa* PA13 was found to be resistant to many of these aminoglycosides. The ability of *Pseudomonas aeruginosa* to grow in the presence of high concentrations of clinically important aminoglycoside antibiotics such as gentamicin, netilmicin and tobramycin suggested the presence of an aminoglycoside-modifying enzyme. These enzymes are the most common mechanism of conferring resistance to aminoglycosides (Vakulenko and Mobashery, 2003). They are present worldwide and are detected in up to 20% of clinical isolates in Europe and Latin America (Poole, 2005). Primers were designed specifically to amplify a range of aminoglycoside modifying enzyme genes that confer resistance to gentamicin. A product was amplified from *P. aeruginosa* PA13 using the primers for the *aac(6')*-IIa gene. This product was sent for sequencing and analysis of the sequence identified an *aac(6')-Ib* gene and not the expected *aac(6')*-IIa gene. The aminoglycoside resistance phenotype of *P. aeruginosa* PA13 suggested low-level production of AAC(6') type IIa and not AAC(6') type Ib , due the fact that the strain was resistant to gentamicin as well as to tobramycin, kanamycin, netilmicin and sisomicin and was not resistant to amikacin. AAC(6')-IIa is commonly found in *Pseudomonas aeruginosa*. It confers resistance to gentamicin, tobramycin, netilmicin and sisomicin (but not to amikacin), whereas AAC(6')-Ib confers resistance to amikacin, tobramycin, kanamycin, netilmicin, and sisomicin (but not to gentamicin).

These two enzymes are highly similar and their amino acid sequences demonstrate 74% similarity (Shaw *et al*., 1993). There are two regions where differences occur between the proteins, at amino acid 53 and between amino acids 115 and 130. The latter region is slightly hydrophobic in AAC(6')-Ib and relatively hydrophillic in AAC(6')-IIa (Rather *et al*., 1992).

Although, three potential start codons were identified on *aac(6')-Ib* open reading frame, only two were located downstream from the recombination site. However, initiation from the ATG codon at position 680 would yield a protein of 19.13 kDa, which is in good agreement with previous values of 20 kDa and 19.5 kDa estimated from immunoblotting experiments (Galimand *et al*., 1993), (Casin *et al*., 1998). The stop codon for this open reading frame was found at posititon 1196 and it was also found downstream of the recombination site Figure 3.7. This ambiguity with the start codons is not uncommon and has been reported in many species including *Pseudomonas fluorescences* (Lambert *et al*., 1994), *Burkholderia cepacia* (Crowley *et al*., 2002), *Enterobacter cloacae* and *Citrobacter freundii* (Casin *et al*. 1998). No typical ribosome binding site was found upstream of the potential start codons.

The *aac(6')*-Ib open reading frame of *P. aeruginosa* PA13 encodes a serine residue instead of the typical leucine residue at position 119. This resulted from a single nucleotide mutation (thymine to cytosine at position 269 [Figure 3.9]) which replaced leucine (TTA) for serine (TCA) (Figure 3.10). This substitution has previously been reported by (Giuliani *et al*., 2005), (Mendes *et al*., 2004), (Crowley *et al*. 2002), (Casin *et al*. 1998), (Mugnier *et al.,* 1998), (Lambert *et al*. 1994) and (Rather *et al*. 1992). In all of these cases the substitution from leucine to serine has been associated with a shift from amikacin resistance to gentamicin resistance. The same case is true for the AAC(6')-Ib from *P. aeruginosa* PA13 as the strain is resistant to gentamicin and susceptible to amikacin. This mutant version of the AAC(6')-Ib enzyme can also be referred to as AAC(6')-Ib_9 or AAC(6')-Ib'.

Research by Rather *et al*. (1992) suggested that a single amino acid substitution $Leu_{119}Ser$ conferred an altered substrate profile, where the AAC(6')-Ib conferred resistance to gentamicin instead of the amikacin resistance seen in the wild-type gene. Rather *et al*., 1992 reported that the amino acid at position 119 in AAC(6')-Ib was critical to the functioning of the enzyme. Casin *et al*., 1998 studied naturally occurring variants of AAC(6')-Ib that had a serine residue instead of a leucine residue at position 119 (numbering of the reference AAC(6')-Ib sequence [Tran Van Nhieu and Collatz, 1987]). A change from leucine to serine at this position conferred an altered substrate profile, where the mutant AAC(6')-Ib conferred resistance to gentamicin instead of the amikacin resistance seen in the wild-type enzyme. Although the enzyme variants had different substrate specificities, both the variants were fully functioning enzymes which would suggest that the amino acid substitution from leucine to serine at this position does not fundamentally alter global protein folding (Casin *et al*., 1998).

The amino acid substitution from leucine to serine has been reported to cause a slight reduction in local hydrophobicity in the aminoglycoside binding domain. (Rather *et al*., 1992). Casin *et al*. (1998) reported that the region around the leucine at position 119 is an α helix in AAC(6')-Ib and stated that there was a strong possibility that the N-terminal portion of this α helix is shortened in variants with serine instead of leucine. Miller *et al*. (1995) suggested that the presence of a free amino group in gentamicin compared to the presence of a hydroxy-amino-butyl group in amikacin at position 1 may be involved in the different substrate specificities of AAC(6')-I and AAC(6')-II and that both amino acid groups could interact with serine. Casin *et al*. (1998) predicted the secondary structure of these enzymes and suggested that the binding domain contains an α helical structure. They believed that the substitution of leucine by serine residue reduced the possibility of this secondary structure. They also stated that serine, a small polar amino acid capable of establishing hydrogen bonding, or leucine, a larger hydrophobic amino acid at position 119, conditioned the conformation of the aminoglycoside binding domain in these enzymes.

Following sequencing, it was noted that this *aac(6')*-Ib gene was located on a Class I integron because it was surrounded by an integrase gene (*intI*) and a quaternary ammonium compound resistance gene (*qacE*Δ1). These genes are typical of Class 1 integrons. Integrons are mechanisms for the acquisition and dissemination of genetic determinants of antimicrobial resistance (Daly and Fanning, 2000). They are genetic elements capable of the acquisition, rearrangement and expression of genes contained in gene cassettes (Collis and Hall, 1995). Class 1 integrons, which are most commonly found in antibiotic-resistant clinical isolates, possess two conserved segments located on either side of the integrated gene cassettes (Fluit and Schmitz, 1999). The 5' conserved segment encodes an integrase gene (*Int1*) and contains *attI1*, the cassette integration site, the promoter P_{ant}, which is found in the integrase gene and is responsible for the expression of downstream-located integrated gene cassettes. It sometimes also contains the secondary promoter P_2 (Fluit and Schmitz, 2004). The 3' conserved segment contains the disinfectant (*qacE*Δ1) and sulphonamide (*sul1*) resistance genes (Fluit and Schmitz, 1999). Gene cassettes generally consist of a promoterless gene associated with a recombination site known as a 59-base element (59-be) (Fluit and Schmitz, 2004). Multiple insertion events can lead to the assembly of large integron-associated cassette arrays. The gene cassettes are located between these two conserved regions (Daly and Fanning, 2000).

Almost all *aac(6')*-Ib genes described to date exist as gene cassettes carried by class 1 integrons (Casin *et al*., 2003). The BLAST searches of genetic databases identified that the integron from *P. aeruginosa* PA13 contained two gene cassettes fused in a head-to-tail arrangement. The first was the 516 bp gene encoding a 172 amino acid protein, known as AAC(6')-Ib (an aminoglycoside modifying enzyme) as described above. The *aac(6')*-Ib gene found in the gene cassette within the integron in *P. aeruginosa* PA13 was 100% similar to *aac(6')*-Ib genes found in several *Pseudomonas aeruginosa* strains (Petroni *et al*., 2002), (Mendes *et al*., 2004) and in a *Burkholderia cepacia* strain (Crowley *et al*., 2001). All of these *aac(6')*-Ib genes were also found on class 1 integrons.

The *aac(6')*-Ib gene was located at the proximal side of this integron with a 798 bp open reading frame encoding a protein of 266 amino acids that displayed similarity with a number of previously identified Class D β-lactamase (oxacillinase) genes on its distal side. The most common mechanism of resistance to β-lactam antibiotics is the production of a β-lactamase (Petroni *et al*., 2002). Integrons with similar gene cassettes in *P. aeruginosa* have been reported previously by (Poirel *et al*., 2002), (Lambert *et al*., 1994) and (Mugnier *et al*., 1998). Crowley *et al.* (2002) identified an integron in a *Burkholderia cepacia* isolate in which the *aac(6')*-Ib gene was located distal to *bla*-$_{OXA}$ gene and adjacent to the 3' region of the Class 1 integron. However, these enzymes occur predominantly in *Pseudomonas aeruginosa* (Crowley *et al*., 2002). These gene cassettes are most likely located together on integrons because a combination of an aminoglycoside and a β-lactam antibiotic are commonly used to treat *P. aeruginosa* infections rather than a single antibiotic class on its own.

Class D β-lactamases (oxacillinases) are characterised by a strong oxacillinase activity but differ widely in their genetic backgrounds and functional features (Bush *et al*., 1995). Oxacillin-hydrolysing enzymes are mostly narrow-spectrum β-lactamases. They usually confer resistance to most penicillins and narrow-spectrum cephalosporins (cephalothin) (Naas and Nordmann, 1999). OXA-type enzymes hydrolyse cloxacillin and oxacillin faster than benzylpenicillin and are generally not inhibited by clavulanic acid (except OXA-18 and OXA-45) (Philippon *et al*., 1997), (Toleman *et al.*, 2003). Potentiation by clavulanate is often poor and many oxacillinase producers are resistant to β-lactamase inhibitor combinations that include this inhibitor. The oxacillinase enzymes however remain susceptible to the β-lactamase inhibitor combination piperacillin-tazobactam (Naas and Nordmann, 1999). They are frequently found in *Pseudomonas aeruginosa*. Most oxacillinase genes that have been identified to date have been located on the variable regions of integrons. The reason for this is unknown but it provides an excellent means for their selection upon β-lactam treatment (Naas and Nordmann, 1999).

BLAST searches showed that an OXA-type enzyme from an uncultured bacterium isolated from activated sludge in Germany (Tennstedt *et al*, 2003) and an OXA-type

enzyme from a *Burkolderia cepacia* strain (Crowley *et al.*, 2002) had the most similar nucleotide sequence to the oxacillinase gene from *P. aeruginosa* PA13 with 99% similarity. The oxacillinase gene also shared 92% sequence identity to the OXA-46 enzyme located on an integron in a clinical isolate of *Pseudomonas aeruginosa* in Belgium (Giuliani *et al.*, 2005). All of these enzymes are members of the OXA-2 sublineage. The oxacillinase was also similar at the level of primary structure with other Class D β-lactamases belonging to the OXA-2 sublineage of oxacillinases (Naas and Nordmann, 1999). Oxacillinases are divided into sublineages depending on their degree of amino acid identity. The largest of these sublineages are OXA-2 and OXA-10. The oxacillinase from *P. aeruginosa* PA13 is a new member of the OXA-2 lineage. It shared 81% amino acid sequence identity with OXA-2 (Rossolini *et al.*, 2000). However, the protein is shorter by nine residues at the carboxy terminus. It also contains identical residues at positions 150 and 164, unlike in OXA-15 and OXA-32 (variants of OXA-2 with extended spectrum activities), which have mutations at these residues. Considering the molecular similarity with the OXA-2 sublineage the oxacillinase from *P. aeruginosa* PA13 may also be assigned to oxacillinases belonging to Group II according to the classification of Sanschagrin *et al.* (1995).

Members of the OXA-2 lineage exhibit a narrow-substrate specificity that is generally limited to penicillins and narrow spectrum cephalosporins. This is similar to the substrate specificities of other narrow-spectrum oxacillinases (Bush *et al.*, 1995). At the genetic level, the similarity between the lineages was not just limited to their coding sequences but also to the *attC* recombination sites (59-be) of the gene cassettes, which suggests a common ancestory for these *bla-$_{OXA}$* cassettes (Figure 3.16).

The deduced amino acid sequence of the oxacillinase enzyme from *P. aeruginosa* PA13 (Figure 3.12) contained a motif characteristically found in serine β-lactamases (Couture *et al.*, 1992) with the serine-threonine-phenylalanine-lysine tetrad (S-T-F-K) motif found at positions DBL 70 to 73. This conserved region is characteristic of β-lactamases which possess a serine at their active site (Couture *et al.*, 1992). DBL is the system used for numbering Class D β-lactamases (Couture *et al.*, 1992). The oxacillinase gene from *P.*

aeruginosa PA13 also included the conserved serine and lysine amino acid residues characteristic of β-lactamases possessing a serine active site (Couture *et al*., 1992). The five structural elements characteristic of Class D β-lactamase were also found: S-X-V at position 118 to120 Y-G-N at position 176 to 180, W-X-E-X-X-L-X-I-S at DBL 164 to 172, Q-X-X-X-L at DBL 176 to 180 and K-T-G at position 216 to 218.

The oxacillinase genes from an uncultured bacterium isolated from activated sludge in Germany (Tennstedt *et al*, 2003) and from a *Burkolderia cepacia* strain (Crowley *et al.*, 2002) were not characterised and were only screened against basic antibiotics. OXA-46 is the most similar characterised oxacillinase to the oxacillinase from *P. aeruginosa* PA13. OXA-46 conferred resistance to most penicillins and the narrow-spectrum cephalosporin, cephalothin. *P. aeruginosa* PA13 had a narrow-spectrum resistance profile that included most penicillins (penicillin G, ampicillin, amoxicillin, oxacillin, cloxacillin, carbenicillin, piperacillin, ticarcillin) the narrow-spectrum cephalosporin, cephalothin. It was also resistant to the β-lactam-β-lactamase inhibitor combination of ticarcillin and clavulanic acid, which is typical of a strain producing an oxacillinase enzyme (Toleman *et al.*, 2003). Although the other isolates were resistant to some of these antibiotics, *P. aeruginosa* PA13 had a higher-level of resistance. In particular it had much higher levels of resistance to oxacillin and cloxacillin, which is typical of strains producing an oxacillinase enzyme (Toleman *et al.*, 2003). It also had an intermediate resistance to the extended-spectrum cephalosporins, ceftizoxime and cefsulodin. The most frequent mechanisms of resistance to extended-spectrum cephalosporins in *P. aeruginosa* are derepression of the chromosomal AmpC β-lactamase (a Class C cephalosporin) and up-regulation of multi-drug efflux (Chen *et al*., 1995). However, the absence of inhibition by cloxacillin (a Class C cephalosporin inhibitor) argued against the presence of an AmpC type β-lactamase (Danel *et al*., 1997). Extended-spectrum variants of OXA-2 type oxacillinases have been identified (OXA-15 and OXA-32) (Danel *et al*., 1997) (Poirel *et al*., 2002), which confer high-level resistance to the extended-spectrum cephalosporins such as, cefpirome, ceftriaxome aztreonam and ceftazidime. The oxacillinase from *P. aeruginosa* PA13 may be an extended-spectrum of the OXA-2 β-lactamase. However, kinetic studies would be required to confirm this. *P. aeruginosa* PA13 has also a low

susceptibility to the β-lactamase inhibitor clavulanic acid but not to the other inhibitor, tazobactam. This is a typical characteristic of strains producing oxacillinase enzymes (Bush *et al*., 1995).

The gene cassettes, *aac(6')-Ib* and the oxacillinase, were surrounded by all of the characteristics typical of a Class 1 integron (Collis and Hall, 1995). The gene cassette containing the *aac(6')*-Ib gene was preceded by a 5' coding sequence containing a Class 1 integrase gene (*IntI1*) (Figure 3.7) and a recombination site, *attI1* (Figure 3.6). The *aac(6')*-Ib gene cassette had a core site (5'-GCCTAAC-3'), a perfect inverse core site (5'-GTTAGGC-3') and a 59-be site made up of 73 base pairs starting inside the 3'-end coding sequence of the gene (Figure 3.15). The oxacillinase gene had a core site (5'-GCCCAAC-3'), an imperfect core site (5'-GTTAGGC-3') and a 59-be site made up off 55 base pairs starting inside the 3'-end coding sequence of the gene (Figure 3.16). Downstream of the latter 59-be site was the 3' conserved segment characteristic of Class 1 integrons which contained the ethidium bromide and quaternary ammonium resistance determinant, *qacΔE1* gene (Figure 3.13). Paulsen *et al*. (1993) described *qacEΔ1* as a defective version of *qacE*, a gene said to encode resistance to quaternary ammonium compounds (QAC) and dyes like ethidium bromide. *qacE* was also found as part of 3'-CS in some integrons in Gram-negative bacteria. This could mean that bacteria harbouring integrons are also resistant to disinfectants like QACs (Kücken *et al*., 2000). QACs, which contain benzalkonium chloride as the most widely used agent, are employed as wound and skin antiseptics and as disinfectants in hospitals. In the same way that the problem with antibiotic resistant bacteria has been increasing in hospitals, bacterial resistance to disinfectants and antiseptics is also on the increase (McDonnell and Russell, 1999). Genes determining resistance to QACs are generally located on plasmids (Kaulfers and Brandt, 1987).

The overall sequence of the integron amplified from *P. aeruginosa* PA13 was compared to other integrons in the GenBank database (Figure 3.14). The most similar integron was found to be 95% similar to an integron in a *Pseudomonas aeruginosa* strain from Italy (Giuliani *et al*., 2005). This integron contained both the *aac(6')*-Ib gene and the

oxacillinase gene, *bla-$_{OXA-46}$*. The integron shared 92% similarity with integrons found in a *Vibrio cholerae* and a *Morganella morganii* strain, both isolated in Buenos Aires, Argentina (Soler Bistué *et al*., 2006) (Power *et al*., 2005). Both these integrons contained the *aac(6')*-Ib *and bla-$_{OXA-2}$* genes. The integron was also 88% similar to an integron found in a *Pseudomonas aeruginosa* strain in France (Naas *et al*., 1998), which contained the *aac(6')*-Ib gene and the *bla-$_{OXA-20}$* genes.

Expression of gene cassettes in class 1 integrons is not uniform. Expression depends on a number of factors such as gene copy number, promoter strength and distance of the gene cassette from the promoter (Martinez-Freijo *et al*., 1998). The promoter is usually found upstream of the gene cassette. The order of a cassette in the integron is related to the level of resistance observed. The relative distance between a gene cassette and the promoter is important regarding expression. Gene cassettes that are closest to the promoter, P_{ant}, are more highly and effectively expressed than distal cassettes (Collis and Hall, 1995). Therefore distal genes may be poorly expressed and have very little effect on the susceptibility of the bacterium to relevant antibiotics (Martinez-Freijo *et al*., 1998). The expression of downstream cassettes can also be affected by the nature of of upstream cassettes. For a higher level of expression, a second promoter adjacent to the first promoter or multiple copies of the same gene is required (Martinez-Freijo *et al*., 1998).

Gene cassettes are generally promoterless. Therefore most gene cassettes are expressed from a common promoter located in the 5'-CS region of integrons. This region contains two potential promoter sites, P_{ant} and P2 (Fluit and Schmitz, 2004). At least five versions of the P_{ant} (also called P_C or P1) promoter have been identified and classified according to their activity (Fluit and Schmitz, 2004). They differ in the sequences of the –35 and/or –10 hexamers and in their strength. There is a weak promoter TGGACA N_{17} TAAGCT, a strong promoter TTGACA N_{17} TAAACT, and hybrid promoters including TGGACA N_{17} TAAACT and TTGACA N_{17} TAAGCT which have an intermediate activity (Bunny *et al.*, 1995). The weak promoter has been found to have 20-fold less activity than the strong promoter (Collis and Hall, 1995). The weaker version of the promoter may be found together with a secondary, compensatory promoter, which increases their

combined activity to expresssion levels which are just threefold lower than the strong P_{ant} promoter (Rowe-Magnis and Mazel, 2002). However, the second potential promoter, P2, is frequently inactive because only 14 out of the optimal 17 nucleotides are present (i.e., TTGTTA-N_{14}-TACAGT instead of TTGTTA-N_{17}-TACAGT) (Fluit and Schmitz, 2004). The second promoter only arises from the insertion of three guanosine residues to increase the spacing between the –10 and –35 sequences to 17 (Levesque *et al*., 1994). Sequence analysis of the integron from *P. aeruginosa* PA13 revealed that it contained both these promoters (Figure 3.6). The P_{ant} contained the sequence associated with weak promoters (TGGACA N_{17} TAAGCT). The P2 promoter (-35 region, TTGTTA; -10 region, TACAGT) had only 14 nucleotides out of the optimal 17 present making it inactive. Therefore expression of the antibiotic resistance genes in this integron was driven by P_{ant}.

Interestingly, the origin of these gene cassettes within the integron in *P. aerginosa* PA13 may not be *Pseudomonas aeruginosa*. Analysis of the genes determined that the G + C content of both is closer to those found in Enterobacteriaceae. The %GC content of *Pseudomonas aeruginosa* is around 67% and the %GC content of *Escherichia coli* is around 50% (Bergey's Manual for Systematic Bacteriology, 2001). The %GC content of the oxacillinase gene from *Pseudomonas aeruginosa* PA13 is 49.6%. The %GC content of *aac6'-Ib* gene from *Pseudomonas aeruginosa* PA13 is 54.3%. This further underlines the mobility of gene cassettes.

It was of interest to clone the oxacillinase gene from *P. aeruginosa* PA13 in order to study its biochemical and kinetic properties. Protein expression is governed by many factors including those that affect transcription, mRNA processing and stability and initiation of translation (Gustafsson *et al*., 2004). With the creation of cloning vectors that contain assorted regulatory elements such as promoters, ribosome binding sites and terminators, these factors have been largely optimized (Gustafsson *et al*., 2004). *Escherichia coli* remains the most attractive strain for heterologous protein production because of its ability to grow rapidly, at high density on inexpensive substrates, its well-characterised genetics and the availability of an increasingly large number of cloning

strains and mutant host strains (Baneyx, 1999). However the fate of foreign proteins expressed in *E. coli* is determined in part by the degradative activities of the host cells (Hanning and Makrides, 1998).

The pET vector, pET-28a, was used for the cloning and expression of the oxacillinase gene. The pET vectors were originally constructed by Studier and Moffatt (1986). The more recent pET vectors have been developed by Novagen and offer enhanced features to allow easier cloning, detection and purification of target proteins. The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under the control of a strong bacteriophage T7 transcription. Expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cells resources are converted to target gene expression (Novagen, 2005). The desired protein can comprise more than 50% of the total cell protein a few hours after induction (Baneyx, 1999). Although this system is extremely powerful, it is also possible to attenuate the expression level simply by lowering the concentration of inducer. Decreasing the expression level may enhance the soluble yield of some target proteins. Another important benefit of this system is the ability to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, target protein expression may be initiated by transferring the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, such as *E. coli* BL21 (DE3). Expression is induced by the addition of IPTG or lactose to the bacterial culture (Novagen, 2005).

Many of the previously discovered oxacillinase genes including OXA-50 (Girlich *et al.*, 2004), OXA-46 (Giuliani *et al*., 2005), OXA-69 (Héritier *et al*., 2005) and OXA-85 (Voha *et al*., 2006) have been successfully expressed in pET vectors. The oxacillinase gene (OXA-57) from *Burkholderia pseudomallei* was successfully expressed using a

pET-28a expression vector (Keith *et al*., 2005). This vector was one expression vector chosen for the study the of the oxacillinase gene from *P. aeruginosa* PA13.

When expression of the oxacillinase protein could not be detected in the soluble fraction of the induced *E. coli* BL21 (DE3) cells containing the pET-28a vector with the oxacillinase insert (pDF2) (Figure 3.27), the insoluble fraction was analysed. Overexpression of foreign proteins in the cytoplasm of *E. coli* is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies. Cellular accumulation of misfolded or unfolded proteins in *E. coli* can result for a number of reasons: spontaneous mutations affecting the folding pathway, exposure of the cells to environmental stress such as high temperatures or expression of recombinant proteins (Hunke and Betton, 2003). In these situations, the polypeptide chain can associate to form unordered aggregates known as inclusion bodies instead of folding into a biologically active state (Betts and King, 1999). Inclusion bodies can accumulate in the cytoplasm or periplasm depending on whether or not a recombinant protein has been engineered for secretion. The target usually accounts for 85-95% of the inclusion body material and is contaminated by outer membrane proteins, ribosomal components and a small amount of phospholipids and nucleic acids (Valax and Georgiou, 1993). Even when the protein is present in a biologically inactive state (in inclusion bodies), it can be detected by analysing the insoluble protein fraction using SDS-PAGE. The oxacillinase protein could not be detected in the insoluble fraction (Figure 3.28).

The pET vectors require a host cell containing a chromosomal copy of the T7 RNA polymerase gene to induce expression. Therefore, the vector is limited to very few host strains such as *E. coli* BL21 (DE3), which carries a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. When expression of the oxacillinase gene could not be detected using the pET-28a vector, a new vector was chosen. The second expression vector chosen for the study of the oxacillinase gene from *P. aeruginosa* PA13 was an in-house vector called pPC. The pPC expression vector contains the P*tac* promoter. DeBoer *et* al. (1983) reported on the high efficiency of P*tac* in expressing

foreign genes in *E. coli* as opposed to the parental promoters. Unlike the pET-28a vector, it could be introduced to many host cells.

When expression of the oxacillinase protein was not detected in the induced *E. coli* XL-10 Gold cells containing the oxacillinase gene in the pPC expression vector (pDF4) at 37°C (Figure 3.38), the cells were induced at 28°C. A traditional approach to reduce protein aggregation (inclusion bodies) is most commonly to decrease the cultivation temperature, as protein folding is often favoured under low temperature cultivation conditions (Baneyx, 1999). The aggregation reaction is usually favoured at higher temperatures due to the strong temperature dependence of hydrophobic interactions that determine the aggregation reaction (Kiefhaber *et al*., 1991). The lower temperature has the combined advantages of slowing down transcription and translational rates and of reducing the strength and rates of hydrophobic reactions that contribute to protein misfolding (Baneyx and Mujacic, 2004). The cells with pDF4 were also cultivated at 150 rpm instead of the usual 200 rpm to provide gentler growth conditions. Both the soluble and insoluble protein fractions were analysed by SDS-PAGE (Figure 3.39 and Figure 3.40). Even at this low temperature the oxacillinase protein was not detected suggesting that the protein was not being expressed in inclusion bodies.

Expression of recombinant proteins in *E. coli* is difficult when the codon usage in the recombinant gene differs from the codon usage in the host cells (Gustafsson *et al*., 2004). Most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. Not all of the mRNA codons are used equally. The degeneracy of the genetic code allows many alternative nucleic acid sequences to encode the same protein. The so-called major codons are those that occur in highly expressed genes, whereas the minor or rare codons tend to be in genes expressed at a low level (Zahn, 1996). Forced high-level expression of a gene with codons that are rarely used by *E. coli* causes depletion of the internal tRNA pools. Insufficient tRNA pools can lead to translational stalling, premature translational termination, translation frameshifting, amino acid misincorporation or inhibition of protein syntheis and cell growth (Novy *et al*., 2001). A subset of codons, namely

AGG/AGA (Arg), CGA/CGG (Arg), AUA (Ile), CUA (Leu), GGA (Gly) and CCC (Pro) are rarely expressed in *E. coli* and appear to cause problems from a translational point of view (Kane, 1995). If the recombinant protein being expressed contains several of these rare codons the protein may not be expressed due to this translational limitation. The *E. coli* RosettaBlue™ strain (Novagen) has been engineered to provide the tRNAs for these rarely expressed codons on a chloramphenicol resistant plasmid, pRARE (Section 2.1.1.3). The use of the *E. coli* RosettaBlue™ strain (Novagen) as an expression host facilitates the expression of proteins that would otherwise be limited by codon bias in *E. coli*. The oxacillinase gene from *Pseudomonas aeruginosa* PA13 contained thirteen of these rare codons. The pPC expression vector containing the oxacillinase gene (pDF4) was transformed into an *E. coli* RosettaBlue™ strain. When these cells were induced and the proteins produced were analysed the oxacillinase protein was not detected in either the soluble or insoluble fractions (Figure 3.41 and Figure 3.42).

The cloned oxacillinase gene and the expression vectors were sequenced and were found to have no errors. The oxacillinase enzyme did not appear to be expressed in inclusion bodies as was shown by reducing the induction temperature and by analysing the insoluble protein fraction of the induced cells. Codon bias did not appear to be the reason why the oxacillinase enzyme was not being expressed as it was induced in a *E. coli* RosettaBlue™ strain, which provides the tRNAs for rarely expressed codons. Therefore, it is possible that the enzyme was unstable in *E. coli* cells and degraded by proteolysis. One of the difficulties associated with the expression of heterologous proteins is inefficient export, which manifests itself by the degradation or aggregation of preproteins in the cytoplasm (Baneyx and Mujacic, 2004). The degradation of misfolded proteins by host proteases guarantees that abnormal polypeptides do not accumulate within the cell and allows amino acid recycling. Targets for degradation include prematurely terminated polypeptides, proteolytically vulnerable folding intermediates that are kinetically trapped off-pathway and partially folded proteins that have failed to reach a native conformation after multiple cycles of interactions with folding modulators (Baneyx and Mujacic, 2004). Although, the host strains, *E. coli* BL21 (DE3) and *E. coli* RosettaBlue™ used were deficient in two proteases, Lon and ompT, these are only two of many proteases in

the cytoplasm. In the cytoplasm, proteolytic degradation is initiated by five heat shock proteases (Lon, ClpYQ/HslUV, ClpAP, ClpXP and FtsH) and completed by peptidases that hydrolyze sequences that are 2-5 residues in length (Baneyx and Mujacic, 2004).

In order to overcome the problem of proteolytic degradation, recombinant proteins can be targeted to the periplasmic space in a Sec-dependent or SRP-dependent fashion by fusing naturally occuring signal sequences, such as PelB to their N-terminus. In *E. coli* cells, the vast majority of proteins destined for export are secreted by the Sec-dependent pathway (Baneyx and Mujacic, 2004). The Sec pathway translocates polypeptides post-translationally, whereas the SRP pathway translocates polypeptides cotranslationally. These two pathways converge at the Sec translocon which transports the polypeptides in an unfolded state across the cytoplasmic membrane (Steiner *et al*., 2006). There are fewer proteases in the periplasm compared to the cytoplasm and many have specific substrates. It may be necessary to add a PelB leader sequence to the N-terminal region of the oxacillinase gene from *P. aeruginosa* PA13. This pelB leader sequence is a sequence of amino acids which when attached to a protein, directs the protein to the periplasmic membrane of *E. coli*, where the sequence is removed by pelB peptidase, which may promote proper folding and protect against proteolytic breakdown (Lee and Raines, 2003). The protein would then be folded into its native conformation by the periplasmic folding helpers, Skp or DsbC (Baneyx and Mujacic, 2004).

5.0 Conclusions

- Thirteen clinical isolates from two Irish hospitals were identified as *Pseudomonas aeruginosa* strains. The methods used included classical methods, API 20NE, Biolog GN and 16S rRNA gene sequencing.

- All of the isolates had similar antibiotic profiles when screened against forty-one antibiotics from eleven antibiotic classes.

- One of the isolates, *P. aeruginosa* PA13, however was much more resistant to the antibiotics tested than the other strains. This isolate was resistant to the same antibiotics as the other isolates but was also resistant to many more antibiotics including: the β-lactams - pipercillin, ticarcillin, carbenicillin, ticarcillin/clavulanic acid, ceftizoxime, cefsulodin, imipenem, the aminoglycosides-gentamicin, netilmicin, streptomycin, tobramycin, sisomicin and the quinolones-ciprofloxacin and ofloxacin. *P. aeruginosa* PA13 was therefore a multiresistant strain.

- Two genes, *aac(6')-Ib* and an oxacillinase gene, conferring antibiotic resistance were located on a Class 1 integron.

- *aac(6')-Ib* was a mutant version of gene. The acetyltransferase *aac(6')-Ib* gene contained the mutant type of the enzyme with a leucine substitution for serine at position 119. This mutation confers gentamicin resistance instead of amikacin resistance.

- A novel oxacillinase gene was identified. The gene was successfully cloned using both the pET-28a and the pPC vectors, however expression of the protein was not detected.

6.0 Future Work

A number of findings have been identified for further study including:

- An aggregative response by *P. aeruginosa* PA13 to high concentrations of gentamicin. This response is thought to contribute to the considerable resistance of the organism to antibiotics.

- Further investigation of the oxacillinase enzyme from *P. aeruginosa* PA13. This would involve adding a pelB leader sequence to the N-terminal region of the oxacillinase gene. This pelB leader sequence is a sequence of amino acids which when attached to a protein, directs the protein to the periplasmic membrane of *E. coli*, where the sequence is removed by pelB peptidase. This modification of the gene may promote proper folding and protect against proteolytic breakdown, thus enhancing protein expression.

- Identification of the location of the integron containing the *aac(6')-Ib* and oxacillinase genes from *P. aeruginosa* PA13.

- If the oxacillinase gene from *P. aeruginosa* PA13 is located on a plasmid then it may be studied by transferring the plasmid to a recipient strain such as *Pseudomonas aeruginosa* PU21 by conjugation.

7.0 Bibliography

Abraham, E. P. and Chain, E. (1940) An enzyme from bacteria able to destroy penicillin. *Nature*, **146**, 837.

Agustín, A. I., Carraminana, J. J., Rota, C. and Herrera, A. (2005). Antimicrobial resistance of *Salmonella* spp. from pigs at slaughter in Spain in 1993 and 2001. *Letters in Applied Microbiology*, **41**, 39-44.

Alberto Pichardo Reyes, E., Bale, M. J., Cannon, W. H. and Matsen, J. M. (1981). Identification of *Pseudomonas aeruginosa* by pyocyanin production on Tech agar. *Journal of Clinical Microbiology*, **13**, 456-458.

Allen, L., Dockrell, D. H., Pattery, T., Lee, D. G., Cornelis, P., Hellewell, P. G. and Whyte, M. K. B. (2005). Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. *The Journal of Immunology*, **174**, 3643-3649.

Altschul, S.F., Gish, W., Miller, W., Meyers, E. W. and Lipman D. J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology*, **215**, 403.

Ambler, R. P. (1980). The structure of β-lactamases. *Philos. Trans. R. Soc. London Ser. B.*, **289**, 321-331.

Aoki, Y. (1994). Bactericidal activity of arbekacin against methicillin-resistant *Staphylococcus aureus*. Comparison with that of vancomycin. *Japanese Journal of Antibiotics*, **47**, 640-646.

Aubert, D., Girlich, D., Naas, T., Nagarajan, S. and Nordmann, P. (2004). Functional and structural characterisation of the genetic environment of extended-spectrum beta-

lactamase *bla-$_{VEB}$* gene from a *Pseudomonas aeruginosa* isolate obtained in India. *Antimicrobial Agents and Chemotherapy*, **48**, 3284-3290.

Babic, M., Hujer, A. M. and Bonomo, R. A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates*, **9**, 142-156.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*, **10**, 411-421.

Baneyx, F. and Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nature Biotechnology*, **22**, 1399-1408.

Barbosa, T. M. and Levy, S. B. (2000). The impact of antibiotic use on resistance development and persistence. *Drug Resistance Updates*, **3,** 303-311.

Barlow, M. and Hall, B. G. (2002). Phylogenetic analysis shows that OXA β-lactamase genes have been on plasmids for millions of years. *J. Mol. Evol*., **55**, 314-321.

Beecher, D. J. and Wong, A. C. L. (2000). Cooperative, synergistic and antagonistic haemolytic interactions between haemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from *Bacillus cereus*. *Pathogenicity and Medical Microbiology*, **146**, 3033-3039.

Bellido, F., Martin, N. L., Siehnel, R. J. and Hancock, R. E. W. (1992). Reevaluation, using intact cells, of the exclusion limit and role of OprF in *Pseudomonas aeruginosa* outer membrane permeability. *Journal of Bacteriology*, **174**, 5196-5203.

Bergey's Manual of Systematic Bacteriology (2001). Vol. 2. Ed. N. R. Krieg and J.G. Holt. Williams and Wilkins Publishers, Baltimore.

Betts, S. and King, J. (1999). There's a right way and a wrong way: *in vivo* and *in vitro* folding, misfolding and subunit assembly of the P22 tailspike. *Structure*, **7**, 131-139.

Biomerieux (1997). Analytical Profile Index (API 20NE) Manual. 6th Edition.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA: a method for preparing plasmid DNA. *Nucleic Acids Research*, **7**, 1513-1523.

Boettger, E. C. (1996). Approaches for the identification of microorganisms. *ASM News*, **62**, 247-250.

Bonfiglio, G., Laksai, Y., Franchino, L., Amicosante, G. and Nicoletti, G. (1998). Mechanisms of β-lactam resistance amongst *Pseudomonas aeruginosa* isolated in an Italian survey. *Journal of Antimicrobial Chemotherapy*, **42**, 697-702.

Brown, V. I. and Lowbury, E. J. L. (1965). Use of an improved cetrimide agar medium and other culture methods for *Pseudomonas aeruginosa*. *J. Clin. Path*., **18**, 752-756.

BSAC (2006). Brithish Society for Antimicrobial Chemotherapy's Methods for Antimicrobial Susceptiblity Testing (Version 5). Available online at http://www.bsac.org.uk/_db/_documents/Version_5_changes_2006.pdf. (Accessed 12th March 2007).

Bunny, K. L., Hall, R. M. and Stokes, H. W. (1995). New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene *catB3*, in an integron in pBWH301. *Antimicrobial Agents and Chemotherapy*, **39**, 686-693.

Bush, K., Jacoby, G. A., Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*, **39**, 1211-1233.

Cameron, F. H., Groot Obbink, D.J., Ackerman, V. P. and Hall, R. M. (1986). Nucleotide sequence of the AAD(2") aminoglycoside adenyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. *Nucleic Acids Research*, **14**, 8625-8635.

Carrio, M. M., Cubarsi, R. and Villaverde, A. (2000). Fine architecture of bacterial inclusion bodies, *FEBS Letters*, **471**, 7–11.

Caselli, E., Powers, R. A., Blasczcak, L. C., Wu, C. Y. E., Prati, F. and Scoichet, B. K. (2001). Energetic, structural, and antimicrobial analyses of β-lactam side chain recognition by β-lactamases. *Chemistry and Biology*, **8**, 17-31.

Casin, I., Bordon, F., Bertin, P., Coutrot, A., Podglajen, I., Brasseur, R. and Collatz, E. (1998). Aminoglycoside 6'-*N*-acetyltransferase variants of the Ib type with altered substrate profile in clinical isolates of *Enterobacter cloacae* and *Citrobacter freundii*. *Antimicrobial Agents and Chemotherapy*, **42**, 209-215.

Casin, I., Hanau-Berçot, B., Podglajen, I., Vahaboglu, H. and Collatz, E. (2003). *Salmonella enterica* serovar Typhimurium bla_{PER-1}-carrying plasmid pSTI1 encodes an extended-spectrum aminoglycoside 6'-*N*-acetyltransferase of type Ib. *Antimicrobial Agents and Chemotherapy*, **47**, 697-703.

Castanheira, M., Toleman, M. A., Jones, R. N., Schmidt, F. J. and Walsh, T. R. (2004). Molecular characterization of a beta-lactamase gene, bla_{GIM-1}, encoding a new subclass of metallo-beta-lactamase. *Antimicrobial Agents and Chemotherapy*, **48**, 4654-4661.

Cerdeno-Tarraga, A. M., Efstratiou, A., Dover, L. G., Holden, M. T., Pallen, M., Bentley, S. D., Besra, G. S., Churcher, C., James, K. D., DeZoysa, A., Chillingworth, T., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Quail, M. A., Rabbinowitsch, E., Rutherford, K. M., Thomson, N. R., Unwin, L.,

Whitehead, S., Barrell, B. G. and Parkhill, J. (2003). The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. *Nucleic Acids Res.*, **31**, 6516-6523.

Chen, C. C., Smith, T. J., Susana, G. K., Wäsch, S., Zawadzke, L. E., Coulson, A. and Herzberg, O. (1996). Structure and kinetics of the β-lactamase mutants S70A and K73H from *Staphylococcus aureus* PC1. *Biochemistry*, **35**, 12251-12258.

Chen, H. Y., Yuan, M. and Livermore, D. M. (1995). Mechanisms of resistance to β-lactam antibiotics amongst *Pseudomonas aeruginosa* collected in the UK in 1993. *J. Med. Microbiol.*, **43**, 300-309.

Chen, W. P., Kuo, T. T. (1993). A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA. *Nucleic Acids Research*, **21**, 2260.

Cladera, A. M., Bannasar, A., Barcelo, M., Lalucat, J. and Garcia-Valdes, E. (2004). Comparative genetic diversity of *Pseudomonas stutzeri* genomovars, clonal structure and phylogeny of the species. *J. Bacteriol.*, 186, 5239-5248.

Clarridge III, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, **17**, 840-862.

Clinical and Laboratory Standards Institute (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard. Seventh edition. *Clinical and Laboratory Standard Institute Document*, **M7-A7,** Wayne, Pennsylvania.

Clinical and Laboratory Standard Institute (2006). Performance standards for antimicrobial disk susceptibility tests; Approved Standard. Ninth Edition. *Clinical and Laboratory Standard Institute Document*, **M2-A9,** Wayne, Pennsylvania.

Clinical and Laboratory Standard Institute (2006). Performance standards for antimicrobial susceptibility testing; Sixteenth Informational Supplement. *Clinical and Laboratory Standard Institute Document*, **M100-S16,** Wayne, Pennsylvania.

Cockerill, F. R. III (1999). Genetic methods for assessing antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, **43**, 199-212.

Collins, C. H. and Lyne, P. M. (1985). Microbiological Methods. 5th edition. Ed. C. H. Collins and P. M. Lyne. Butterworths, London.

Collis, C. M. and Hall, R. M. (1995). Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Antimicrobial Agents and Chemotherapy*, **39**, 155-162.

Collis, C. M., Kim, M., J., Stokes, H. W. and Hall, R. M. (1998). Binding of the purified integron DNA integrase IntI 1 to integron and cassette-associated recombination sites. *Molecular Microbiology*, **29**, 477-490.

Costas, M., Holmes, B., On, S. L. W. and Stead, D. E. (1992). Identification of medically important *Pseudomonas* species using computerised methods, 1-20. *In*- Identification methods in applied and environmental microbiology, Eds. R. G. Board, D. Jones and F. A. Skinner. Blackwell Scientific Publications, Oxford.

Courvalin, P. (1996). The Garrod Lecture: evasion of antibiotic action by bacteria. *Journal of Antimicrobial Chemotherapy*, **37**, 855-869.

Couture, F., Lachapelle, J. and Levesque, R. C. (1992). Phylogeny of LCR-1 and OXA-5 with class A and class D β-lactamases. *Molecular Microbiology*, **6**, 1693-1705.

Cowan and Steel's Manual for the Identification of Medical Bacteria (1993). 3rd Edition. Ed. G. I. Barrow and R. K. A. Feltham. Cambridge University Press, Cambridge, New York.

Craig, W. A. and Gudmundsson, S. (1996). Postantibiotic effect, 296-329. *In* Antibiotics in laboratory medicine, 4th Edition, Ed. V. Lorian. Williams and Wilkins, Baltimore, Md.

Crowder, M. W., Wang, Z., Franklin, S. L., Zovinka, E. P., Benkovic, S. J. (1996). Characterisation of the metal-binding sites of the beta-lactamase from *Bacteroides fragilis*. *Biochemistry*, **35**, 12126-12132.

Crowley, D., Daly, M., Lucey, B., Shine, P., Collins, J. J., Cryan, B., Moore, J. E, Murphy, P., Buckley, G. and Fanning, S. (2002). Molecular epidemiology of cystic fibrosis-linked *Burkholderia cepacia* complex isolates from three national referral centres in Ireland. *Journal of Applied Microbiology*, **92**, 992-1004.

Dagert, M. and Ehrlich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene*, **6**, 23-28.

Dajani, A. S. (2002). Beta-lactam resistance: clinical implications for pediatric patients. *J. Int. Med. Res*., **30**, 2-9.

Dale, J. W., Godwin, D., Mossakowska, D., Stephenson, P., Wall, S. (1985). Sequence of the OXA-2 beta-lactamase: comparison with other penicillin-reactive enzymes. *FEBS Letters*, **191**, 39-44.

Daly, M and Fanning, S. (2000). Characterisation and chromosomal mapping of antimicrobial resistance genes in *Salmonella enterica* serotype Typhimurium. *Applied and Environmental Microbiology*, **66**, 4842-4848.

Daly, M., Villa, L., Pezzella, C., Fanning, S. and Carattoli, A. (2005). Comparison of multidrug resistance gene regions between two geographically unrelated *Salmonella* serotypes. *Journal of Antimicrobial Chemotherapy*, **55**, 558-561.

Danel, F., Hall, L. M. C., Gur, D. and Livermore, D. M. (1997). OXA-15 an extended-spectrum variant of OXA-2 β-lactamase, isolated from a *Pseudomonas aeruginosa* strain. *Antimicrobial Agents and Chemotherapy*, **41**, 785-799.

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science*, **264**, 375-382.

De Angelis, J., Gastel, J., Klein, D. C. and Cole, P. A. (1998). Kinetic analysis of the catalytic mechanism of serotonin *N*-acetyltransferase (EC 2.3.1.87). *J. Biol. Chem.*, **273**, 3045-3050.

De Boer, H. A., Comstock, L. J. and Vasser, M. (1983). The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. *Proc. Natl. Acad. Sci.*, **80**, 21-25.

De Vos, D., Lim, A., Pirnay, J.P., Duinslaeger, L., Revets, H., Vanderkelen, A., Hamers, R. and Cornelis, P. (1997). Analysis of epidemic Pseudomonas aeruginosa isolates by isoelectric focusing of pyoverdine and RAPD-PCR: modern tools. *Burns*, **23**, 379.

Dickenson, D. A. and Forman, H. J. (2002). Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.*, 64, 1019-1026.

Di Conza, J., Ayala, J. A., Power, P., Mollerach, M. and Gutkind, G. (2002). Novel class 1 integron (InS21) carrying *bla*$_{\text{CTX-M-2}}$ in *Salmonella enterica* serovar infantis. *Antimicrobial Agents and Chemotherapy*, **46**, 2257-2261.

Dobrindt, U., Hochhut, B., Hentschel, U. and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology*, **2**, 414-424.

Drancourt, M, Bollet, C., Carlioz, A., Martelin, R., Gayral, J. P. and Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol*., **38**, 3623-3630.

Drenkard E. and Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, **416**, 740-743.

Dyda, F., Klein, D. C., Burgess Hickman, A. (2000). GCN5-related N-acetyltransferases: a structural overview. *Annual Review of Biophysics and Biomolecular Structure*, **29**, 81-103.

Edgar, R. and Bibi, E. (1997). Mdfa, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. *Journal of Bacteriology*, **179**, 2274-2280.

Eliopoulos, G. M. and Moellering, Jr., R. C. (1996). Antimicrobial combinations, 330-396. *In* Antibiotics in laboratory medicine, 4th Edition, Ed. V. Lorian. Williams and Wilkins, Baltimore, Md.

El-Deeb, B. A., Gherbawy, Y. A., El-Sharouny, H. M. and Fahmy, N. (2006). Plasmid incidence, antibiotic and heavy metal resistance patterns of endophytic bacteria isolated from aquatic plant *Echhornia crassipe*. Genebank submission

Fabiane, S. M., Sohi, M. K., Wan, T., Payne, D. J., Bateson, J. H., Mitchell, T., Sutton, B. J. (1998). Crystal structure of the zinc-dependent beta-lactamase from Bacillus cereus at 1.9Å resolution: binuclear active site with features of a mononuclear enzyme. *Biochemistry*, **37**, 12404-12411.

Fang, G., Rocha, E., Danchin, A. (2005). How essential are non essential genes? *Mol. Biol. Evol.*, **22**, 2147-2156.

Fishman, N. (2006). Antimicrobial stewardship. *Am. J. Infect. Control*, **34**, 55-73.

Fluit, A. C. and Schmitz, F. J. (1999). Class 1 integrons, gene cassettes, mobility and epidemiology. *Eur. J. Clin. Microbiol. Infect. Dis.*, **18**, 761-770.

Fluit, A. C. and Schmitz, F. J. (2004). Resistance integrons and super-integrons. *Clinical Microbiology and Infection*, **10**, 272-288.

Forkner, C.E. (1960). *Pseudomonas aeruginosa* infections. *In* Modern medical monographs No. 22, Ed. I. S. Wright. Grune and Stratton, New York and London.

Forster, S. Snape, J. R., Lappin-Scott, H. M. and Porter, J. (2002). Simultaneous fluorescent Gram staining and activity assessment of activated sludge bacteria. *Appl. Env. Microbiol.*, **68**, 4772-4779.

Fox, G. E., Stackebrandt, E., Hespel, R. B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magrum, L. J., Zablen, L. B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Leuhrsen, K. R., Chen, K. N. and Woese, C. R. (1980). The phylogeny of prokaryotes. *Science*, **209**, 457-463.

Fuqua, C., Parsek, M. R. and Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annual Review of Genetics*, **35**, 439.

Galimand, M., Lambert T., Gerbaud, G. and Courvalin, P. (1993). Characterization of the *aac(6')*-Ib gene encoding an aminoglycoside 6'-*N*-acetyltransferase in

Pseudomonas aeruginosa BM2656. *Antimicrobial Agents and Chemotherapy*, **37**, 1456-1462.

García-Martínez, J., Bescós, I., Rodríguez-Sala, J. J. and Rodríguez-Valera, F. (2001). RISSC: a novel database for ribosomal 16S-23S RNA genes spacer regions. *Nucleic Acids Research*, **29**, 178-180.

Giamarellou, H. (2002). Prescribing guidelines for severe *Pseudomonas* infections. *Journal of Antimicrobial Chemotherapy*, **49**, 229-233.

Girlich, D., Naas, T. and Nordmann, P. (2004). Biochemical characterization of naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **48**, 2043-2048.

Girlich, D., Poirel, L., Leelaporn, A., Karim, A., Tribuddharat, C., Fennewald, M. and Norddmann, P. (2001). Molecular epidemiology of the integron-located VEB-1 extended-spectrum ß-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. *Journal of Clinical Microbiology*, **39**, 175-182.

Giuliani, F., Docquier, J. D., Riccio, M. L. Pagani, L. and Rossolini, G. M. (2005). OXA-46, a new class D β-lactamase of narrow substrate specificity encoded by a $bla_{\text{VIM-1}}$-containing integron from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrobial Agents and Chemotherapy*, **49**, 1973-1980.

Gobel, U. B., Geiser, A. and Stanbridge, E. J. (1987). Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *Journal of General Microbiology*, **133**, 1969-1971.

Golemi, D., Maveyraud, L., Vakulenko, S., Samama, J. P. and Mobashery, S. (2001). Critical involvement of a carbamylated lysine in catalytic function of Class D β-lactamases. *Biochemistry*, **98**, 14280-14285.

Golemi-Kotra, D. (2002). Mechanistic details of class A and D β-lactamases. Ph.D. Thesis, Wayne State University, Detroit, Michigan.

Gombac, F., Riccio, M. L., Rossolini, G. M., Lagatolla, C., Tonin, E., Monti-Bragadin, C., Lavenia, A. and Dolzani, L. (2002). Molecular characterization of integrons in epidemiologically unrelated clinical isolates of *Acinetobacter baumannii* from Italian hospitals reveals a limited diversity of gene cassette arrays. *Antimicrobial Agents and Chemotherapy*, **46**, 3665-3668.

Gonzalez, L. S. and Spencer, J. P. (1998). Aminoglycosides: A practical review. *American Family Physician*, **58**, 1811-1820.

Goto, S. and Enomoto, S. (1970) Nalidixic acid cetrimide agar. A new selective plating medium for the selective isolation of *Pseudomonas aeruginosa*. *Japan Journal of Microbiology* **14**, 65-72.

Green, R. and Noller, H. F. (1997). Ribosomes and translation. *Annual Reviews in Biochemistry*, **66**, 679-716.

Greenwood, D. (1995). *In* Antimicrobial Chemotherapy, 32-48. Ed. D.Greenwood. Oxford University Press, Oxford, U. K.

Greenwood, D. and Whitley R. (2003). Modes of action p.11-24. *In* Antibiotic and Chemotherapy (8th edition) Eds. R.G. Finch, D. Greeenwood, S.R. Norrby and R.J. Whitley, Churchill Livingstone, New York.

Griffith, O. W. (1999). Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radical Biol. Med.*, **27**, 922-935.

Gustafsson, C., Govindarajan, S. and Minshull, J. (2004). Codon bias and heterologous protein expression. *Trends Biotechnology*, **22**, 346-353.

Gutell, R. R. (1994). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Research*, **22**, 3502-3507.

Hall, L. M., Livermore, D. M., Gur, D., Akova, M. and Akalin, H. E. (1993). OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **37**, 1637-1644.

Hall, R. M. and Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular Microbiology*, **15**, 593-600.

Hall, R. M. and Recchia, G. D. (1995). Gene cassettes: a new class of mobile element. *Microbiology*, **141**, 3015-3027.

Hall, R. M. and Stokes, H. W. (2004). Integrons or super integrons? *Microbiology*, **150**, 3-4.

Hampton, K. D. and Wasilauskas, B. L. (1979). Isolation of oxidase-negative *Pseudomonas aeruginosa* from sputum culture. *Journal of Clinical Microbiology*, **9**, 632-634.

Hanahan, D. (1985). Techniques for transformation of *E. coli*. *In* D.M. Glover (Ed.). *DNA cloning: a practical approach*, **1**, 91-109. IRL Press Ltd., Oxford, England.

Hancock, R. E. W. (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. *Clin. Infect. Dis*., **27**, 93-9.

Hancock, R. E. W. and Brinkman, F. S. L. (2002). Function of P*seudomonas* porins in uptake and efflux. *Annual Reviews in Microbiology*, **56**, 17-38.

Hancock, R. E. W., Siehnel, R. and Martin, N. (1990). Outer membrane proteins of *Pseudomonas*. M*ol. Microbiol*., **4**, 1096-1075.

Hanning, G and Makrides, S. C (1998). Strategies for optimising protein expression in *Escherichia coli*. *Trends in Biotechnology*, **16**, 54-60.

Harmsen, D., Karch, H. (2004). 16S rDNA for diagnosing pathogens: a living tree. *ASM News*, **70**, 19-24.

Hart, C. A. (1998). Antibiotic resistance: an increasing problem? *British Medical Journal*, **316**, 1255-1256.

Hassan, H. M. and Fridovich, I. (1980). Mechanism of antibiotic action of pyocyanin. *J. Bacteriol*., **141**, 156-163.

Health Protection Surveillence Centre (2006). The management of invasive group A streptococcal infections in Ireland. Available online at http://www.hpsc.ie/hpsc/AZ/Other/GroupAStreptococcalDiseaseGAS/Publications/File,2080,en.pdf (Accessed 11th November 2006)

Hegedus, Z. L. (2000). The probable involvement of soluble and deposited melanins, their intermediates and the reactive oxygen side-products in human diseases and aging. *Toxicology*, **145**, 85-101.

Heinaru, E., Truu, J., Scottmeister, U. and Heinaru, A. (2000). Three types of phenol and p-cresol catabolism in phenol and p-cresol degrading bacteria isolated from river water continuously polluted with phenolic compounds. *FEMS Microbiol Ecol*., **31**, 195-205.

Heinzl, B., Eber, E., Oberwaldner, B., Haas, G. and Zach, M. S. (2002). Effects of inhaled gentamicin prophylaxis on acquisition of *Pseudomonas aeruginosa* in children with cystic fibrosis: a pilot study. *Pediatric Pulmonology*, **33**, 32-37.

Heiss, S., Benoit, Y., Marechaux, C. and Monot, F. (2004) GenBank Submission.

Héritier, C., Poirel, L., Fournier, P. E., Claverie, J. M., Raoult, D. and Nordmann, P. (2005). Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, **49**, 4174-4179.

Hernandez-Valladares, M., Felici, A., Weber, G., Adolph, H. W., Zeppezauer, M., Rossolini, G. M., Amicosante, G., Frere, J. M., Galleni, M. (1997). Zn(II) dependence of the *Aeromonas hydrophila* AE036 metallo-beta-lactamase activity and stability. *Biochemistry*, **36**, 11534-11541.

Herzberg, O. (1991). Refined Crystal Structure of Beta-Lactamase from *Staphylococcus aureus* PC1 at 2.0Å resolution. *J. Mol. Biol*., **217**, 701-719.

Heuer, H., Krögerrecklenfort, E., Wellington, E. M. H., Egan, S., van Elsas, J. D., van Overbeek, L., Collard, J.-M., Guillaume, G., Karagouni, A. D., Nikolakopoulou, T. L. and Smalla, K. (2002). Gentamicin resistance gene in environmental bacteria: prevalence and transfer. *FEMS Microbiology Ecology*, **42**, 289-302.

Hickman, A. B., Namboodiri, M. A. A., Klein, D. C., Dyda, F. (1999). The structural basis of ordered substrate binding by serotonin *N*-acetyltransferase: enzyme complex at 1.8 Å resolution with a bisubstrate analogue. *Cell*, **97**, 361-369.

Hunke, S. and Betton, J. M. (2003). Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli*. *Molecular Microbiology*, **10**, 1-11.

Huovinen, P., Huovinen, S., Jacoby, G. A. (1988). Sequence of PSE-2 beta-lactamase. *Antimicrobial Agents and Chemotherapy*, **32**, 134-136.

Iglewski, B. H. (1996). Pseudomonas. *In: Baron's Medical Microbiology,* S. Barron (Ed.), *4th ed*., Univ of Texas Medical Branch, U. S. (via NCBI bookshelf).

Jacoby, G. (2006). β-lactamase nomenclature. *Antimicrobial Agents and Chemotherapy*, **50**, 1123-1129.

Jacoby, G., Bush, K. Amino acid sequences for TEM, SHV, and OXA extended-spectrum and inhibitor resistant β-lactamases. (Accessed May 23rd 2006 at http:www.lahey.org/studies/webt.htm).

Jeannot, K., Sobel, M. L., El Garch, F., Poole, K. and Plésiat, P. (2005). Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. *Journal of Bacteriology*, **187**, 5341-5346.

Joris, B., Ghuysen, J. M., Dive, G., Renard, A. Dideberg, O., Charlier, P., Frére, J. M., Kelly, J. A., Boyington, J. C. and Moews, P. C. (1988). The active-site-serine penicillin-recognizing enzymes as members of the Streptomyces R61 DD-peptidase family. *Biochem. J*., **250**, 313-324.

Kaleli, I., Cevahir, N., Demir, M., Yildirim, U. and Sahin, R. (2006) Anticandidal activity of *Pseudomonas aeruginosa* strains isolated from clinical specimens. *Mycoses*, **50**, 74-78.

Kandela, S. A., al-Shibib, A. S. and al-Khayat, B. H. (1997) A study of purified pyorubin produced by local *Pseudomonas aeruginosa*. *Acta. Microbiol. Pol*., **46**, 37-43.

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology*, **6**, 494-500.

Kanner, D., Gerber, N. N. and Bartha, R. (1978). Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa. Journal of Bacteriology*, **134**, 690-692.

Kaulfers, P. M. and Brandt, D. (1987). Isolation of a conjugative plasmid in *Escherichia coli* determining formaldehyde resistance. *FEMS Microbiol. Lett.*, **43**, 161-163.

Keane, T. M., Naughton, T. J. and McInerney, J. O. (2007) MultiPhyl: A high-throughput phylogenomics webserver using distributed computing, *Nucleic Acids Research*. In press.

Keith, K. E., Oyston, P. C., Crossett, B., Fairweather, N. F., Titball, R. W., Walsh, T. R. and Brown, K. A. (2005). Functional characterisation of OXA-57, a Class D β-lactamase from *Burkholderia pseudomallei*. *Antimicrobial Agents and Chemotherapy*, **49**, 1639-1641.

Kelly, J. A., Dideberg, O., Charlier, P., Wery, J. P., Libert, M., Moews, P. C., Knox, J. R., Duez, C., Fraipont, C., Joris, B. (1986). On the origin of bacterial resistance to a penicillin target. *Science*, **231**, 1429-1431.

Kerr, J. R., Taylor, G. W., Rutman, A., Hoiby, N., Cole, P. J. and Wilson, R. (1999). *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J. Clin. Pathol.*, **52**, 385-387.

Kiefhaber, T., Rudolph, R., Kohler, H. H. and Buchner, J. (1991). Protein aggregation *in vitro* and *in vivo*: a quantitative model of the kinetic competition between folding and aggregation. *Biotechnology*, **9**, 825-829.

King, E. O., Ward, M. K. and Raney, E. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med*., **44**, 301.

Kobashi, Y., Hasebe, A., Nishio, M. and Uchiyama, H. (2007). Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. *Microbes Environ*., **22**, 44-51.

Koch, A. L. (2003). Bacterial wall as target for attack: past present and future research. *Clinical Microbiology Reviews*, **16**, 673-678.

Köhler, T., Kok, M., Michea-Hamzehpour, M., Plesiat, P.Gotoh, N. Nishino, T., Curty, K. C. and Pechere, J. C. (1996). Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **40**, 2288-2290.

Kolbert, C. P. and Persing, D. H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current Opinion in Microbiology*, **2**, 299-305.

Kücken, D., Feucht, H. H. and Kaulfers, P. M. (2000). Association of *qacE and qacEΔ1* with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. *FEMS Microbiology Letters*, **183**, 95-98.

Laemelli, U. K. (1970). Cleavage of structural proteins during the assemble of the head bacteriophages T4. *Nature*, **227**, 680-685.

Lambert P. A. (2002) Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J R Soc Med*, **95**, S 41, 22-26.

Lambert, T., Ploy, M. C. and Courvalin, P. (1994). A spontaneous point mutation in *aac(6')-Ib* gene results in altered substrate specificity of aminoglycoside 6'-*N*-acetyltransferase of a *Pseudomonas fluorescens* strain. *FEMS Microbiology Letters*, **115**, 297-304.

Lamont, I. L. and Martin, L. W. (2003) Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*. *Microbiology*, **149**, 833-842.

Laraki, N., Galleni, M., Thamm, I., Riccio, M. L., Amicosante, G., Frere, J. M., Rossolini, G. M. (1999). Structure of In31, a *bla*IMP-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. *Antimicrobial Agents and Chemotherapy*, **43**, 890-901.

Lau, G. W., Hassett, D. J., Ran, H. and Kong, F. (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends in Molecular Medicine*, **10**, 599.

Ledent, P., Raquet, X., Joris , B., Van Beeumen, J. and Frere, J. M. (1993). A comparative study of the class D β-lactamases. *Biochem. J*., **292**, 555-562.

Lee, B., Haagensen, J. A. J., Ciofu, O., Andersen, J. B., Hoiby, N. and Molin, S. (2005) Heterogeneity of Biofilms Formed by Nonmucoid *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis. *J. Clin. Microbiol*., **43**, 5247-5255.

Lee, E. L., Raines, R. T. (2003). Contribution of active site residues to the function of onconase, a ribonuclease with antitumurol activity. *Biochemistry*, **42**, 11443-11450.

Levesque, C., Brassard, S., Lapointe, J. and Roy, P. H. (1994). Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integron, *Gene*, **142**, 49-54.

Li, X. Z., Livermore, D. M. and Nikaido, H. (1994) (a). Role of efflux pump(s) in intrinsic of *Pseudomonas aeruginosa*: Resistance to tetracycline, chloramphenicol and norfloxacin. *Antimicrobial Agents and Chemotherapy*, **38**, 1732-1741.

Li, X. Z., Ma, D., Livermore, D. M. and Nikaido, H. (1994) (b). Role of efflux pump(s) in intrinsic of *Pseudomonas aeruginosa*: Active efflux as a contributing factor to β-lactam resistance. *Antimicrobial Agents and Chemotherapy*, **38**, 1742-1752.

Lindstedt, B-A., Heir, E., Nygård, I. and Kapperud, G. (2003). Characterization of class 1 integrons n clinical strains of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis from Norwegian hospitals. *Journal of Medical Microbiology*, **52**, 141-149.

LiPuma, J. J., Dulaney, B. J., McMenamin, J. D., Whitby, P. W., Stull, T. L., Coenye, T. and Vandamme, P. (1999). Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patients. *Journal of Clinical Microbiology*, **133**, 1969-1971.

Liu, P. (1976). Biology of *Pseudomonas aeruginosa*. *Hosp. Pract*., **11**, 139-147.

Livermore, D. M. (1995). β-lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*, **8**, 557-584.

Livermore, D. M. (2002). Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin. Infect. Dis.*, **34**, 634-640.

Loeb, M. B., Craven, S., McGeer, A. J., Simor, A. E., Bradley, S. F., Low, D. E., Armstrong-Evans, M., Moss, L. A. and Walter, S. D. (2003). Risk factors for

resistance to antimicrobial agents among nursing home residents. *American Journal of Epidemiology*, **157**, 40-47.

Lowbury, E. J. L. and Collins, A. G. (1955). The use of a new cetrimide product in a selective medium for *Pseudomonas pyocyanea*. *Journal of Clinical Pathology*, **8**, 47-48.

Lu, W.P., Kincaid, E., Sun, Y. and Bauer, M. D. (2001). Kinetics of beta-lactam interactions with penicillin-susceptible and resistant penicillin-binding protein 2x proteins from *Streptococcus pneumoniae*: Involvement of acylation and deacylation in beta-lactam resistance. *J. Biol. Chem.*, **276**, 31494-31501.

Lyczak, J. B., Cannon, C. L. and Pier G. B. (2002). Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews*, **15**, 194-222.

Maiden, M. C. J. (1998). Horizontal genetic exchange, evolution and spread of antibiotic resistance in bacteria. *Clin. Infect. Dis*., **27**, 12-20.

Majiduddin, F. K., Materon, I. C. and Palzkill, T. G. (2002). Molecular analysis of beta-lactamase structure and function. *Int. J. Med. Microbiol*, **292**, 127-137.

Mandaville S. M. (2002). Bacterial Source Tracking (BST) – A review. Project H-2, Soil and Water Conservation Society of Metro Halifax. Accessed online at http://lakes.chebucto.org/H-2/bst.html (10th January 2007).

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*, **64**, 795-799.

Martinez, J. L. and Baquero, F. (2000). Mutational frequencies and antibiotic resistance. *Antimicrobial Agents and Chemotherapy*, **44**, 1771-1777.

Martinez-Freijo, P., Fluit, F. J., Schmitz, V. S., Grek, V.S.C., Verhoef and Jones, M. E. (1998). Class 1 integrons in Gram-negative isolated and association with decreased susceptibility to multiple antibiotic compounds. *Journal of Antimicrobial Chemotherapy*, **42**, 689-696.

Masahisa, H., Hiroyuki, S., Keiko, A., Tadao, T., Kiyoshi, A., Toshiyuki, O., Takako, I., Shizuka, L. and Tetsuro, M. (2005). A bacteriological study of multiple-drug-resistant *Pseudomonas aeruginosa* isolates derived from 2 patients. *J. Univ. Occup. and Environ. Health*, **27**, 209.

Masterton, R. (2003). A quick guide to antibiotic resistance-antibiotic resistance in noscomial infections. Available online at http://www.infectionacademy.org (18th August 2005).

Massova, I. And Mobashery, S. (1998). Kinship and diversification of bacterial penicillin-binding protein and β-lactamases. *Antimicrobial Agents and Chemotherapy*, **42**, 1–17.

Matagne, A. Lamotte-Brasseur, J. Frere, J. M. (1998). Catalytic properties of Class A beta-lactamases: efficiency and diversity. *Biochem. J*., **330**, 581-598.

Maveyraud, L., Golemi, D., Kotra, L. P.,Tranier, S., Vakulenko, S., Mobashery, S., Samama, J. P (2000). Insights into class D beta-lactamases are revealed by the crystal stucture of the OXA10 enzyme from *Pseudomonas aeruginosa*. *Structure*, **8**, 1289-1298.

Mavrodi, D. V., Bonsall, R. F., Delaney, S. M., Soule, M. J., Philips, G. and Thomashow, L. S. (2001). Functional analysis of genes for biosynthesis of pyocyanin

and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology*, **183**, 6454-6465.

Mazel, D. and Davies, J. (1999). Antibiotic resistance in microbes. *Cellular and Molecular Life Sciences*, **56**, 742-754.

Mazel, D. (2004). Integrons and the origin of antibiotic resistance gene cassettes. *ASM News*, **70**, 520-525.

Mellon, M., Benbrook, C., Benbrook, K. (2001). *In* Estimates of antimicrobial abuse in livestock, p.60. Ed. M. Mellon.Union of concerned scientists. Cambridge, MA.

Mendes, R. E., Toleman, M. A., Ribeiro, J., Sader, H. S., Jones, R. N. and Walsh, T. R. (2004). Integron carrying a novel metallo-beta-lactamase gene, *bla*$_{IMP}$-16, and a fused form of aminoglycoside-resistant gene *aac(6')*-30/*aac(6')*-Ib': report from the SENTRY Antimicrobial Surveillance Program. *Antimicrobial Agents and Chemotherapy*, **48**, 4693-4702.

Merck (2007). β-lactams. *The Merck Online Medical Library*. Available online at http://www.merck.com/mmpe/sec14/ch170/ch170c.html#S14_CH170_T008. (Accessed 3rd March 2007).

Merimaa, M., Heinaru, E., Liivak, M., Vedler, E. and Heinaru, A. (2006). Grouping of phenol hydroxlase and catechol 2, 3 dioxygenase genes among phenol and p-cresol degrading *Pseudomonas* species and biotypes. *Arch. Microbiol.*, **186**, 287-296.

Mesaros, N., Nordmann, P., Plésiat, P., Roussel-Devallez, M., Van Eldere, J., Glupczynski, Y., Van Laethem, Y., Jacobs, F., Lebecque, P., Malfroot, A., Tulkens, P. M. and Van Bambeke, F. (2007). *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millenium. *Clinical Microbiology and Infection*, **13**, 560-578.

Michel, B., Proudfoot, A. E. I., Wallace, C. J. A. and Bosshard, H. R. (1989). The cytochrome c oxidase-cytochrome c complex: spectroscopic analysis of conformational changes in the protein-protein interaction domain. *Biochemistry*, **28**, 456-462.

Miller , G. H., Sabatelli, F. J., Naples, L., Hare, R. S. and Shaw, K. J. (1995). The changing nature of aminoglycoside resistance mechanisms and the role of isepamicin. A new broad spectrum aminoglycoside. *Journal of Chemotherapy*, **7**, S31-S44.

Mingeot-Leclercq, M-P., Glupczynski, Y. and Tulkens, P. M. (1999). Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy*, **43**, 727-737.

Mingeot-Leclercq, M-P. and Tulkens, P. M. (1999). Aminoglycosides: Nephrotoxicity. *Antimicrobial Agents and Chemotherapy*, **43**, 1003-1012.

Moellering, R. C. Jr. (2006). Vancomycin: a 50-year reassessment. *Clin. Infect. Dis*., **42**, 3-4.

Mordoch, S. S., Granot, D., Lebendiker, M. and Schulinder, S. (1999). Scanning cysteine accessibility of EmrE, an H^+-coupled multidrug transporter from *Escherichia coli,* reveals a hydrophobic pathway for solutes. *J. Biol. Chem.*, **274**, 19480-19486.

Mugnier, P., Casin, I., Bouthors, A. T. and Collatz, E. (1998). Novel OXA-10-derived extended spectrum beta-lactamases selected in vivo or in vitro. *Antimicrobial Agents and Chemotherapy*, **42**, 3113-3116.

Mulvey, M.R., Boyd, D. A., Baker, L., Mykytczuk, O., Reis, E. M., Asensi, M. D., Rodrigues, D. P and Ng, L. K. (2004). Characterization of a *Salmonella enterica* serovar Agona strain harbouring a class 1 integron containing novel OXA-type beta-

lactamase (*bla*$_{OXA}$-53) and 6'-N-aminoglycoside acetyltransferase gene *aac(6')*-I30. *Journal of Antimicrobial Chemotherapy*, **54**, 354-359.

Mac Gowan, A. P., Wise, R. (2001). Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *Journal of Antimicrobial Chemotherapy*, **48**, S1, 17-28.

McDonnell, G. and Russell A. (1999). Antiseptics and disinfectants: activity, action and resistance. *Clin. Microbiol. Rev.,* **12**, 147-197.

Naas,T., Sougakoff,W., Casetta,A. and Nordmann,P. (1998). Molecular characterization of OXA-20, a novel class D beta-lactamase, and its integron from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **42**, 2074-2083

Naas, T. and Nordmann, P. (1999). OXA-type β-lactamases. *Current Pharmaceutical Design*, **5**, 865-879.

Nešvera, J. Hochmannová, J. Pátek, M. (1998). An integron of class 1 is present on the plasmid pCG4 from Gram-positive bacterium *Corynebacterium glutamicum*. *FEMS Microbiology Letters*, **169**, 391-395.

Neu, H. C. (1992). The crisis in antibiotic resistance. *Science*, **257**, 1064-1073.

Neuwald, A. F., Landsman, D. (1997). GCN5-related histone *N*-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem. Sci.*, **22**, 154-155.

Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, **33**, 1831-1836.

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science*, **264**, 382-387.

Nikaido, H. (1998). Antibiotic resistance caused by Gram-negative multidrug efflux pumps. *Clin. Infect. Dis*., **27**, S32-S41.

Nordenberg, T. (1998). Miracle drugs vs. Superbugs – Preserving the usefulness of antibiotics. *FDA Consumer Magazine*, **32**, 6.

Norrby, S. R. (2005). Integrons: adding another threat to the use of antimicrobial therapy. *Clin. Infect. Dis*., **41**, 10-11.

Nosanchuk, J. D. and Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cell Microbiology*, **5**, 203-223.

Novagen (2005). pET Systems Manual, 11th Edition.

Novy, R., Drott, D., Yaeger, K. and Mierendorf, R. (2001). Overcoming the codon bias of E. coli for enhanced protein expression. *Innovations Newsletter*, **12**, 1-3.

Nunez, B., Cabezas, M. and de la Cruz, F. (2001). GenBank submission.

Obritsch, M. D., Fish, D. N., MacLaren, R. and Jung, R. (2005) Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. *Pharmacotherapy*, **25**, 1353-1364.

Ogle, J. M., Brodersen, D. E., Clemons Jr., W. M., Tarry, M. J., Carter, A. P., Ramarkrisnan (2001). Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science*, **292**, 897-902.

Ogunnariwo, J. and Hamilton-Miller, J. M. (1975). Brown and red-pigmented *Pseudomonas aeruginosa*: differentiation between melanin and pyorubin. *J. Med. Microbiol*., **8**, 199-203.

Okii, M., Iyobe, S., Mitsuhashi, S. (1983). Mapping of the gene specifying aminoglycoside 3'-phosphptransferase II on *Pseudomonas aeruginosa* chromosome. *Journal of Bacteriology*, **155**, 643-649.

Oliphant, C. M. and Green, G. M. (2002). Quinolones: a comprehensive review. *American Family Physician*, **65**, 455-464.

Ouellette, M., Gerbaud, G., Lambert, T. and Courvalin, P. (1987). Acquisition by a Campylobacter-like strain of aphA-1, a kanamycin resistance determinant from members of the family *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy*, **31**, 1021-1026.

O'Callaghan, E. M., Tanner, M. S. and Boulnois, G. J. (1994). Development of a PCR probe test for identifying *Pseudomonas aeruginosa* and *Pseudomonas* (*Burkholderia*) *cepacia*. *Journal of Clinical Pathology*, **47**, 222-224.

O'Cuív, P. (2003). Identification and characterisation of novel iron acquisition mechanisms in *Sinorhizobium meliloti* 2011 and *Pseudomonas aeruginosa*. PhD Thesis, Dublin City University.

O'Malley, Y. Q., Reszka. K. J., Spitz, D. R., Denning, G. M. and Britigan, B. E. (2004). *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol*, **287**, 94-103.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science*, **276**, 734-740.

Paetzel, M., Danel, F., de Castro, L., Mosimann, S. C., Page, M. G., Strynadka, N. C. (2000). Crystal structure of the class D beta-lactamase OXA-10. *Nat. Struct. Biol*., **7**, 918-925.

Page, M. G. P. (2000). β-lactamase inhibitors. *Drug Resistance Updates*, **3**, 109-125.

Palleroni, N. J. (1986). Taxonomy of the Pseudomonads, 3-26. *In* The bacteria - A treatise on structure and function, Vol. X. The Biology of *Pseudomonas*. Ed. J. R. Sokatch. Academic Press, Orlando, Diego.

Partridge, S. R., Collis, C. M., Hall, R. M. (2002). Class 1 integron containing a new gene cassette, aadA10, associated with Tn1404 from R151. *Antimicrobial Agents and Chemotherapy*, **46**, 2400-2408.

Paster, B. J., Dewhirst, F. E., Socransky, S. S. and Haffajee, A. (2001). GenBank submission.

Patterson, D. L. (1999) Reduced susceptibility of *Staphylococcus aureus* to vancomycin -- a review of current knowledge. *Commun Dis Intell*., **24**: 69-73.

Paulsen, I. T., Brown, M. H., Skurray, R. A., (1996). Proton-dependent multidrug efflux systems. *Microbiological Reviews*, **60**, 575-608.

Paulsen, I. T., Littlejohn, T.G., Radström, P., Sundström, L., Sköld, O., Swedberg, G. and Skurray, R. (1993). The 3'conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrobial Agents and Chemotherapy*, **37**, 761-768.

Pernot, L., Frenois, F., Rybkine, T., L'Hermite, G., Petrella, S., Delettre, J., Jarlier, V., Collatz, E., Sougakoff, W. (2001). Crystal structures of the class D beta-lactamase

OXA-13 in the native form and in complex with meropenem. *J. Mol. Biol*., 310, 859-874.

Petroni, A., Corso, A., Melano, R., Cacace, M. L., Bru, A. M., Rossi, A. and Galas, M. (2002). Plasmidic extended-spectrum β-lactamases in Vibrio cholerae O1 El Tor isolates in Argentina. *Antimicrobial Agents and Chemotherapy*, **46**, 1462-1468.

Petrosino, J., Cantu III, D., Palzkill, T. (1998). Beta-lactamases: protein evolution in real time. *Trends Microbiol*., **6**, 323-327.

Philippon, L. N., Naas, D., Bouthors, A.T., Barakett, V. and Nordmann, P. (1997). OXA-18, a class D clavulanic acid-inhibited extended spectrum β-lactamase from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **41**, 2188-2195.

Ploy, M. C., Courvalin, P. and Lambert, T. (1998). Characterisation of In40 of *Enterobacter aerogenes* BM2688, a class 1 integron with two new gene cassettes, *cmlA2* and *qacF*. *Antimicrobial Agents and Chemotherapy*, **42**, 2557-2563.

Poirel, L., Gerome, P., De Champs, C., Stephanazzi, J., Naas, T. and Nordmann, P. (2002). Integron-located *oxa-32* gene cassette encoding and extended-spectrum variant of OXA-2 beta-lactamase from *P.aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **46**, 566-569.

Poirel, L., Girlich, D., Naas, T. and Nordmann, P. (2001) (a). OXA-28, an extended-spectrum variant of OXA-10 β-lactamase from *Pseudomonas aeruginosa* and its plasmid and integron-located gene. *Antimicrobial Agents and Chemotherapy*, **45**, 447-453.

Poirel, L., Weldhagen, T., Naas, C., de Champs, M. G. and Nordmann, P. (2001) (b). GES-2, a class A beta lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. *Antimicrobial Agents and Chemotherapy*, **45**, 2598-2603.

Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **49**, 479-487.

Poole, K. and Srikumar, R. (2001). Multidrug efflux systems in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr. Top. Med. Chem.*, **1**, 59-71.

Power, P., Galleni, M., Di Conza, J., Ayala, J. A. and Gutkind, G. (2005). Description of In116, the first blaCTX-M-2-containing complex class 1 integron found in *Morganella morganii* isolates from Buenos Aires, Argentina. *Journal Antimicrob. Chemother*., **55**, 461-465

Pratt, R. F. (2002). Functional evolution of the serine β-lactamase active site. *J. Chem. Soc. Perkin. Trans.*, **2**, 851-861.

Prieto, M. A., Galán, B. Torres, B., Férrández, A., Férrández, C., Minambres, B., Garcia, J. L. and Diaz, E. (2004). Aromatic metabolism versus carbon availability: the regulatory network that controls catabolism of less-preferred carbon sources in *Escherichia coli*. *FEMS Microbiology Reviews*, **28**, 503.

Qinghu, R., Chen, K. and Paulsen, I. T. (2006). TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Research*, **35**, 274-279.

Rather, P. N., Munayyer, H., Mann, P. A., Hare, R. S., Miller, G. H. and Shaw, K. J. (1992). Genetic analysis of bacterial acetyltransferases: identification of amino acids determining the specificities of the aminoglycoside acetyltransferase Ib and IIa proteins. *Journal of Bacteriology*, **174**, 3196-3203.

Rayner, C. F. J, Cole, P. J. and Wilson R. (1994). The management of chronic bronchial sepsis due to bronchiectasis. *Clin. Pulm. Med.,* **1**, 348-355.

Recchia, G.D. and Hall, R. M. (1995). Gene cassettes: a new class of mobile element. *Microbiology*, **141**, 3015-3027.

Rice, L. B., Willey, S. H., Papanicolaou, G. A., Medeiros, Eliopoulos, G. M., Moellering Jr., R. C. and Jacoby, G. A. (1990). Outbreak of ceftazidime resistance caused by extended-spectrum beta-lactamases at a Massachusetts chronic-care facility. *Antimicrobial Agents and Chemotherapy*, **34**, 2193-2199.

Richardson, J. S. (1981). The anatomy and taxonomy of protein structure. *Adv. Protein Chem.*, **34**, 167-330.

Rodnina, M. V., Beringer, M. and Wintermeyer, W. (2007). How ribosomes make peptide bonds. *Trends. Biochem. Sci*., **32**, 20-26.

Rojas, J. R., Trievel, R. C., Zhou, J., Mo, Y and Li, X. (1999). Crystal structure of *Tetrahymena* GCN5 with bound coenzyme-A and histone H3 peptide. *Nature*, **401**, 93-98.

Rolinson, G. N. (1971). Bacterial resistance to penicillins and cephalosporins. *Proc. R. Soc. Lond. B*., **179**, 403-410.

Rosser, S. J. and Young, H. K. (1999). Identification and characterisation of class 1 integrons in bacteria from an aquatic environment. *Journal of Antimicrobial Chemotherapy*, **44**, 11-18.

Rossolini, G. M. and Mantengoli (2005). Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clinical Microbiology and Infectious Disease*, **11**, S4, 17-32.

Rowe-Magnis, D. A., Guerout, A. M. and Mazel, D. 2002. Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Molecular Microbiology*, **43**, 1657-1669.

Rowe-Magnis, D. A. and Mazel, D. (2002). The role of integrons in antibiotic resistance gene capture. *Int. J. Med. Microbiol*., **292**, 115-125.

Roy, P. H. (1999). Horizontal transfer of genes in bacteria. *Microbiology Today*, **26**, 168-170.

Russell, A. D. and Chopra, I. (1990). Understanding antibacterial action and resistance. Ed. M. H. Rubinstein. Ellis Horwood, New York.

Sacchi, C. T., Whitney, A. M., Mayer, L. W, Morey, R. M., Steigerwait, A., Boras, A., Weyant, R. S. and Popovic T. (2002). Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. *Emerging Infectious Diseases*, **8**, 1117-1123.

Salyers, A. A. and Amábile-Cuevas, C. F. (1997). Why are antibiotic resistance genes so resistant to elimination? *Antimicrobial Agents and Chemotherapy*, **41**, 2321-2325.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual (2nd edition). Cold Springs Harbour, New York.

Samer, Q. (2005). *Pseudomonas aeruginosa* Infections. *Web MD*. Available online (http://www.emedicine.com/med/topic1943.htm) Accessed 16th January 2007.

Sanchez-Amat, A., Ruzafa, C. and Solano, F. (1998). Comparative tyrosine degradation in *Vibrio cholerae* strains. The strain ATCC 14035 as a prokaryotic melanogenic model of homogentisate-releasing cell. Comparative Biochemistry and Physiology Part B: *Biochemistry and Molecular Biology*, **119**, 557.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.

Sanschagrin, F., Couture, F. and Levesque, R. C. (1995). Primary structure of OXA-3 and phylogeny of oxacillin-hydrolyzing class D beta-lactamases. *Antimicrobial Agents and Chemotherapy*, **39**, 887-893.

Schaber, J. A., Carty, N. L., MacDonald, N. A., Graham, E. D., Cheluvappa, R., Griswold, J. A. and Hamood, A. N. (2004). Analysis of quorum sensing-dificient isolates of *Pseudomonas aeruginosa*. *J. Med. Microbiol*., **53**, 841-853.

Schatz, A. and Waksman, S. A. (1944). Effect of streptomycin and other antibiotic substances upon *Mycobacterium tuberculosis* and related organisms. Proc. Soc. Exp. Biol. Med., **57**, 244-248.

Schmitz, F. R., Fluit, A. C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H. P. and Jones, M. E. (1999). The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *Journal of Antimicrobial Chemotherapy*, **43**, 253-259.

Schopf, J. W. and Packer, B. M. (1987). Early archaen microfossils from Warrawoona group, Australia. *Science*, **237**, 70-73.

Schulz, G. (1993). Bacterial porins: structure and function. *Curr. Opin. Cell. Biol*., **5**, 701-707.

Schwarz, S. and Nobel, W. C. (1999). Aspects of bacterial resistance to antimicrobials used in veterinary dermatological practice. *Veterinary Dermatology*, **10**, 163-176.

Seol, B., Naglic, T., Madic, J. and Bedekovic, M. (2002). *In vitro* antimicrobial susceptibility of 183 *Pseudomonas aeruginosa* strains isolated from dogs to selected antipseudomonal agents. *Journal of Veterinary Medicine*, **49**, 188-192.

Seppälä, H., Klaukka, T., Voupio-Varkila, J., Moutiala, A., Helenius, H., Lager, K. and Houvinen, P. (1997). The effect of changes in the consumption of macrolide antibiotics of erythromycin resistance in group A streptococci in Finland. *New England Journal of Medicine*, **337**, 441-446.

Shaw, K. J., Rather, P. N., Hare, R.S and Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiology and Molecular Biology Reviews*, **57**, 138-163.

Shi, L., Fujihara, K., Sato, T., Ito, H., Garg, P., Chakrabarty, R., Ramamurthy, T., Nair, G. B., Takeda, Y. and Yamasaki, S. (2006). Distribution and characterisation of integrons in various serogroups of *Vibrio cholerae* strains isolated from diarrhoeal patients between 1992 and 2000 in Kolkata, India. *Journal of Medical Microbiology*, **55**, 575-593.

Sigmund, C. D., Ettayebi, M., Morgan, E. A. (1984). Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*. *Nucleic Acids Res*., **12**, 4653-4663.

Slade, H. D. (1954). Metabolism of amino acids by streptococci. *In* Streptococcal Infections. Ed. M. McCarthy, Columbia University Press, New York.

Smeal, B. C., Bender, L., Jungkind, D. L. and Hastie, A. T. (1987). Simultaneous production of rhamnolipids, 2-alkyl-4-hydroxyquinolones and phenazines by clinical isolates of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology*, **25**, 1308-1310.

Smith, C.A. and Baker, E. N (2002). Aminoglycoside antibiotic resistance by enzymatic deactivation. *Current Drug Targets – Infectious Disorders*, **2**, 143-160.

Soler Bistué, A. J. C., Martín, F. A., Petroni, A., Faccone, D., Galas, M. Tolmasky, M. E. and Zorreguieta, A. (2006). *Vibrio cholerae* In117, a Class 1 integron harboring *aac(6')*-Ib and *bla*$_{\text{CTX-M-2}}$, is linked to transpostion genes. *Antimicrobial Agents and Chemotherapy*, **50**, 1903-1907.

Sonnenshein, C. (1927). Die mucous-form des pyocyaneus-bakteriums, *bacterium pyocyaneum mucosum. Zentralbl. Bakteriol. Parasitenk. Infectionskr. Hyg. Abt. I, Orig.*, **104**, 365-373.

Spilker, T., Coenye, T., Vandamme, P. and LiPuma, J. J. (2004). PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *Journal of Clinical Microbiology*, **42**, 2074-2079.

Stanier, R. Y., Palleroni, N. J. and Duodorff, M. (1966). The aerobic Pseudomonads: a taxonomic study. *Journal of General Microbiology*, **43**, 159-271.

Stapleton, P. Shannon, K. and French, G. L. (1999). Construction and characterisation of mutants of the TEM-I β-lactamase containing amino acid substitution associated with both extended-spectrum ressistance and resistance to β-lactamase inhibitors. *Antimicrobial Agents and Chemotherapy*, **43**, 1881-1887.

Steiner, D., Forrer, P., Stumpp, M. T. and Plückthun, A. (2006). Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display. *Nature Biotechnology*, **24**, 823-831.

Stintzi, A., Johnson, Z., Stonehouse, M., Ochsner, U., Meyer, J. M., Vasil, M. L. and Poole, K. (1999). The *pvc* gene cluster of *Pseudomonas aeruginosa*: Role in synthesis

of pyoverdine chromophore and regulation by PtxR and PvdS. *Journal of Bacteriology*, **181**, 4118-4124.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K. S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S. and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, **406**, 959-964.

Studemeister, A. E. and Quinn, J. P. (1988). Selective imipenem resistance in *Pseudomonas aeruginosa* associated with diminished outer membrane permeability. *Antimicrobial Agents and Chemotherapy*, **32**, 1267-1268.

Studier, F. W. and Moffatt, B. A. (1986). Use of bacteriophages T7 RNA polymerase to direct selective high level expression of cloned genes. *Journal of Molecular Biology*, **189**, 113-130.

Sun, T., Nukaga, M., Mayama, K., Braswell, E. H. and Knox, J. R. (2003). Comparison of β-lactamases of class A and D: 1.5-Å crystallographic structure of the class D OXA-1 oxacillinase (2003). *Protein Science*, **12**, 82-91.

Sundstrom, L. Radstrom, P. Swedberg G. and Skold, O. (1988). Site-specific recombination promotes linkage between trimethoprim and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus Tn21. *Mol. Gen. Genet*., **213**, 191-201.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A. and Schlüter, A. (2003). Occurrence of integron-associated resistance gene cassettes located on antibiotic

resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiology Ecology*, **45**, 239-252.

Tercero, J. C., Riles, L. E., Wickner, R. B. (1992). Localized mutagenesis and evidence for post-transcriptional regulation of MAK3. *J. Biol. Chem*, **267**, 20270-20276.

Thi Dao, K. H., Hamer, K. E., Clark, C. L. and Harshman, L. G. (1999). Pyoverdine production by *Pseudomonas aeruginosa* exposed to metals or an oxidative stress agent. *Ecological Applications*, **9**, 441-448.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

Thornley, M. J. (1960). The differentiation of Pseudomonas from other Gram-negative bacteria on the basis of arginine metabolism. *J. Appl. Bacteriol*., **23**, 37-52.

Todar, K. (2002). Antimicrobial agents used in the treatment of infectious disease. Todar's online textbook of bacteriology. Available online at http://textbookofbacteriology.net/antimicrobial.html. Accessed 14th November 2006.

Todar, K. (2004). *Pseudomonas* and related bacteria. Todar's Online Textbook of Bacteriology. Available online at: http://textbookofbacteriology.net/pseudomonas.html. Accessed 5th July 2006.

Toleman, M. A., Rolston, K., Jones, R. N. and Walsh, T. R. (2003). Molecular and biochemical characterization of OXA-45, an extended-spectrum Class 2d' β-lactamase in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **47**, 2859-2863.

Tosini, F., Visca, P., Luzzi, I., Dionisi, A. M., Pezzella, C., Petrucca, A. and Carattoli, A. (1998). Class 1 integron–borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype Typhimurium. *Antimicrobial Agents and Chemotherapy*, **42**, 3053-3058.

Tran Van Nhieu, G. and Collatz, E. (1987). Primary stucture of aninoglycoside 6'-*N*-acetyltransferase, AAC(6')-4, fused in vivo with the signal peptide of the Tn3-encoded beta-lactamase. *J. Bacteriol*., **196**, 5708-5714.

Ueda, K., Seki, T., Kudo, T., Yoshida, T. and Kataoka, M. (1999). Two distinct mechanisms cause heterogeneity of 16S rRNA. *Journal of Bacteriology*, **181**, 78-82.

Vaara, M., Plachy, W. Z. and Nikaido, H. (1990). Partitioning of hydrophobic probes into lipopolysaccharide bilayers *Biochim. Biophys. Acta*., **1024**, 152-158.

Vakulenko, S. B. and Mobashery, S. (2003). Versatility of aminoglycosides and prospects for their future. *Clinical Microbiology Reviews*, **16**, 430-450.

Valax, P. and Georgiou, G. (1993). Molecular characterization of β-lactamase inclusion bodies produced in *Escherichia coli*. *Composition. Biotechnol. Prog*., **9**, 539-547.

Van Bambeke, F., Balsi, E., Tulkens, P. M., (2000). Antibiotic efflux pumps. *Biochemical Pharmacology*, **60**, 457-470.

Van Bambeke, F., Glupczynski, Y., Plésiat, Pechère, J. C. and Tulkens, P. M. (2003). Antibiotic efflux pumps in prokaryotic cell: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. *Journal of Antimicrobial Chemotherapy*, **51**, 1055-1065.

Van Belkum, A., Goessens, C., Van der Schee, Lemmens den Toom, N., Vos, M. C., Cornelissen, J., Lugtenburg, E., de Marie, Siem, Verbrugh, H., Löwenberg, B and Endtz, H. (2001). Rapid emergence of ciprofloxacin-resistant enterobacteriaceae containing multiple gentamicin resistance-associated integrons in a Dutch hospital. *Emerging Infectious Diseases*, **7**, 862-871.

Vedel, G. (2005). Simple method to determine β-lactam resistance phenotypes in *Pseudomonas aeruginosa* using the disc agar diffusion test. *Journal of Antimicrobial Chemotherapy*, **56**, 657-664.

Véron, M. and Berche, P. (1976). Virulence et antigènes de *Pseudomonas aeruginosa. Bull Inst Pasteur* **74**, 295-337.

Voha, C., Docquier, J. D., Rossolini, G. M. and Fosse, T. (2006). Genetic and biochemical characterization of FUS-1 (OXA-85), a narrow-spectrum Class D β-lactamase from *Fusobacterium nucleatum* subsp. *polymorphum*. *Antimicrobial Agents and Chemotherapy*, **50**, 2673-2679.

Walker, P. D. and Duggin, G. G. (1988). Drug nephrotoxicity in rats. *Annual Review of Pharmacology and Toxicology*, **28**, 331-345.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, **406**, 775-781.

Walsh, C. (2003). Antibiotics: actions, origins, resistance. ASM Press, Washington D.C.

Walther-Rasmussen, J. and Høiby, N. (2006). OXA-type carbapenemases. *Journal of Antimicrobial Chemotherapy*, **57**, 373-383.

Wang, X., Feng, J. and Jiang, Y. (2007). The isolation, differentiation and degradation characteristics of petroleum-degrading strains. Genbank submission.

Weldhagen, G. F. (2004). Integrons and β-lactams – a novel perspective on resistance. *International Journal of Antimicrobial Agents*, **23**, 556-562.

Wick M., Frank, D., Storey, D. and Iglewski, B. (1990). Structure, function and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annual Review of Microbiology*, **44**, 335-363.

Wickens, H. and Wade, P. (2005). Understanding antibiotic resistance. *The Pharmaceutical Journal*, **274**, 501-504.

Wiedmann, M., Weilmeirer, D., Dineen, S. S., Ralyea, R. and Boor, K. J. (2000). Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Appl. Environ. Microbiol*., **66**, 2085-2095.

Williams, R. A. D., Lambert, P. A. and Singleton, P. (1996). Antimicrobial drug action. BIOS scientific publishers, Oxford, UK.

Wilson, R. and Dowling R. (1998). *Pseudomonas aeruginosa* and other related species. *Thorax*, **53**, 231-219.

Wimberly, B. T., Brodersen, D. E., Clemons Jr., W. M., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartsch, T., Ramakrishnan, V. (2000). The structure of the 30S ribosomal subunit. *Nature*, **407**, 327-339.

Woese, C. R. (1987). Bacterial evolution. *Microbial. Rev*., **512**, 221-271.

Woo, P. C. Y., Leung, K. W. and Yuen, K.Y. (2000). Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow

transplant recipient. *Journal of Clinical Pathology: Molecular Pathology*, **53**, 211-215.

World Health Organisation (2001). WHO Global strategy for containment of antimicrobial resistance. Available online at http://whqlibdoc.who.int/hq/2001/WHO_CDS_CSR_DRS_2001.2a.pdf

Wright, G. D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews*, **57**, 1451-1470.

Wright, G. D. and Thompson P. R. (1999). Aminoglycoside phosphotransferases: proteins, structure and mechanism. *Frontiers in Bioscience*, **4**, 9-21.

Wybenga-Groot, L. E., Draker, K., Wright, G. D. and Berghuis, A. M. (1999). Crystal structure of an aminoglycoside 6'-*N*- acetyltransferase:defining the GCN5-related *N*-acetyltransferase superfamily fold. *Structure*, **7**, 497-507.

Yabuuchi, E and Ohyama, A (1972). Characterisation of 'pyomelanin'-producing strains of *Pseudomonas aeruginosa*. *International Journal of Systematic Bacteriology*, **22**, 53-64.

Yokoyama, K., Doi, Y., Yamane, K., Kurokawa, H., Shibata, N., Shibayama, K., Yagi, T., Kato, H. and Arakawa, Y. (2003). Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet*, **362**, 1888-1893.

Yoshimura, F. and Nikaido, H. (1982). Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J. Bacteriol*., **152**, 636-642.

Yoshimura, F. and Nikaido, H. (1985). Diffusion of β-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrobial Agents and Chemotherapy*, **27**, 84-92.

Yum, J. H., Shin, H. B., Roh, K. H., Yong, D., Lee, K. and Chong, Y. (2007). *bla*$_{IMP-1}$ metallo-beta-lactamase gene-containing integron in an *Acinetobacter baumannii* from a clinical specimen. Genbank submission.

Zahn, K. (1996). Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. *Journal of Bacteriology*, **178**, 2926-2933.

Zhang, Z., Wilson, R. C. and Fox, G. E. (2002). Identification of characteristic oligonucleotides in the bacterial 16S ribosomal RNA sequence dataset. *Bioinformatics*, **18**, 244-250.

Zhao, K and Chen, K. (2006). Isolation and identification of a high efficiency phosphate-solubilizing bacterium. GenBank submission.