Selected β-lactam resistance in selected species of Gram-negative bacteria

University of Cape Town

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Selected β-lactam resistance in selected species of Gram-negative bacteria

THESIS PRESENTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
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Lastly I wish to thank the Almighty for giving me the strength to persevere.

ABBREVIATIONS

$\times g$	times gravity
$\times$	times
μg	microgram(s)
μl	microlitre(s)
A	adenine
bp(s)	base pair(s)
C	cytosine
cDNA	complimentary DNA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dH_20	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ESBL(s)	extended spectrum β-lactamase(s)
g	gram(s)
G	guanine
GSH	Groote Schuur Hospital
ICU	intensive care unit
IPTG	isopropyl-β-D-thio-galactopyranoside
kb	kilobase(s)
M	molar
mg	milligram(s)
MIC(s)	minimal inhibitory concentration(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar

mRNA	messenger RNA
NaCl	sodium chloride
NB	nutrient broth
NCBI	National Center for Biotechnology Information
nm	nanometer(s)
O/N	overnight
°C	degrees Celsius
OD	optical density
OMP(s)	outer membrane protein(s)
ORF(s)	open reading frame(s)
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pmol	picomole(s)
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
S	Svedberg units
SDS	sodium dodecyl sulphate
SSC	saline-sodium citrate
T	thymine
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA
TES	Tris-EDTA Saline
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
U	unit(s)
UV	ultra-violet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

YT	Yeast tryptone
α	alpha
β	beta
σ	sigma

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ABSTRACT

The molecular basis of β-lactam resistance was investigated in this study. Selected Gram-negative clinical isolates potentially displaying therapeutic problems were chosen as the subjects. Clinical diagnostic tests are not always very accurate and informative about the molecular nature of β-lactam resistance, thus molecular investigations are carried out to more fully and comprehensibly explain the clinically observed phenomena.

In the view of Enterobacteria being some of the most commonly isolated, clinically problematic, bacteria, *E. coli* and *K. pneumoniae* isolates with unusual β-lactam resistance profiles were investigated. The first cluster of *E. coli* isolates were investigated with respect to their resistance to the cephalosporins and the cephamycin, cefoxitin. β-lactamase promoter mutations were identified and expression studies were performed to corroborate overexpression of the AmpC enzyme.

The second group of *E. coli* isolates displayed resistance to the inhibitor combination, amoxicillin-clavulanate. In this group, the resistance genes were identified, with their promoters. Resistance was attributed to TEM overproduction and overproduction of a specially adapted TEM (IRTs) β-lactamase. Primer extension was used to determine the transcriptional start site of the IRT.

The *K. pneumoniae* isolates were investigated for their resistance to the cephalosporins, cefoxitin and amoxicillin-clavulanate. The resistance was attributed to an interplay of β-lactamase production and the loss of permeability.

Since *A. baumannii* is becoming an increasingly popular opportunistic pathogen in the clinical setting, strain RAN was also investigated. The presence of β-lactamase resistance genes was investigated. The chromosomal *ampC* gene was confirmed to be present, cloned and sequenced. The regulation of the *ampC* gene was examined. The *ampC* gene was also studied in terms of its genetic context. Sequence analysis suggests that the *ampC* might be associated with a stabilized transposition event.

CHAPTER 1

Literature review

GENERAL INTRODUCTION

1.1 β-lactam antibiotics

1.1.1 penicillins

1.1.2 cephalosporins

1.1.3 carbapenems

1.1.4 monobactams

1.1.5 β-lactamase inhibitors

1.2 Mechanism of action of β-lactams

1.3 Mechanisms of resistance to the β-lactams

target alteration

β-lactamases

mode of action

classification of β-lactamases

plasmid and chromosome location

β-lactamase mediated resistance in *E. coli* and *K. pneumoniae*

β-lactamase mediated resistance in *A. baumannii*

outer membrane proteins, in general

efflux, in general

1.4 AIM OF STUDY

GENERAL INTRODUCTION

Since the discovery of penicillin, the growth of antimicrobial agents based on the β-lactam nucleus has increased tremendously (Rolinson 1998). Due to resistance occurring to the penicillins, which was the first group of β-lactam antimicrobial agents discovered, the search for better agents able to withstand and overcome the resistance, has lead to the discovery of an assortment of different β-lactam agents with structural and chemical modifications altering their specificity and activity. β-lactams have also been developed to improve properties such as stability, prolonged action, absorption, and resistance to extended spectrum β-lactamases (ESBLs).

1.1 β-lactam antibiotics

1.1.1 Penicillins

The natural occurring benzylpenicillin, a product of *Penicillium notatum*, is the nucleus on which most of the analogues are based per se. 6-Aminopenicillanic acid (β-lactam ring (Figure 1.1) and a thiozolidine ring) can be obtained from benzylpenicillin and most of the semi-synthetic penicillins are based on the structure of 6-Aminopenicillinic acid. The penicillins can be divided into six groups (Table 1.1) (Lambert and O'Grady).

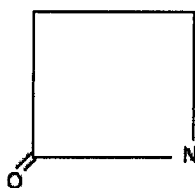


Figure 1.1 The β-lactam ring, the common molecular structure of all β-lactam compounds.

TABLE 1.1 Examples of penicillins and their categories (Lambert and O'Grady, 1995)

Group	Properties	Examples
1	Benzylpenicillin and its long lasting parenteral forms	Benethamine penicillin Procaine penicillin Clemizole penicillin
2	Orally absorbed penicillins resembling benzylpenicillin	Azidocillin Phenithicillin Propicillin
3	Penicillins resistant to staphylococcal β-lactamases	Cloxacillin Oxacillin Methicillin
4	Extended spectrum penicillins	Ampicillin Amoxycillin(amoxicillin) Mecillinam
5	Penicillins active against *P. aeruginosa*	Azlocillin Piperacillin Carbenicillin Ticarcillin
6	β-lactamase-resistant penicillins	Foramidocillin Temocillin

1.1.2 Cephalosporins

The cephalosporins are based on the structure of cephalosporin C which was first identified in *Cephalosporium acremonium*, as one of its fermentation products. The cephalosporins are very similar to the penicillins in structure except that the five-membered thiazolidine ring is replaced by a six-membered dihydrothiazine ring. In some cases the sulphur in this ring is replaced by an oxygen atom, forming the oxacephems. The nucleus of all cephalosporins is the 7-aminocephalosporanic acid molecule which is a derivative of cephalosporin C. Cephamycins are a special group within the cephalosporins having modifications at carbon 7 with an α-methoxy group forming 7-

methoxycephalosporin. Most chemical modifications occur at carbon positions C-3 and C-7. Cephalosporins were divided into 3 groups namely, first generation, second generation and third generation, each group being, early compounds, compounds resistant to β-lactamases, and compounds resistant to β-lactamases and displaying enhanced antimicrobial activity, respectively. These groups have further been subdivided into six groups (Table 1.2) to distinguish oral from parenteral compounds and to separate some of the third generation compounds that do not share the same properties as the rest (Lambert and O'Grady). However the nomenclature is not as clear cut as outlined in Table 1.2 eg. cefepime is quite frequently referred to as a fourth generation cephalosporin (Erwin *et al.*, 1997; Pitout *et al.*, 1997; Stürenburg and Mack, 2003). A new class or fifth generation of cephalosporins with a catechol substitution has been proposed and one of these has been tested as an antimicrobial agent (Erwin *et al.*, 1997).

TABLE 1.2 Examples of cephalosporins and their categories (Lambert and O'Grady)

Group	Properties	Examples
1	Parenteral compounds with moderate antimicrobial activity (narrow spectrum)	Cephaloridine Cephalothin Cefazolin
2	Oral compounds with moderate antimicrobial activity (narrow spectrum)	Cephalexin Cephradine Cefaclor
3	Parenteral compounds resistant to a wide range of β-lactamases with moderate antimicrobial activity (expanded spectrum)	Cefuroxime Cefoxitin(cephamycin) Cefotetan(cephamycin)
4	Parenteral compounds resistant to a wide range of β-lactamases and with potent antimicrobial activity (broad spectrum)	Cefepime Cefotaxime Ceftazidime Ceftriaxone Cefpodoxime
5	Oral compounds resistant to a wide range of β-lactamases and with potent antimicrobial activity (broad spectrum)	Cefixime Ceftibuten
6	Oral compounds resistant to a wide range of β-lactamases and with moderate antimicrobial activity against enterobacteria and active against *Pseudomonas aeruginosa* (broad spectrum)	Cefsulodin Cefoperazone Cefpimizole

Groups 1 and 2 belong to the first generation, group 3 to the second generation and groups 4, 5 and 6 to the third generation.

1.1.3 Carbapenems

Carbapenems have been isolated from various streptomyces and currently have the widest spectrum of activity of the β-lactams. Two generic names are given to the carbapenems, thienamycins and olivanic acid. Their chemical structures comprise the same rings as penicillin, except that the sulphur atom is replaced by a CH_2 and a double bond exists between carbon 2 (C-2) and carbon 3 (C-3) in the 'thiazolidine ring'. The two most common carbapenems are imipenem and meropenem. Unfortunately, imipenem is hydrolyzed by the mammalian renal dehydropeptidase I and is therefore administered with the dehydropeptidase I inhibitor, cilastatin, as an imipenem-cilastatin combination. Meropenem is more resistant to the dehydropeptidase I enzyme (Lambert and O'Grady).

1.1.4 Monobactams

Monobactams are β-lactams comprised of a single monocyclic ring to which various side chains are appended. Aztreonam, carumonam and tigemonam are examples of the monobactams (Lambert and O'Grady).

1.1.5 β-lactamase inhibitors

It has been known that certain β-lactam compounds, cephalosporin C and cloxacillin for example, inhibit certain β-lactamases but that the quantities required to be useful in the clinical setting would be too high. Searches for more potent β-lactamase inhibitors have resulted in agents such as clavulanic acid, sulbactam and tazobactam (Livermore 1998; Bonomo and Rice 1999). These three inhibitors are currently available and used in clinical therapy. Clavulanic acid is administered in a fixed ratio with amoxicillin and could reverse resistance to amoxicillin (Lambert and O'Grady; Therrien and Levesque, 2000). Ratios are 1:2 for oral preparations and 1:5 for intravenous administration (Lambert and O'Grady) of amoxicillin-clavulanate.

Clavulanate is a naturally occurring clavam whilst tazobactam and sulbactam are penicillanic sulphone derivatives. These inhibitors are mostly used in combination with penicillins but also with cephalosporins. The partner antibiotics for clavulanate are

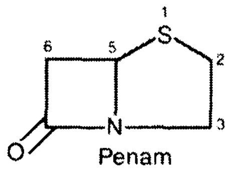
amoxicillin and ticarcillin; tazobactam is combined with piperacillin; and sulbactam is combined either with ampicillin or cefoperazone (Livermore 1998; Bonomo and Rice, 1999).

These inhibitors display poor antimicrobial activity by themselves and are used in conjunction with a suitable β-lactam. They compete with the β-lactam for the active site of the β-lactamase, suicidally, inactivating the β-lactamase protecting and leaving the β-lactam free to find its target (Bonomo and Rice 1999; Yang *et al.*, 1999; Therrien and Levesque, 2000). The effectiveness of a β-lactamase inhibitor depends on many factors, one of which is the penetration of the inhibitor into the periplasm of Gram-negative bacteria (Farmer *et al.*, 1999). Clavulanate compared with tazobactam, sulbactam and BRL42715 is superior in its penetrating ability (Farmer *et al.*, 1999).

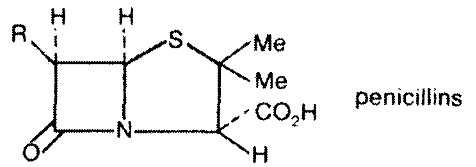
Resistance to the inhibitor combination amoxicillin-clavulanic acid can occur as a result of overproduction of the AmpC enzyme, TEM-1 overexpression or TEM enzyme derivatives (inhibitor resistant TEM [IRT] enzymes) and the production of oxacillinases (Nicolas-Chanoine, 1997; Livermore 1998; Yang *et al.*, 1999; Bonomo and Rice 1999; Wang *et al.*, 2002).

Methicillin is a non-competitive inhibitor, binding to a site outside of the active site of the β-lactamase. An example of a non-β-lactam based inhibitor of β-lactamases is the sulphated macrolide, izumenolide (Lambert and O'Grady).

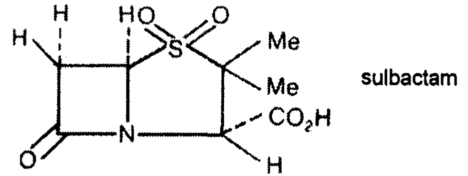
SKELETON | **EXAMPLE**



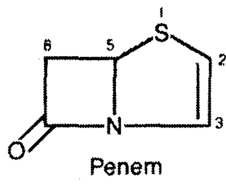
Penam



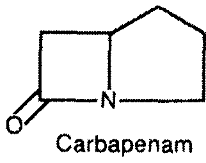
penicillins



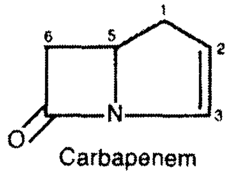
sulbactam



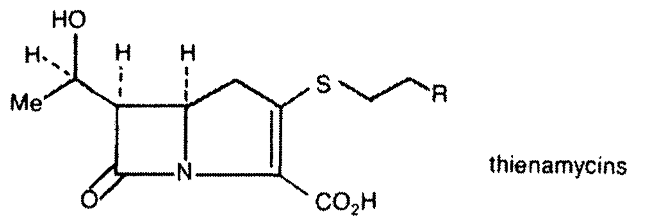
Penem



Carbapenam



Carbapenem



thienamycins

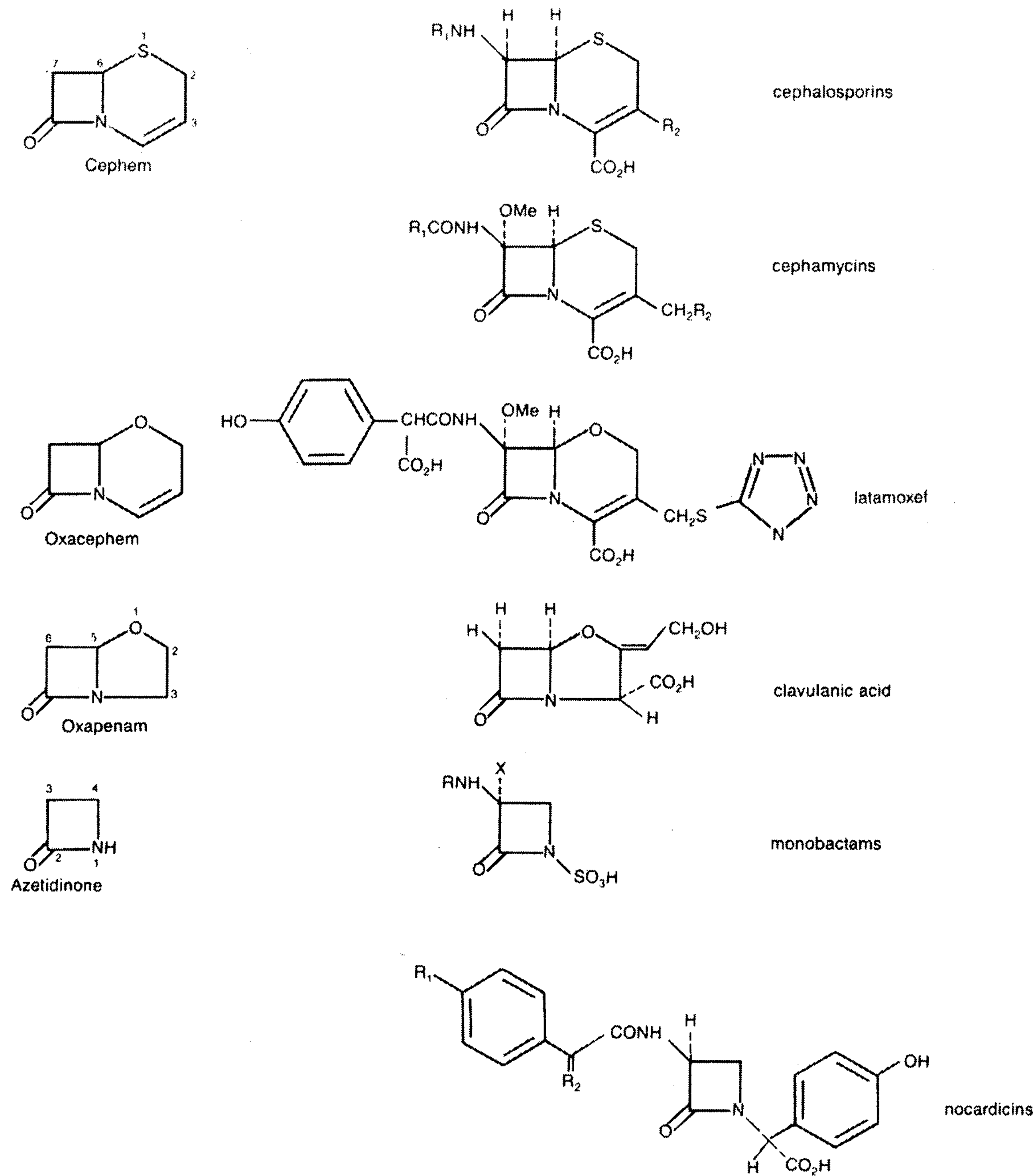


Figure 1.2 β-lactam skeletons and the different kinds of β-lactams (Lambert and O'Grady).

1.2 Mechanism of action of β-lactams

The way in which β-lactams inhibit bacterial growth is to inhibit cell wall synthesis. The cell wall peptidoglycan building blocks are *N*-acetylglucosamine alternating with *N*-acetylmuramic acid. *N*-acetylmuramic acid has a pentapeptide chain affixed to it, ending in D-alanine-D-alanine. The sugar moieties are joined by the transglycosylases and the pentapeptides are crosslinked by transpeptidases (Ghuysen 1991; Goffin and Ghuysen 1998). It is this final crosslinking of the peptides that is inhibited by the β-lactam which covalently and irreversibly binds to the PBP, inactivating its physiological function and resulting in a poorly formed peptidoglycan that cannot support cell growth.

1.3 Mechanism of resistance to the β-lactams

In the previous section only a few of the β-lactam antibiotics from this diverse group of antimicrobials were discussed. Although these compounds are structurally related, having the β-lactam ring as a common molecular feature, does not necessarily simplify the molecular mechanism of resistance.

Target alterations

Penicillin binding proteins (PBPs) are generally considered to be the targets of the β-lactam antibiotics (Georgopapadakou and Liu, 1980; Ghuysen 1991; Goffin and Ghuysen 1998). The β-lactam covalently binds to the PBP thereby inactivating their physiological function.

There is a wide variety of PBPs within the Gram-negative species and the Gram-positive species. The number and amount of PBPs differ for different bacteria (Georgopapadakou and Liu, 1980), such that each species has its own unique set of PBPs. However, PBPs can be divided mainly into two groups, namely the low molecular weight (M_r) and the high molecular weight (M_r) PBPs (Georgopapadakou and Liu, 1980; Ghuysen 1991; Goffin and Ghuysen 1998). The low M_r PBPs are peptidases (Ghuysen 1991), catalyzing transpeptidation. The high M_r weight PBPs are transglycosylases and lack serine

transferase activity on Ac-L-Lys-D-Ala-D-Ala, the standard substrate analogue of the low M_r PBPs.

Bifunctional PBPs combine in a single polypeptide chain, the penicillin binding (PB) transpeptidase and the non-penicillin binding (n-PB) transglycosylase functions (Goffin and Ghuysen 1998). Other multimodular PBPs are similar in their modular design to the bifunctional PBPs (transpeptidase and glycosylase) but contain a non-penicillin binding (n-PB) domain which is not a transglycosylase combined with a serine transferase (peptidase) PB domain (Goffin and Ghuysen 1998).

High M_r PBPs were divided into two classes A and B derived from homology searches (Ghuysen 1991). Recently, Goffin and Ghuysen (1998) further classified multimodular PBPs Class A and Class B into orthologs and paralogs. Paralogs normally have different functions and cannot substitute for each other whereas orthologs can be substituted for one another.

The affinity with which a β-lactam binds various PBPs within an organism varies. At least one of the targeted PBPs should be essential for cell survival, and can be referred to as the 'killing site' (Georgopapadakou and Liu, 1980), for a particular β-lactam to be effective as a bacteriocidal agent.

Resistance to the β-lactams as a result of reduced expression (Tajima (2003)) and of altered PBPs whose PB domain has acquired a decrease affinity for the drug have recently been reported especially in isolates of *Streptococcus pneumoniae* (Di Guilmi *et al.*, 1998; Smith *et al.*, 2001; Bennett *et al.*, 2003; Ohsaki *et al.*, 2003; Smith *et al.*, 2003) and *Staphylococcus aureus* (Katayama *et al.*, 2004; Malouin *et al.*, 2003). Alterations in PBPs have been reported to play a role in β-lactam resistance in *Helicobacter pylori* (Okamoto *et al.*, 2002; Kwon *et al.*, 2003). Pierre *et al.*, (1990) reported alteration of PBPs to result in imipenem resistance in *Listeria monocytogenes*.

Recently, a new cephalosporin was tested for its affinity against PBPs of *Staphylococcus aureus* (Malouin *et al.*, 2003). A screen to identify transglycosylase and peptidase inhibitors was developed in *Escherichia coli* by Chandrakala *et al.*, 2004. Fortunately, this mechanism of resistance is not as common in Gram-negative bacteria as it is in Gram-positive bacteria.

β-lactamases

β-lactamases are enzymes that inactivate the β-lactam antibiotics and thereby cause resistance. The inactivation of the compound occurs when the β-lactam ring is hydrolyzed. Essentially there are only two hydrolytic mechanisms employed by these enzymes. The first mechanism involves a catalytic serine residue and the second involves a zinc residue.

Location, kinetics, enzyme quantity and physiochemical conditions are all determinants for a β-lactamase to confer resistance (Livermore 1995). The location of β-lactamases in Gram-positive organisms is mainly extracellular although some enzyme may be associated with the cytoplasmic membrane. In *Mycobacterium fortuitum* the production of β-lactamase was reported to be both intracellular and extracellular (Fattorini *et al.*, 1991). In Gram-negative organisms, β-lactamases are largely found in the periplasm (Curtis *et al.*, 1972; Minsky *et al.*, 1986; Bowden and Georgiou, 1990), although some enzyme may be associated with the inner membrane (Plückthun and Pfitzinger, 1988). 'Illegitimate secretion' in the absence of a leader (signal) peptide, may cause β-lactamase to be bound to the cytoplasmic membrane and be partially exposed in the periplasm (Bowden *et al.*, 1992). Mature cytoplasmic β-lactamase is thus able to interact with the cytoplasmic membrane and be translocated to the periplasm in the absence of a leader peptide (Bowden *et al.*, 1992), albeit with a low efficiency. Mature β-lactamase may also be found in periplasmic inclusion bodies rather than cytoplasmic inclusion bodies (Georgio *et al.*, 1986) in β-lactamase overproducing strains of *E. coli.*

β-lactamases normally follow standard Michaelis-Menten kinetics for most substrates (Livermore 1995). Exceptions are the zinc-metallo enzymes, Class B, which rely on zinc

atoms for efficient catalysis (Wang *et al.*, 1999; Murphy *et al.*, 2003). The enzyme kinetics of OXA-14 was recently investigated (Danel *et al.*, 2001) and suggests possible dimerisation among Class D enzymes.

Higher levels of enzyme normally dictate higher levels of resistance (Livermore 1997; Wu *et al.*, 1994). An increase in enzyme quantity was associated with an increase in MICs for most β-lactams, except for cefoxitin and imipenem, in *Mycobacterium fortuitum* (Fattorini *et al.*, 1991) strains. The relationship between type and amount of enzyme produced, was investigated in *Enterobacteriaceae* (Thomson *et al.*, 1990) and followed the same trend of higher enzyme amount dictating higher β-lactam resistance, although this relationship does not hold for all β-lactamases.

Physiochemical conditions, eg media type and growth conditions (Fattorini *et al.*, 1991), may influence the way resistance is ascribed in the laboratory. A higher pH (8.0) was shown to increase the sensitivity of TEM-type β-lactamases to piperacillin used in combination with tazobactam or sulbactam, resulting in decreased MICs to these β-lactam inhibitor combinations (Fornara *et al.*, 1997). This phenomenon was shown to be dependent on enzyme quantity (Fornara *et al.*, 1997) and could only be observed for strains expressing copious amounts of β-lactamase. Lower temperatures of incubation (<37°C) reduced the amount of periplasmic β-lactamase inclusion bodies and increased the total amount of β-lactamase activity in *E. coli* β-lactamase overproducing strains (Chalmers *et al.*, 1990).

Mode of action of β-lactamases

In Gram-negative bacteria, β-lactamases are located mostly in the periplasm, between the inner and outer membrane, thus requiring only small amounts of β-lactamase to be produced to inactivate readily hydrolyzed substrates (Pitout *et al.*, 1997). β-lactamases react with the β-lactam compound forming a serine ester (acyl enzyme), that is readily hydrolyzed, resulting in the splitting of the β-lactam ring and thereby inactivating the antibiotics' effectiveness (Figure 1.3). However, the metallo-β-lactamases utilizes zinc to disrupt the β-lactam ring. When the β-lactam binds to its target (PBPs), the ester is not

readily hydrolyzed, leaving the β-lactam compound covalently attached to its target. The β-lactamase inhibitors on the other hand, (eg clavulanate) covalently binds to the β-lactamase and irreversibly remains in its acylated state.

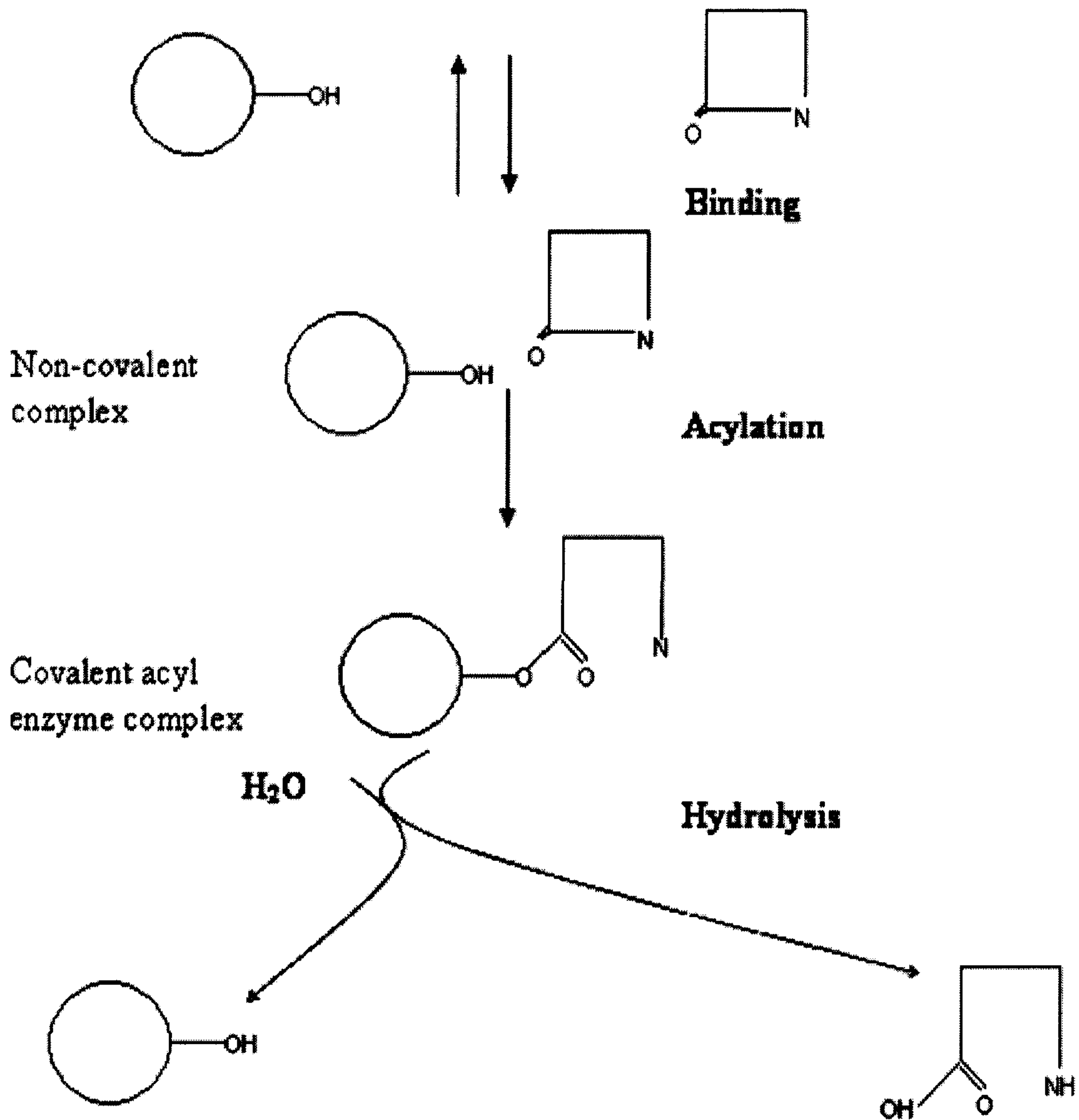


Figure 1.3 Molecular action of serine β-lactamases. The enzyme first associates non-covalently with the β-lactam ring. The β-lactam ring is then attacked by the free hydroxyl group of the serine residue in the active site of the enzyme forming the acyl enzyme complex. The nucleophilic attack by the water molecule causes the disassociation of the enzyme and inactivates the β-lactam. For PBPs the reaction stops at the acylation step or very slow hydrolysis occurs. (Figure taken from Livermore 1995; Livermore 1998).

Classification of β lactamases

β-lactam antibiotics are the most frequently prescribed antibiotics. β-lactamases are the most common reason for resistance to the β-lactam antibiotics. These enzymes operate by inactivating the β-lactam ring of the β-lactam compound. There are four major classes of β-lactamases in the Ambler grouping, Class A, B, C, and D (Livermore, 1995; Jones 1998; Livermore 1998), where Class A, C and D comprise of the serine enzymes and Class B the metallo-zinc enzymes. Class A generally refers to TEM and SHV enzymes and other enzymes usually inhibited by the inhibitor (clavulanate), Class C contains the chromosomal enzymes usually resistant to the cephalosporins and not inhibited by clavulanate, Class D mostly contains the OXA type enzymes which have a preferred substrate specificity for oxacillin, and Class B, the metallo-zinc enzymes. The Bush classification system is based on the substrate preference and functional group (Table 1.3). It is a more elaborate way of classifying the β-lactamases. Bush Group 2 is equivalent to Ambler Class A except for 2d which is equivalent to Ambler Class D, the chromosomal AmpC type enzymes of Ambler Class C is equivalent to group 1, and the metallo enzymes of Ambler Class B is equivalent to group 3 (Livermore 1995; Livermore 1998). Bush group 4 enzymes are penicillinases not inhibited by clavulanic acid (Pitout *et al.*, 1997).

TABLE 1.3 Classification of β-lactamases

Class/Group		Characteristics	Representative enzyme
[a]Ambler	[b]Bush-Jacoby-Medeiros		
C	1	Cephalosporinases not inhibited by clavulanic acid	AmpC
A	2a	Penicillinases inhibited by clavulanic acid	PC1 (*S. aureus*)
A	2b	Broad-spectrum enzymes inhibited by clavulanic acid	TEM-1, TEM-2, SHV-1
A	2be	Extended broad-spectrum enzymes inhibited by clavulanic acid (ESBLs)	TEM-3 to 28, SHV-2 to 6
A	2br	Broad-spectrum enzymes with reduced binding to clavulanic acid (IRTs)	TEM-30 to 36, TRC-1
A	2c	Carbenicillin-hydrolyzing enzymes inhibited by clavulanic acid	PSE-1, CARB-3
D	2d	Cloxacillin-hydrolyzing enzymes inhibited by clavulanic acid	OXA-1, PSE-2
A	2e	Cephalosporinases inhibited by clavulanic acid	*Proteus vulgaris*
A	2f	Carbapenem-hydrolyzing non-metallo β-lactamases	IMI-1, NMC-A, Sme-1
B	3	Zinc metallo-β-lactamases	L1 (*Stenotrophomonas maltophilia*)
Undetermined	4	Penicillinases not inhibited by clavulanic acid	*P. cepacia*

Table modified from Pitout *et al.*, 1997 and Livermore (1995)
[a]Ambler 1980
[b]Bush *et al.*, 1995

Plasmid and Chromosomal location

It is well known that most of the Class A β-lactamases that confer β-lactam resistance and which are subsequently reported, are plasmid encoded (Vuye *et al.*, 1989; Livermore 1995; Livermore 1998). Class B and Class D (Medeiros *et al.*, 1985) β-lactamases were found on both plasmid and on the chromosome (Livermore 1998). Class C β-lactamases have always been associated with a chromosomal location until the discovery of a plasmid-mediated *ampC* gene (MIR-1) of *Enterobacter cloacae* (Papanicolaou *et al.*, 1990). Since 1990, the number of these enzymes has increased at an approximate rate of 1 to 2 new enzymes per year (Bauernfeind *et al.*, 1998). These plasmid-mediated Class C β-lactamases have been discovered worldwide in a variety of organisms (Phillipon *et al.*, 2002), but remain difficult to detect in the clinical laboratory (Thomson 2001).

β-lactamase mediated resistance in *E. coli* and *K. pneumoniae*

Many of the Gram-negative organisms contain a chromosomal *ampC* gene. Among the Enterobacteriaceae the *ampC* gene is frequently present, except for *Salmonella* (Morosini *et al.*, 2000) in which it is absent. In *Citrobacter freundii* and *Enterobacter cloacae* this gene is inducible and is regulated by the *ampR* gene, which encodes the transcriptional regulator AmpR, which 'responds' to a good β-lactam inducer. Regulation of AmpC expression has been described to be affected by AmpD, an amidase (Höltje *et al.*, 1994; Jacobs *et al.*, 1995). Mutations in *ampD* result in constitutive AmpC overproduction (Kopp *et al.*, 1993). Overexpression of AmpC is due to changes in *ampR* or absence of *ampD* and is called derepression (Sanders 1987) or permanent hyperproduction of AmpC. The signal for AmpC induction in *Enterobacter cloacae* was found in the cytoplasm and in the periplasm (Dietz *et al.*, 1996) and identified as the anhydromuramyl-pentapeptide, (Dietz *et al.*, 1997) a component of cell wall recycling. However, in *E. coli* the *ampC* gene is not located adjacent to the *ampR* gene and is not regulated by *ampR* either. Instead, the AmpC in *E. coli* is produced constitutively at low basal levels and regulated by a growth-rate dependent attenuator (Jaurin *et al.*, 1981). Only in very rare instances (2 %) is the AmpC in *E. coli* overexpressed, giving rise to clinical resistance (Livermore, 1995; Forward *et al.*, 2001; [Chapter 2]). *Klebsiella pneumoniae* has a Class A

chromosomal β-lactamase, SHV-1. SHV-1 was suggested to be intrinsic to *K. pneumoniae* (Babini and Livermore, 2000; Chaves *et al.*, 2001).

MIR-1, isolated from *K. pneumoniae*, was one of the first plasmid-mediated *ampC* (Jacoby and Tran, 1999) β-lactamases to be characterized (Papanicolaou *et al.*, 1990; Bauernfeind *et al.*, 1998). *Klebsiella pneumoniae* expressing the plasmid-mediated, MIR-1 conferred resistance to cefotaxime, ceftazidime, ceftriaxone among other extended spectrum β-lactams. It also conferred resistance to the cephamycin, cefoxitin, (Papanicolaou *et al.*, 1990). There are also other types of plasmid-mediated *ampC* genes first characterized in *K. pneumoniae*, isolated worldwide, which encode CMY-1, MOX-1, FOX-1, LAT-1, ACC-1 and DHA-2, (DHA-1 was first isolated in *Salmonella enteritidis*) (Bauernfeind *et al.*, 1996; Bauernfeind *et al.*, 1998; Philippon *et al.*, 2002) and are able to hydrolyze the cephamycins. ACT-1 was simultaneously identified in *K. pneumoniae* and *E. coli* isolates (Bradford *et al.*, 1997). BIL-1, another plasmid-mediated Class C β-lactamase (Fosberry *et al.*, 1994) with biochemical properties similar to MIR-1 was first identified in *E. coli* (Woodford *et al.*, 1990; Payne *et al.*, 1992). Subsequently, other CMY-type β-lactamases were found in *E. coli* too (Zhao *et al.*, 2001).

Variants of TEM-1 and SHV-1 giving rise to an extended spectrum of β-lactam hydrolysis, particularly the cephalosporins, are termed extended spectrum β-lactamases (ESBLs) (Bush and Jacoby, 1997; Livermore 1998). *E. coli* and *K. pneumoniae* are two of the most frequent ESBL producers. *K. pneumoniae* ESBL producing strains have been identified worldwide (Ben Redjeb *et al.*, 1990; Hibbert-Rogers *et al.*, 1995; Gniadkowski *et al.*, 1998; Bedenic and Zagar, 1998; Laksai *et al.*, 2000; Yang *et al.*, 1999; Gulay *et al.*, 2000; Essack *et al.*, 2001; Morris *et al.*, 2003). ESBLs are mostly found on plasmids (Quinn *et al.*, 1989; Ben Redjeb *et al.*, 1990; Rasmussen *et al.*, 1993; Urban *et al.*, 1994; Benedic and Zagar, 1998; Laksai *et al.*, 2000; Gulay *et al.*, 2000) and have been associated with outbreaks (Urban *et al.*, 1994; Prodinger *et al.*, 1996; Yang *et al.*, 1999). TEM and SHV derived ESBLs are the more commonly found β-lactamases in *K. pneumoniae*. Besides the TEM and SHV derived ESBLs, there are at least seven other distinct ESBL families: CTX-M, PER, VEB, GES, TLA, BES and OXA, indicated by

two recent reviews (Gniadkowski 2001; Stürenberg and Mack, 2003). A dendogram (Bradford 2001) indicates that the Class A ESBLs are more closely related to each other than the Class D OXA-type enzymes which have ESBL activity.

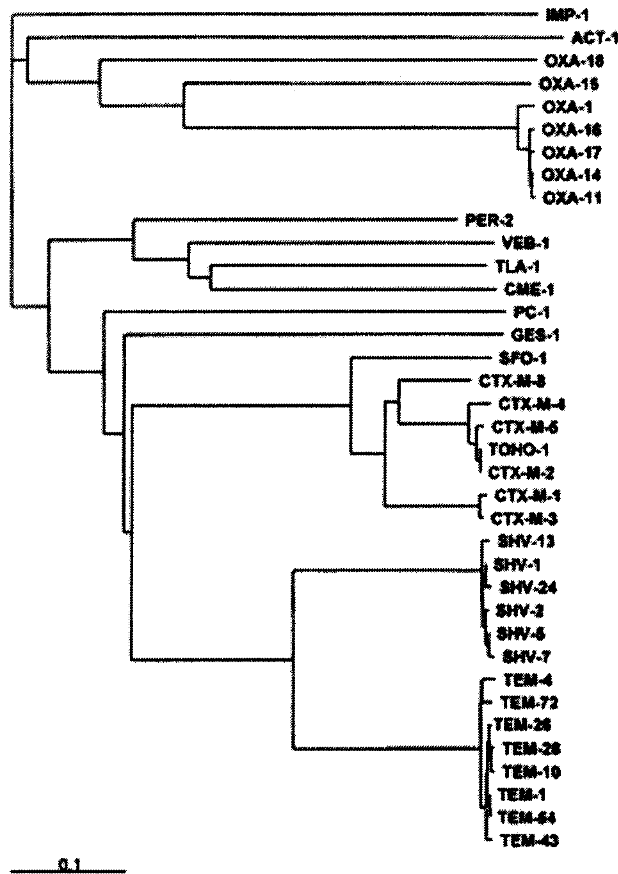


Figure 1.4 (Figure taken from Bradford 2001) Phylogeny of ESBLs. Representative sequences of various ESBLs were obtained from GenBank. The PC1 (class A, *S. aureus* enzyme), IMP-1 (class B, metallo-enzyme), and ACT-1 (class C, AmpC-type enzyme) β-lactamases were included for comparison. Signal peptides were identified with SPSScan and removed prior to alignment. Sequences were aligned using Clustal X. Trees were constructed with Clustal X, which uses the neighbor-joining method, with a bootstrap value of 1,000. The IMP-1 sequence was used to root the tree. Trees were visualized with TREEVIEW

There are also other less frequently reported ESBLs, seemingly isolated and not part of any major family eg the cefuroximase FUR (Vuye *et al.*, 1989) isolated from *K. pneumoniae*; the '*Pseudomonas*' specific enzyme -1 (PSE-1) (Huovinen and Jacoby, 1991) which was found in naturally occurring clinical isolates of *E. coli* (Medeiros *et al.*,

1982); *E. coli* expressing FEC-1 was isolated from fecal flora from dogs (Matsumoto *et al.*, 1988) and DJP-1 isolated from *K. pneumoniae* (Payne *et al.*, 1990). The nomenclature of ESBLs is constantly changing. The ESBL, MEN-1 was isolated from *E. coli* and renamed CTX-M-1 in 1996 (Bauernfeind *et al.*, 1996). A few of the CAZ (Vuye *et al.*, 1989), CTX and YOU enzymes were renamed and placed within the TEM and SHV families (Jacoby and Medeiros, 1991; Sirot 1995; http://www.lahey.org/studies/). Similarly, PSE-2 is now called OXA-10 (http://www.lahey.org/studies/). In an attempt to keep up to date with the constantly changing nomenclature and the addition of new ESBLs, a website was established at http://www.lahey.org/studies/). The cephalosporins most commonly hydrolyzed by ESBLs are ceftazidime and cefotaxime. ESBLs to date do not hydrolyze the cephamycin, cefoxitin, (Livermore 1998). ESBLs are mostly susceptible to the inhibitor clavulanate (Bush *et al.*, 1995). However, the TEM enzymes have evolved to confer resistance to the inhibitors. The inhibitor resistant TEM (IRT) [Chapter 3] enzymes were identified in *E. coli* more frequently than in *K. pneumoniae.*

β-lactamase mediated resistance in *A. baumannii*

β-lactamases found in *A. baumannii* to date are able to hydrolyze penicillins, cephalosporins and carbapenems (Urban *et al.*, 2003). β-lactamases that hydrolyze the cephalosporins are generally the chromosomal enzymes (Sanders 1987; Bergogne-Bérézin and Towner, 1996) and ESBLs. A chromosomal *ampC* gene from *A. baumannii* was cloned and characterized (Bou *et al.*, 2000). Biochemical studies characterized AmpC (Perilli *et al.*, 1996; Bou *et al.*, 2000; López-Hernández *et al.*, 2000; Danes *et al.*, 2002) whilst more recent studies determined the genetic properties of *ampC* (Mammeri *et al.*, 2003; Corvec *et al.*, 2003).

A β-lactamase hydrolyzing both oxacillin and showing hydrolytic activity towards the carbapenem, imipenem, was identified by Hornstein *et al.* (1997) in France. ARI-1 (Donald *et al.*, 2000), now termed OXA-23 is such a Class D carbapenemase which confer resistance to imipenem, was obtained from a clinical isolate in Edinburgh, UK, in 1985 and its corresponding nucleotide sequence determined in 2000 (Donald *et al.*, 2000). OXA-24 was isolated in a strain of *A. baumannii* by Bou *et al.*, (2000) and was

involved in an outbreak of carbapenem resistant *A. baumannii*. Class D OXA-23/27 and OXA-24/25/26 (Bou *et al.*, 2000; Afzal-Shah *et al.*, 2001) are serine carbapenemases identified in *A. baumannii* that weakly hydrolyze the carbapenems *in vitro*. More recently OXA-40, a variant of the OXA-24/25/26 cluster, was involved in carbapenem resistance in *A. baumannii* (Lopez-Otsoa *et al.*, 2002; Héritier *et al.*, 2003).

A. baumannii strains were previously found to contain TEM-1 and CARB-5 (Aubert *et al.*, 1996) penicillinases. TEM-1 and OXA-type β-lactamases have been identified both in isolation (Danes *et al.*, 2002) and combination (Bou *et al.*, 2000; Danes *et al.*, 2002). TEM-1, OXA-21 and a presumptive chromosomal cephalosporinase combination was detected in a clinical strain of *A. baumannii* by Vila *et al.*, 1997. In a previous study they (Vila *et al.*, 1993) reported that TEM-1 was present in 16 % of their clinical isolates. A TEM-1, and an OXA derived gene was reported by Bou *et al.*, (2000), in an isolate from which they cloned the chromosomal *ampC*. By further cloning studies they showed that the OXA derived gene was OXA-24 (Bou *et al.*, 2000). TEM-1 was putatively identified in *A. baumannii* isolates expressing the chromosomal AmpC in conjunction with other unknown β-lactamases (Fernández-Cuenca *et al.*, 2003).

Interestingly, TEM-derived and SHV-derived ESBLs, to date, have not been identified in *A. baumannii*. However, two outbreaks of *A. baumannii* producing non-TEM and non-SHV derived ESBLs have been reported, PER-1 (Vahaboglu *et al.*, 1997) in Turkey and more recently VEB-1 (Poirel *et al.*, 2003) in France. PER-1 was subsequently detected in 62 % of *Acinetobacter* spp. (Vahaboglu *et al.*, 1998).

Zinc-metallo carbapenemases, IMP-1, IMP-2, IMP-3, IMP-4, IMP-5, VIM-1 and VIM-2 have all been identified worldwide in *A. baumannii* (Livermore and Woodford, 2000; Chu *et al.*, 2001; Da Silva *et al.*, 2002; Gales *et al.*, 2002; Tysall *et al.*, 2002; Urban *et al.*, 2003). Two isolates from separate studies yielding a PCR product when tested for bla_{IMP} showed low level meropenem resistance and remained susceptible to imipenem when tested for their respective MICs (Henwood *et al.*, 2002; Tysall *et al.*, 2002). Thus the carriage of the bla_{IMP} gene did not result in the characteristic phenotypic expression

of carbapenem resistance. This finding indicated the importance of early identification and detection of an isolate that may become resistant to the carbapenems. More bla_{IMP} alleles were detected in imipenem resistant *A. baumannii* isolates (Cornaglia *et al.*, 1999; Takahashi *et al.*, 2000). Riccio *et al.*, 2000 identified a bla_{IMP-2} gene on a integron-borne cassette in *A. baumannii*. bla_{VIM-2} was also found on an integron related cassette in *A. baumannii* (Yum *et al.*, 2002). These findings may have implications in the spread of metallo-β-lactamase resistance genes.

Outer membrane proteins, in general

Gram-negative bacteria have both an inner membrane layer and an outer membrane layer, separated from each other by a periplasm. It is the outer membrane that is in constant exposure to the environment and thus the outer membrane serves both as a portal of entry for nutrients and as barrier against harmful chemicals (Hancock 1987; Benz 1988; Nikaido 1994). Outer membrane proteins basically come in two types, α-helical and β-barrel proteins (Schulz 2002). The outer membrane proteins (OMPs) associated with antibiotic resistance are of the β-barrel protein type (Dutzler *et al.*, 1999; Koebnik *et al.*, 2000) which associate as trimers. It is thought that at the narrowest part of the porin trimer, the charge distribution complements that of the ampicillin molecule, thereby creating a region of attraction and facilitating translocation of the β-lactam through the constriction zone, resulting in higher permeability rates (Nestorovich *et al.*, 2002). Impermeability arise when the antibiotics can no longer penetrate the bacterium, either due to reduced or total loss of expression of the porin through which the agent normally enter, or when the porin is altered in structure (Dé *et al.*, 2001). After β-lactamases, this is probably the next most common mechanism of β-lactam resistance in Gram-negative bacteria. This type of resistance is investigated in Chapter 4.

Efflux, in general

Extrusion has been reported as a mechanism of resistance to antibiotics. Efflux pumps, have been categorized and placed into five major groups (Poole 2000), all of which require either the proton motive force or ATP as a source of energy. β-lactam resistance has been attributed to efflux in *Pseudomonas aeruginosa* (Li *et al.*, 1994; Nikaido 1996;

Nikaido 1998; Nikaido *et al.*, 1998; Masuda *et al.*, 1999; Nakae *et al.*, 1999; Okamoto *et al.*, 2002; Borges-Walmsley and Walmsley 2001; Schweizer 2003). β-lactamase inhibitors were also reported to be substrates of multidrug efflux pumps in *Pseudomonas aeruginosa* (Li *et al.*, 1998). In *Salmonella typhimurium* only β-lactam antibiotics with lipophilic side chains were effluxed (Nikaido *et al.*, 1998). However, not all pumps are able to extrude β-lactams as substrates.

Insertional inactivation studies have shown the presence of an RND-type pump involved in aminoglycoside resistance in a clinical resistant strain of *A. baumannii* (Magnet *et al.*, 2001). The possibility of other efflux pumps being present in several *A. baumannii* clinical strains, was investigated by Ribera *et al.* (2002) using a novel pump inhibitor (Renau *et al.*, 1999; Mamelli *et al.*, 2003; Chevalier *et al.*, 2004). Recently, the *tet*(A) gene, encoding a tetracycline efflux pump, was found in combination with the *tet*(M) determinant, in a clinical strain of *A. baumannii* (Ribera *et al.*, 2003). However, efflux as a mechanism of resistance to the β-lactams has not been reported very commonly in *A. baumannii.*

1.4 AIM OF STUDY

The aim of this study was to investigate the molecular mechanisms of resistance to selected β-lactams in selected Gram-negative bacteria isolated from the diagnostic laboratory in Groote Schuur Hospital (GSH).

CHAPTER 2

The molecular basis of AmpC hyperproduction in *E. coli* clinical isolates

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2.3.2 DNA sequence analysis of the regulatory region of *ampC*

2.3.3 Expression of *ampC*

2.3.4 *ampC* mRNA studies

2.4 CONCLUSION

2.1 INTRODUCTION

The chromosomal cephalosporinase gene, *ampC*, of *E. coli* is different from its counterparts in other members of the family Enterobacteriaceae in that it is not inducible (Jaurin and Grundström, 1981). *E. coli* strains carrying the wild type gene produce low basal amounts of AmpC and are inherently susceptible to ampicillin (Livermore 1995).

Analysis of promoters of *E. coli* genes identified consensus promoter sequences comprising a -35 hexamer (TTGACA) optimally separated (17 bp) from a -10 hexamer (TATAAT) (Hawley and McClure, 1983; Ozoline *et al.*, 1997). The promoter sequences, TTGTCA (-35) and TACAAT (-10), for *E. coli ampC* are separated by 16 bp and show poor homology to their corresponding consensus sequences (Olsson *et al.*, 1983; Caroff *et al.*, 1999; Caroff *et al.*, 2000; Forward *et al.*, 2001; Siu *et al.*, 2003). It is this weak promoter, in combination with an attenuator containing a hairpin loop, which decreases the efficiency of transcription of *ampC*, leading to a low level production of the enzyme (Jaurin *et al.*, 1981).

In some strains AmpC is hyperproduced and these strains are resistant to cefoxitin and β-lactamase inhibitors (clavulanate), and have reduced susceptibility to the newer β-lactams such as the oxyiminocephalosporins (Bergström and Normark, 1979; Cooksey *et al.*, 1990; Jacoby and Sutton, 1985; Jaurin *et al.*, 1982; Livermore 1995). Studies on the molecular basis of AmpC hyperproduction have shown that some hyperproducers contain more than one copy of *ampC* (Edlund *et al.*, 1979; Edlund and Normark 1981), while others have acquired an insertion element, IS2, which provides a strong promoter for the transcription of *ampC* (Jaurin and Normark, 1983). In addition, mutations in the regulatory regions and/or the attenuator of *ampC* have been identified as the molecular basis of hyperproduction of AmpC (Olsson *et al.*, 1983; Caroff *et al.*, 1999; Caroff *et al.*, 2000; Forward *et al.*, 2001; Corvec *et al.*, 2002).

During 1997, a cluster of *E. coli* strains with antibiotic resistance profiles suggestive of AmpC hyperproduction was isolated from patients in hospitals in Cape Town. Isolation

of these strains was sporadic, indicating that they were not related to an outbreak, nevertheless, this was unusual: *E. coli* AmpC hyperproducers were rarely isolated in the Groote Schuur Hospital (GSH) diagnostic laboratory. This chapter will describe studies carried out to determine the genetic basis of β-lactam resistance in the cluster of *E. coli* strains. Part of this work was published in Antimicrobial Agents and Chemotherapy, (1999) 43: 957-959.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Bacterial strains and plasmids

Six clinical isolates of *E. coli*, strains E1 to E6, were isolated and identified in the diagnostic laboratory in Groote Schuur Hospital, Cape Town. Two of the strains were isolated from urine, two were isolated from wounds and one was isolated from an abscess. The sixth isolate was selected as a susceptible control. *E. coli* DH5α was used as a recipient in transformation studies. Plasmid pUC19 was used as vector in cloning experiments.

2.2.2 Antibiotic susceptibility testing

Antibiotic susceptibility profiles were determined by the Kirby Bauer disk diffusion method using National Committee for Clinical Laboratory Standards criteria (NCCLS). MICs were determined using Etests (AB Biodisk, Solna Sweden).

2.2.3 Methods used in the characterization of the *ampC* promoters

2.2.3.1 Isolation and quantitation of genomic and plasmid DNA

Bacterial genomic DNA was extracted from *E. coli* according to the CTAB method described by Ausabel *et al.*, (1987). The final DNA was pellet washed with 70 % ethanol, air dried for five minutes and resuspended in 50-100 μl TE buffer or dH_20. Small amounts of plasmid DNA were isolated using the alkaline lysis method of Ish-

Horowicz and Burke (1981). Large-scale plasmid DNA was isolated using Nucleobond AX 100 PC-kit (Macherey-Nagel, Germany). DNA was quantitated using spectrophotometric analysis using an ultraviolet source set to 260 nm. One OD unit relating to 50 μg/ml.

2.2.3.2 Polymerase Chain Reaction (PCR) amplification of promoter sequences

DNA sequences containing the promoter region of *ampC* were amplified using *Taq* (Biotaq) and primers, 5′-CTACGGTCTGGCTGCTA-3′ and 5′-TGGAGCAAGAGGCGGTA-3′ which anneal to the nucleotides -61 to -45 and +92 to +108, of the *ampC* functional gene, respectively. PCR was performed by initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 50 sec, annealing at 60°C for 50 sec and extending at 72°C for 50 sec, and a final extension at 72°C for 7 min. Amplicons were sequenced directly.

2.2.3.3 Agarose gel eletrophoreses (AGE)

Amplicons, restricted plasmids and DNA fragments were separated in horizontal agarose gels of 0.8-1 % (w/v) in TAE, containing ethidium bromide (0.5 μg/μl) and visualized over UV light. When necessary, DNA was gel purified using either the method described by Seth (1984), or the Qiagen gel purification kit.

2.2.3.4 Sequencing

Sequencing of PCR products and recombinant plasmids was performed using automated sequencing facilities at the Core Facility, University of Cape Town. Recombinant plasmids, following site-directed mutagenesis, were sequenced manually, using [α-^{32}P]dATP and the SequiTherm EXCEL™ *II* DNA sequencing kit. DNA sequence alignments were performed by DNAMAN version 4.0 (Lynnon BioSoft) software.

2.2.4 Methods used to study the expression of AmpC

2.2.4.1 PCR of *ampC* regulatory region

DNA was extracted [2.2.3.1] subjected to AGE and purified [2.2.3.3]. The promoter regions were amplified using the primers 5′-TCCGAATTCCTACGGTCTGGCTGCTA-

3′, (-61 to -45) and 5′-TTTGGATCCAGGGTCTGGTTTCCAT-3′, (+44 to +59), where underscores represent *Eco*RI and *Bam*HI restriction sites, respectively. The PCR assay was carried out as described [2.2.3.2].

2.2.4.2 Cloning and transformation studies

PCR generated promoter fragments were cloned into pUC19, using the *Bam*HI and *Eco*RI restriction sites (in the opposite orientation to the *lac* promoter). Clones were selected for on 2 × YT agar plates containing ampicillin 50 (μg/ml), IPTG (2.5 mg/L) and X-gal (0.5 %). A *Bam*HI and *Sal*I fragment containing the luciferase gene was restricted from pGEM-*luc* (Promega) and ligated downstream of the promoter sequences previously ligated into pUC19. The recombinant plasmids were introduced into competent *E. coli* DH5α (Dagert and Ehrlich, 1979) by transformation. Clones containing the lucifersase gene were identified following restriction enzyme digestion for a *Bam*HI and *Sal*I fragment containing the luciferase gene.

2.2.4.3 Site-directed mutagenesis

Nucleotide changes were introduced in the promoter sequences contained in the recombinant plasmids [2.2.4.2] by site-directed mutagenesis using a Stratagene QuikChange® Site-Directed Mutagenesis kit. The primers used were designed to change the -42 T→C (at nucleotide -42) and a -1 T→C (position -1) of the *ampC* promoter region.

2.2.4.4 Luciferase assays

Cultures of *E. coli* were grown to mid-log phase (optical density at 600 nm, 0.7 to 0.8). Cells were harvested from 1 ml of culture, resuspended in 100 μl of 25 mM Tris-HCl (pH 7.8)-2 mM EDTA-10 % glycerol-1 % Triton X-100, sonicated in a Branson waterbath sonicator for 20 s, and placed on ice for 20 s. Sonication and cooling were repeated, after which, the lysate was centrifuged for 5 min at 14000 × g in an Eppendorf centrifuge. The supernatant was collected, and the protein concentration was determined using a Bio-Rad *DC* protein assay. Luciferase activity was measured in a BioOrbit 1253 luminometer after 100 μl of luciferin (Promega) had been mixed with 20 μl of cell extract.

2.2.5 Methods used to study stability of mRNA transcripts

2.2.5.1 Extraction and quantitation of RNA

The strains were cultured until exponential phase. At this point a 10 ml aliquot was removed and kept on ice. To inhibit further initiation of transcription, rifampin (0.2 mg/ml) was added to the cells. At selected time intervals a 10 ml aliquot was withdrawn and chilled on ice. Cells were harvested and total RNA was extracted using hot acidic phenol (Elisha and Steyn, 1991). RNA was quantitated by spectrophotometric analysis using an ultraviolet source set to 280 nm. One OD unit related to 33 μg/ml.

2.2.5.2 Preparation of *ampC* and 16S rRNA probes

Primers 5′-TACTGGCGTGCTTGGTG-3′ and 5′-GACTCTTCGCTGGATTGG-3′ corresponding to nucleotides +314 to +330 and+1141 to +1157, respectively were used to amplify an 844 bp internal portion of *ampC* with conditions described [2.2.3.2]. The 840 bp fragment of *ampC* was purified using a Qiagen spin column and labeled with [α-^{32}P]dCTP using Ready-To-Go DNA labeling Beads (Pharmacia Biotech). Similarly, an internal fragment of the 16S rRNA gene was amplified from *E. coli* DNA using the universal primers (Edwards *et al.*, 1989) and labeled.

2.2.5.3 Preparation of Northern blot

Total RNA (15 μg) was electrophoresed in 1.2 % agarose-0.66 M formaldehyde with 40 mM morpholinepropanesulfonic acid-10 mM sodium acetate-1 mM EDTA (pH 7.7) and transferred to Hybond N+ (Amersham International) nylon membrane in 20 × SSC.

2.2.5.4 DNA-RNA hybridization

Hybridization procedures used and conditions for washing were those recommended by the manufacturer of Hybond.

2.3 RESULTS AND DISCUSSION

2.3.1 Antimicrobial susceptibility testing

Using disc susceptibility testing, all the isolates were resistant to cefoxitin and cefuroxime and susceptible to cefotaxime. Only E2 showed reduced susceptibility to cefotaxime. The MICs of ceftazidime, cefuroxime, ceftriaxone, and cefotaxime were determined and are presented in Table 2.1. Each of the antibiotics was less active against E1, E2, E3, E4, and E5 than against the susceptible isolate E6 (Table 2.1). This pattern of resistance and reduced susceptibility is consistent with an increased expression of AmpC in *E. coli.*

TABLE 2.1 MICs for *E. coli* strains E1 to E6

MIC (µg/ml) of:				
Strain	Ceftazidime	Cefuroxime	Ceftriaxone	Cefotaxime
E1	32	>256	3	3
E2	6	>256	2	3
E3	24	>256	4	4
E4	32	>256	4	4
E5	16	>128	1.5	2
E6	0.25	4	0.0047	0.0047

2.3.2 DNA sequence analysis of the regulatory region of *ampC*

To investigate the regulatory region of *ampC* in the resistant strains, the promoter/attenuator sequences were amplified and sequenced directly. The sequences

corresponding to nucleotides -44 to +91 (Jaurin and Grundström, 1981) from E1, E2, E3, E4, E5 and E6 are shown in Fig. 2.1.

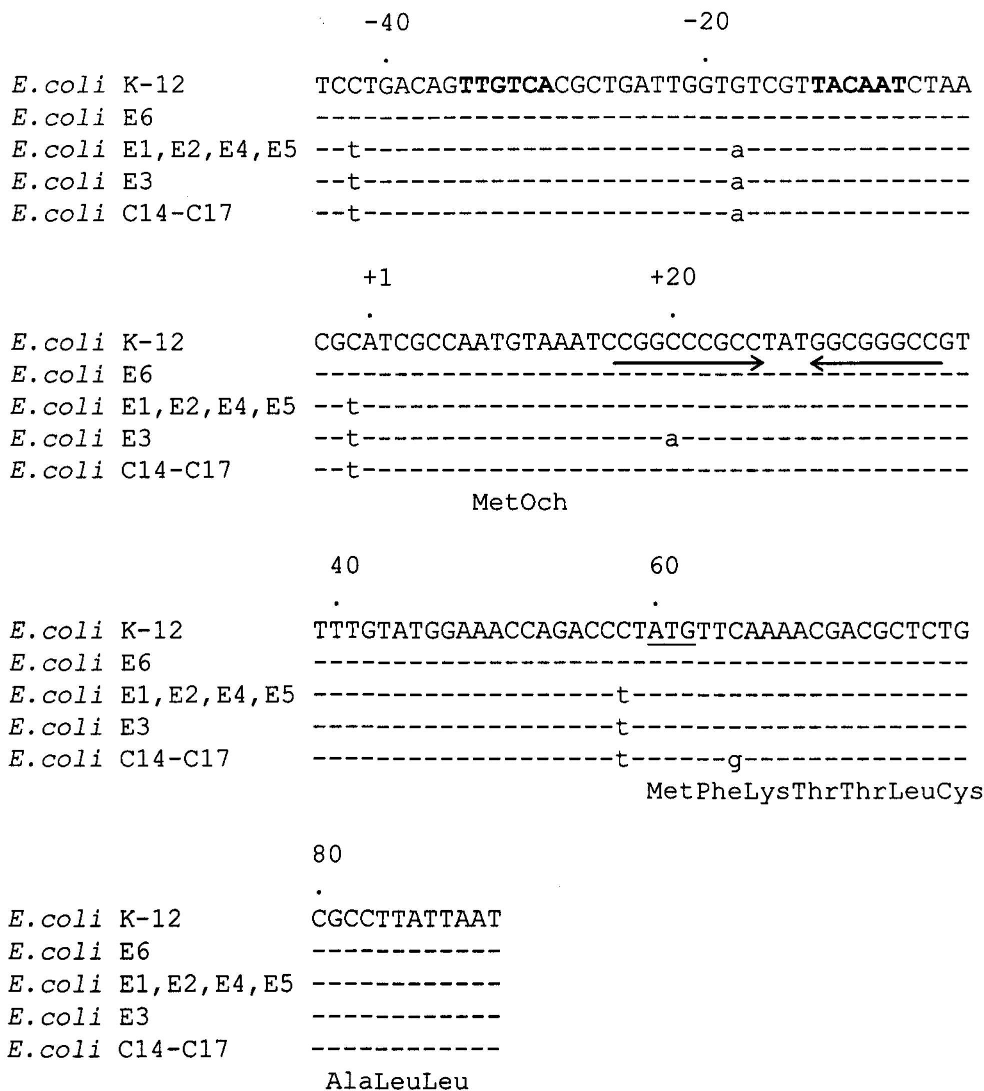

Figure 2.1 Comparison of DNA sequence (135 bp) of the *ampC* control region and signal peptide from susceptible and AmpC-hyperproducing *E. coli* strains. The sequences from *E. coli* K-12, hyperproducing strains C14 to C17, are from Olsson *et al.*, 1983. A dot above the sequence represents every 20 nucleotides. Identical nucleotides are indicated by dashes. Nucleotides that differ from the sequences of the susceptible *E. coli* strains (K-12 and E6) are in lowercase. The prototype -35 and-10 regions are in boldface lettering and the attenuator is indicated by the arrows. The initiation codon is underlined, and the three letter amino acid code is directly below the partial sequence of the signal peptide.

The sequences from E1, E2, E3, E4 and E5 contain a T at -42, a G at -18, a T at -1, and a T at +58, whereas C, A, T, and C are present in the corresponding positions in the sequence from the susceptible strain E6. The mutation at -42 changes a C to a T, creating a new hexamer that has perfect homology to the consensus -35 hexamer (TTGACA) recognized by the major RNA polymerase ($E\sigma^{70}$) in *E. coli* (Hawley and McClure, 1983). A concomitant mutation at -18 (G→A) generates an optimally spaced (17 bp) -10 hexamer (TATCGT), with good homology to the consensus sequence (TATAAT), creating a strong promoter in the *ampC* regulatory region (Fig. 2.1). In this respect the promoters are the same as those of clinical isolates of *E. coli* that over express AmpC (Olsson *et al.*, 1983; Caroff *et al.*, 1999; Caroff *et al.*, 2000; Forward *et al.*, 2001). Interestingly the same promoter has been described for the *S. sonnei ampC* (Olsson *et al.*, 1983). It was previously suggested that *E. coli* strains had acquired or retained regulatory regions from *S. sonnei*, generating a small *E. coli* sub population that contains the *ampC* regulatory region of *Shigella sonnei*, which have the potential to mutate to high-level ampicillin resistance (Olsson *et al.*, 1983).

Different mutations (Table 2.2) resulting in strong promoters have been described (Olsson *et al.*, 1983; Caroff *et al.*, 1999; Caroff *et al.*, 2000; Forward *et al.*, 2001; Corvec *et al.*, 2002; Siu *et al.*, 2003). A transversion at -32 (T→A) changes the -35 hexamer towards consensus, with a concomitant increase in cefoxitin MICs (Forward *et al.*, 2001) and promoter activity based on CAT reporter gene assays (Caroff *et al.*, 2000). Mutations in the -10 hexamer alone, appear to be less frequent events (Corvec *et al.*, 2002). A C→T transition in the -10 hexamer resulted in only a moderate increase in promoter strength (Corvec *et al.*, 2002), underlining the importance of the degree of homology of the -35 hexamer to the consensus sequence with respect to docking of the RNA polymerase. Recently, a 2 bp insertion (GT) between nucleotide -14 and -15 resulted in two new -35 and -10 hexamers used in the transcription of *ampC* (Siu *et al.*, 2003).

TABLE 2.2 Mutations identified in the regulatory regions (promoter and/or attenuator) of *ampC* in *E. coli*

Reference and Strains	-42 mutation	**-35 hexamer** **TTGTCA** **-35 to -30**	Spacer region **-29 to -14**	**-10 hexamer** **TACAAT** **-13 to -8**	Upstream of attenuator	Attenuator (hairpin loop)	Downstream of attenuator
From Olsson *et al.*, 1983							
C14, C15, C16, C17	C→T		-18G→A		-1C→T		+58C→T
From Caroff *et al.*, 1999							
95003941	C→T		-18G→A		-1C→T	+31C→G	+58C→T
96002328	C→T		-18G→A		-1C→T		+58C→T
96004153		-32T→A	-18G→A		-1C→T +5C→A	+24C→A	+58C→T
96001256		-32T→A	-18G→A		-1C→T		+58C→T
96007791		-32T→A	-18G→A		-1C→T		+58C→T
95004674			G insertion -13 and -14 17 bp spacer			+24C→A	
From Caroff *et al.*, 2000							
96004153	C→T	-32T→A	-18G→A		-1C→T +5C→A	+24C→A	+58C→T
96010266	C→T		-18G→A		-1C→T		+58C→T

Reference and Strains	-42 mutation	**-35 hexamer** **TTGTCA** **-35 to -30**	Spacer region **-29 to -14**	**-10 hexamer** **TACAAT** **-13 to -8**	Upstream of attenuator	Attenuator (hairpin loop)	Downstream of attenuator
From Forward *et al.*, 2001							
20, 60, 47	C→T		-18G→A		-1C→T		+58C→T
62	C→T		-18G→A		-1C→T	+23G→A +37G→A	+49A→G +58C→T
45	C→T		-18G→A		-1C→T	+33G→A	+58C→T
52, 40, 53, 79			CT insertion -13 and -14 18 bp spacer			22C→T 26,27TA→GT 32G→A	
65			T insertion -13 and -14 17 bp spacer		+5C→T	20C→G	
28			T insertion -13 and -14 17 bp spacer		+5C→T		
71			T insertion -13 and -14 17 bp spacer			36,37CG→GT	39T→C
74			T insertion -13 and -14 17 bp spacer				
2, 17		-32T→A					+58C→T
54, 58		-32T→A				22C→T 26,27TA→GT 32G→A	
30		-32T→A					+58C→T

Reference and Strains	-42 mutation	**-35 hexamer** **TTGTCA** **-35 to -30**	Spacer region **-29 to -14**	**-10 hexamer** **TACAAT** **-13 to -8**	Upstream of attenuator	Attenuator (hairpin loop)	Downstream of attenuator
23			-18G→A				+58C→T
31			-18G→A		-1C→T		+58C→T
42			-18G→A		-1C→T	22C→T 26,27TA→GT 32G→A	
59			-28G→A				+58C→T
6, 11							+58C→T
35, 41, 34, 67						22C→T 26,27TA→GT 32G→A	
From Corvec *et al.*, 2002							
96010266M	C→T		-18G→A		-1C→T		+58C→T
99004202			-18G→A	-11C→T	-1C→T		+58C→T
From Siu *et al.*, 2003							
EC38			-28G→A GT insertion -14 and -15 18 bp spacer		-1C→T		+58C→T
EC44			-28G→A T insertion -19 and -20 17 bp spacer				

Author and reference in yellow highlighted cells

Wild type promoter hexamers from *E. coli* K12 in red bolded font

Nucleotide numbering from Jaurin and Grundström, 1981

Apart from the sequences of the -35 and -10 hexamers, the distance between the two plays an important role in the strength of the promoter (Hawley *et al.*, 1983; Ozoline *et al.*, 1997). Alteration of the two hexamers from 16 to 17-bp resulted in a cefoxitin MIC of 32 μg/ml (Forward *et al.*, 2001).

The expression of *E. coli* AmpC is regulated also by an attenuator located immediately upstream of the *ampC* transcriptional start site (Jaurin *et al.*, 1981; Caroff *et al.*, 1999; Forward *et al.*, 2001). All of the *ampC* regulatory regions investigated in this study contained mutations (C→T) at position -1 and +58, the nucleotides flanking the attenuator. It is interesting to speculate on the roles of these mutations in the increased transcription of *ampC*. It has been shown that DNA sequences found downstream of pause sites influence the pause half-life of RNA polymerase (Feng *et al.*, 1994) and that a bound ribosome prevents the formation of stem-loop structures (Platt, 1986). It may be that the mutations at -1 and +58, together with the attenuator, cause the RNA polymerase to pause, allowing the ribosome to stay close behind, thereby negating the formation of the attenuator stem and loop and facilitating transcriptional readthrough.

Only one of the *ampC* regulatory regions (E3) contained an additional mutation (C→A at +20), which disrupted the hairpin loop of the attenuator. However, this mutation did not result in an increase in promoter activity when compared with the activity of the regulatory regions from strains E1, E2, E4 and E5 [Table 2.3]. A number of different mutations have been identified in the attenuator (Forward *et al.*, 2001). In most cases mutations in the attenuators are associated with mutations in the promoter sequences (Forward *et al.*, 2001).

2.3.3 Expression of *ampC*

To study the expression of the *ampC* genes, the luciferase gene was cloned downstream of the *ampC* promoters amplified from E1 to E6, ligated into pUC19 [2.2.4]. The recombinants designated pLucE1, pLucE2, pLucE3, pLucE4, pLucE5 and pLucE6 were expressed in *E. coli* DH5α and promoter activity was determined by measuring luciferase activity [2.2.4]. When compared with luciferase activity obtained with *E. coli* (pLucE6), an 8- to 18- fold increase in activity was obtained with *E. coli* containing pLucE1, pLucE2, pLucE3, pLucE4 and pLucE5. These data suggest that the nucleotide changes at -42 and possibly -1 upregulate the expression of AmpC.

To understand more fully the role of each mutation in the increased expression, site-directed mutagenesis was performed [2.2.4]. Changing the -42 residue from T→C resulted in luciferase activity levels identical to that of pLucE6 (Table 2.3). This accords with the data of Caroff *et al.* (2000). On the other hand, reverting the -1 residue (T→C), did not result in any significant reduction in luciferase expression (Table 2.3). When both positions (-42 and -1) were reverted, the expression of luciferase was similar to that observed when only -42 was reverted. Thus, the -1 mutation can be excluded as being a major factor in the overexpression of AmpC, as has been suggested previously (Caroff *et al.*, 2000); the -42 mutation is the key to the increased levels of AmpC expression.

TABLE 2.3 Effect of the mutations on the strength of the promoters

Constructs	Luciferase activity (Units/mg protein)
pLucE1	1121
pLucE2	1079
pLucE3 (attenuator +20)	505
pLucE4	675
pLucE5	914
pLucE6 (susceptible promoter)	61
pLucE5 with -42 T→C	44
pLucE5 -1 T→C	643
pLucE5 with -42 T→C and -1 T→C	23
pLuc with no promoter	6

2.3.4 *ampC* mRNA studies

To ascertain whether the stability of mRNA transcripts played a role in resistance, Northern hybridizations [2.2.5] were carried out. When the membranes were probed with an internal portion of *ampC*, no signal was detected with RNA from the susceptible isolate E6 (Fig. 2.2). A strong signal was obtained when the membrane was probed with the 16S rRNA probe, indicating that RNA has been transferred to the membrane. The *ampC* probe hybridized to a transcript of the expected size (1.2 kb) in each of the resistant strains signifying an increase in transcription of *ampC* in the E1–E5 (Fig. 2.2). The amounts of transcripts began to decrease immediately after the addition of rifampin. Four minutes after the addition of this antibiotic the signal was barely visible, indicating that the transcript half-life is typical of that of *E. coli* messages (Belasco *et al.*, 1986). These findings show that mRNA stability is not responsible for the increase in AmpC, implying that the gene is transcriptionally regulated.

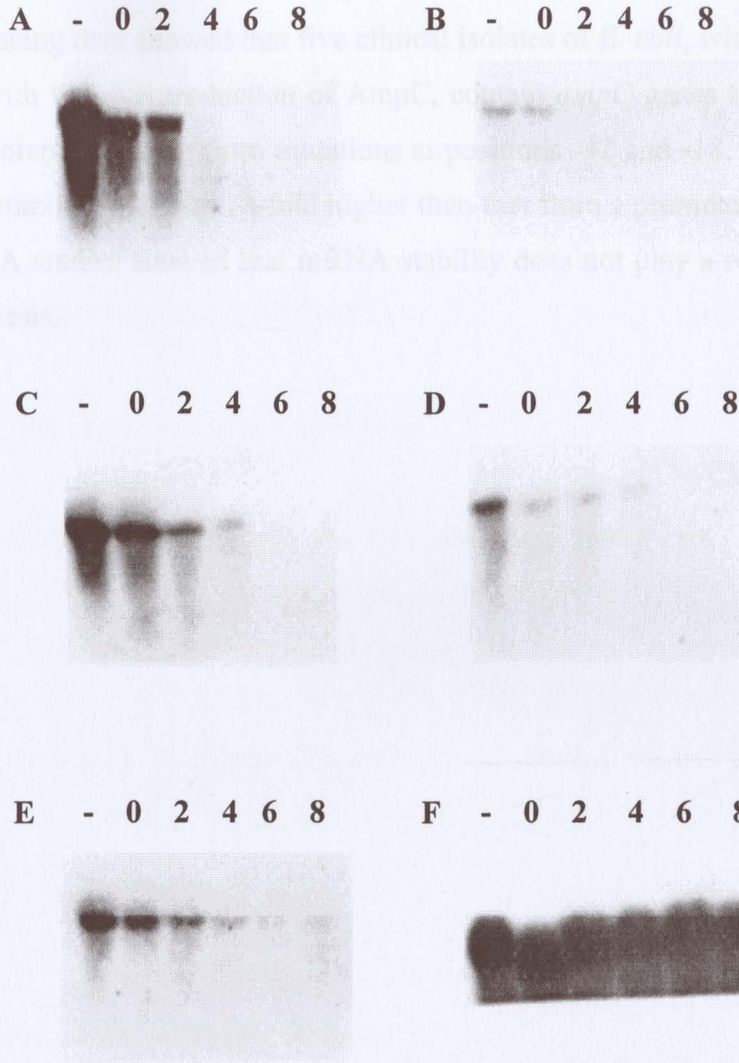

Figure 2.2 Hybridization of the Northern blots prepared from RNAs extracted from *E. coli* E1 to E6 and probed either with the *ampC* or 16S rRNA probe. The strains were cultured until exponential phase. At this point (lane -) a 10 ml aliquot was removed and RNA was extracted. Rifampin (0.2 mg/ml) was added, a 10 ml aliquot was withdrawn immediately (lane 0), and RNA was extracted. Additional aliquots were removed after 2, 4, 6, and 8 min, and RNA was extracted. Panels: A, RNA from E1 probed with *ampC* probe; B, RNA from E2 probed with the *ampC* probe; C, RNA from E3 probed with the *ampC* probe; D, RNA from E4 probed with the *ampC* probe; E, RNA from E5 probed with the *ampC* probe; F, RNA from E6 probed with 16S rRNA probe.

2.4 CONCLUSIONS

DNA sequencing data showed that five clinical isolates of *E. coli*, with resistance profiles consistent with the overproduction of AmpC, contain *ampC* genes that are preceded by strong promoters, resulting from mutations at positions -42 and -18. Transcription from the strong promoter was 8-to 18-fold higher than that from a promoter from a susceptible isolate. RNA studies showed that mRNA stability does not play a role in the control of AmpC synthesis.

CHAPTER 3

An investigation of amoxicillin-clavulanate resistance in *E. coli*

3.1 INTRODUCTION

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Bacterial strains and plasmids

3.2.2 Methods used to detect and characterize β-lactamase genes

3.2.2.1 PCR detection of bla_{TEM} related genes

3.2.2.2 Sequencing and sequence analysis

3.2.3 Method to determine transcriptional start site(s) of $bla_{TEM-30A}$

3.3 RESULTS

3.3.1 Antibiotic susceptibility testing

3.3.2 Detection and characterization of bla_{TEM} genes

3.3.3 Primer extension analysis

3.4 DISCUSSION

3.1 INTRODUCTION

In Chapter 2, I discussed how mutations in the *ampC* promoter of *E. coli* resulted in an increase of AmpC and a concomitant increase in resistance to the oxyiminocephalosporins and cefoxitin. In 1999, *E. coli* strains exhibiting resistance to amoxicillin-clavulanate were isolated in the clinical microbiology laboratory at Groote Schuur Hospital (GSH). As the strains were susceptible to the oxyiminocephalosporins and cefoxitin, the resistance to amoxicillin-clavulanate could not be attributed to the overproduction of AmpC.

Clavulanate is a natural product, which protects the accompanying amoxicillin from degradation by binding irreversibly to the catalytic site of serine β-lactamases (Yang *et al.*, 1999; Wang *et al.*, 2002; [Chapter 1]). Resistance to the inhibitor combination, amoxicillin-clavulanate, was reported from as early as 1987 (Martinez *et al.*, 1987). A subsequent report of amoxicillin-clavulanate resistance was made in 1989 in clinical *E. coli* and *K. pneumoniae* strains from hospitalized patients in Spain (Roy *et al.*, 1989). Resistance to the combination is now prevalent (Blazquez *et al.*, 1993; Zhou *et al.*, 1994; Stapleton *et al.*, 1995; Henquell *et al.*, 1995; Leflon-Guibout *et al.*, 2000) and probably results from the extensive use of amoxicillin-clavulanate in both the community and hospitals. In *E. coli,* mechanisms responsible for this resistance include hyperproduction of AmpC [Chapter 2], inhibitor resistant TEM β-lactamases (IRTs) (Zhou *et al.*, 1994; Nicolas-Chanoine, 1997; Livermore 1998; Yang *et al.*, 1999; Bonomo and Rice 1999; Wang *et al.*, 2002; Miró *et al.*, 2002), overproduction of TEM-1 (Wu *et al.*, 1994; Stapleton 1995), oxacillinases (Zhou *et al.*, 1994; Ling *et al.*, 1994; Miró *et al.*, 2002) and decreased uptake (Reguera *et al.*, 1991). The first three mechanisms account for the majority of resistance to amoxicillin-clavulanate.

The gene encoding TEM-1 was first identified on a plasmid from *E. coli* (Datta and Kontomichalou, 1965). Since then a plethora of TEM-derived β-lactamases, including Extended Spectrum β-lactamases (ESBLs) and IRTs have been identified (Livermore 1998; Bonomo and Rice, 1999).

```
3601   AGGAAGCAAAGCTGAAAGGAATCAAATTTGGCCGCAGGCGTACCGTGGACAGGAACGTCG

3661   TGCTGACGCTTCATCAGAAGGGCACTGGTGCAACGGAAATTGCTCATCAGCTCAGTATTG

3721   CCCGCTCCACGGTTTATAAAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTT
                                                          T(32) Pa/Pb
3781   ATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAA

3841   TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT
                                                G(141) P5
3901   GAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATG
         T(162) P4
```

Figure 3.1 Nucleotide sequence of the promoter region, including the start codon (dark green), of the *bla*$_{TEM-1A}$ gene. The -35 and -10 hexamers of *Pa* are indicated in bright green, the -35 hexamer of *Pb* is underscored and in bold (italics are used where the -35 hexamer of *Pb* overlaps with the -10 hexamer of *Pa*), -35 and -10 hexamers of *P3* are highlighted in pink. The mutations giving rise to promoters *Pa*/*Pb*, *P5* and *P4*, are indicated directly below the nucleotide bases of *bla*$_{TEM-1A}$. The nucleotide numbering in brackets is according to Sutcliffe, 1978.

Early studies on the regulation of *bla*$_{TEM}$ identified three promoters for initiation of transcription (Figure 3.1), the weak *P3* promoter (Russell and Bennett, 1981) and two overlapping promoters (*Pa* and *Pb*) (Chen and Clowes, 1984). Following a C→T substitution at position 32, transcription from *Pa*/*Pb* resulted in a 10-fold increase in expression from *bla*$_{TEM-1}$ (Chen and Clowes, 1984). More recently, two additional promoters have been described. A G→T substitution in the -10 hexamer (position 162) of the *P3* promoter, results in a strong promoter, designated *P4* (Leflon-Guibout *et al.*, 2000). A C→G transversion in the -35 hexamer of *P3* results in promoter *P5* (Lartigue *et al.*, 2002). Thus, up mutations in either of the weak *P3* promoter hexamers generates a strong promoter.

Based on the recent TEM gene nomenclature, which takes into account the nucleotide sequence of the coding region and the promoter sequences (Goussard and Courvalin, 1999; Leflon-Guibout *et al.*, 2000), sometimes called sequence framework (Caniça *et al.*, 1997; Pomba-Féria and Caniça, 2003) seven *bla*$_{TEM}$ genes (*bla*$_{TEM-1A}$, *bla*$_{TEM-1B}$, *bla*$_{TEM-1C}$, *bla*$_{TEM-1D}$, *bla*$_{TEM-1E}$, *bla*$_{TEM-1F}$, and *bla*$_{TEM-1G}$) encoding TEM-1 β-lactamase have been described. As shown in Table 3.1 the different designations are based on the nucleotide

sequences of the functional genes, using *bla*$_{TEM-1A}$ as the reference sequence. All the mutations in the structural genes are silent mutations.

TABLE 3.1 [a]Nucleotide mutations in genes encoding TEM-1

Strain or reference	bla_{TEM} (promoter)	**32**	**141**	**162**	**175**	226 (6)	346 (48)	407 (69)	436 (78)	604 (134)	682 (160)	925 (242)	929 (244)
Leflon-Guibout *et al.*, 2000b	1A (*P3*)	C	C	G	A	C	A	A	C	G	T	G	C
Lartigue *et al.*, 2002	1B (*Pa/Pb*)	T				T			T	T			
Pomba-Féria and Caniça, 2003	1B (*P3*)				G	T			T	T			
Lartigue *et al.*, 2002	1B (*P4*)			T		T			T	T			
Lartigue *et al.*, 2002	1B (*P5*)		G			T			T	T			
Leflon-Guibout *et al.*, 2000b	1C (*P3*)								T				
Leflon-Guibout *et al.*, 2000b	1D (*P3*)								T				
Leflon-Guibout *et al.*, 2000b	1E (*P3*)				G	T	G		T	T			
Leflon-Guibout *et al.*, 2000b	1F (*P4*)			T			G	T			C		
Pomba-Féria and Caniça, 2003	1G (*P3*)								T	T			

[a]Nucleotide numbering is according to the method of Sutcliffe, 1978
bla_{TEM-1} framework is used as a reference , differences are indicated below the bla_{TEM-1A} gene
The numbering in bold refers to the promoter region
Amino acid numbering is shown in brackets below the corresponding nucleotide number

Overproduction of the TEM-1 type enzyme probably accounts for most amoxicillin-clavulanate resistance in *E. coli* (Ling *et al.*, 1994; Stapleton *et al.*, 1995). Nevertheless, a significant number of *E. coli* strains harbour IRTs (Blazquez *et al.*, 1993; Zhou *et al.*, 1994; Henquell *et al.*, 1995; Bret *et al.*, 1997). These enzymes were first identified in amoxicillin-clavulanate resistant *E. coli* from France (Vedel *et al.*, 1992; Henquell *et al.*, 1994; Bremer-Melchior *et al.*, 1995; Girlich *et al.*, 2000; Leflon-Guibout *et al.*, 2000). Characterization of the mechanism of resistance revealed TEM-1 and TEM-2 variants. Initially the enzymes were given the moniker IRT, but subsequently they have been renamed with numerical TEM descriptions, which take into account the origins of the gene and its promoter (Leflon-Guibout *et al.*, 2000). Like TEM-1, the IRTs belong to the Ambler molecular class A and Bush-Jacoby-Medeiros group 2br [Chapter 1, Table 1]. There are at least 22 distinct IRTs with designations ranging between TEM-30 and TEM-80 (www.lahey.org/studies/temtable.stm).

Point mutations that lead to IRT phenotype occur at specific amino acids within the TEM enzyme (Bonomo and Rice, 1999). A number of amino acid substitutions have been reported (Zhou *et al.*, 1994; Henquell *et al.*, 1995; Bret *et al.*, 1997; Leflon-Guibout, 2000). The structural modifications caused by the substitutions have been determined, and the kinetic properties of the altered enzymes have been established (Knox, 1995; Bret *et al.*, 1997). It is important to note that point mutations leading to IRT phenotype are different from those which lead to ESBL phenotype. Thomson *et al.*, 1998 made an interesting observation that mutations leading to IRT phenotype can be reversed *in vitro* and suggest that amoxicillin-clavulanate selective pressure can lead to IRT forward mutations.

IRTs have been found mainly in clinical isolates of *E. coli* and to a lesser extent in *Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* and *Citrobacter freundii* (Roy *et al.*, 1989; Lemozy *et al.*, 1995; Bret *et al.*, 1997). While the majority of reports on IRT producing *E. coli* strains have come from France (Bemer-Melchior *et al.*, 1995; Leflon-Guibout *et al.*, 2000), they have also been isolated from other European countries

(Caniça *et al.*, 2002; Féria *et al.*, 2002; Miró *et al.*, 2002). The number of IRT producing *E. coli* strains in most countries is unknown.

This Chapter will describe the studies carried out to determine the genetic basis of resistance to amoxicillin-clavulanate in four clinical isolates of *E. coli* from Groote Schuur Hospital.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Bacterial strains and plasmids

Three strains of *E. coli*, (AC1, AC2 and AC3) were isolated from patients in Groote Schuur Hospital (GSH), Cape Town and one strain was isolated (AC4) from a patient attending a community-based clinic associated with GSH during 1999. AC1 was isolated from a pus swab; AC2 from blood; AC3 and AC4 were isolated from urine. Antibiotic disk susceptibility testing using National Committee for Clinical Laboratory Standards, showed that two of the strains (AC3 and AC4) were resistant to the inhibitor combination amoxicillin-clavulanic acid while the remaining strains (AC1 and AC2) had intermediate susceptibility to this combination. MICs for amoxicillin-clavulanate were performed using Etest [2.2.2]. pUC19 DNA was used as a positive control for TEM PCR.

3.2.2 Methods used to detect β-lactamase genes

3.2.2.1 PCR detection of bla_{TEM} related genes

An internal portion of the bla_{TEM} gene was amplified using the TEMA and TEMB primers, respectively (Whitelaw 1999). For further characterization of functional TEM genes, the 3601TEM primer 5′-AGGAAGCAAAGCTGAAAGGAATCAAATTTGG-3′ which anneals to the Tn*3* repressor gene sequences (NCBI accession number J01749) normally associated with bla_{TEM}, and the DEB primer 5′-ATGAGTAAACTTGGTCTGAC-3′ (Caniça *et al.*, 1997) were used in PCR assays. Assays were carried out in a final volume of 50 μl containing 20 pmol of each primer, 200 μM of each dNTP and 2.5 units of Taq DNA polymerase in the buffer supplied. PCR was performed by initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extending at 72°C for 60 sec and a final extension at 72°C for 7 min.

3.2.2.2 Sequencing and sequence analysis

Sequencing of PCR products and recombinant plasmids was performed using automated sequencing facilities at the Core Facility, University of Cape Town and at the central analytical facility, University of Stellenbosch. DNA sequence alignments were performed by DNAMAN version 4.0 (Lynnon BioSoft) software.

3.2.3 Method to determine transcriptional start site(s) of *bla*$_{TEM-30A}$

RNA was extracted according to the method described [2.2.5.1]. Primer extension analysis was carried out as follows. One hundred micrograms of total RNA was precipitated and resuspended in 100 μl HP buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, pH 8.0; 400 mM NaCl; 80 % formamide). Primer 5′-GCGCAACTCTAGGTCAAGC-3′, that annealed to sequences 134 bp (Figure 3.1) downstream of the ATG initiation codon of *bla*$_{TEM-1}$ was synthesized and labeled with (indodicarbocyanine) Cy5 in the Department of Molecular and Cell Biology, University of Cape Town. The primer was mixed with the RNA, heated to 95°C and left to anneal to the mRNA template at 43°C O/N. The RNA/primer was precipitated, washed, dried, and resuspended in 20 μl RTB containing, 4ul AMV Transcriptase buffer, Promega; 10 mM dNTP, 40 U RNAse inhibitor, Roche Diagnostics, 14 μl sterilised dH_2O. The primer was extended using 20 U AMV Reverse Transcriptase (Promega Madison, USA) at 42°C for 2 hours. The reaction was stopped by addition of 1 μl 0.5 M EDTA (pH 8.0). Ribonuclease A (1 μl of 10 mg/ml) (Roche Diagnostics) was added and left at 37°C for 30 minutes to remove the RNA. The primer extension product was precipitated with 150 μl TES after which the pellet was reconstituted in 5 μl TE and 8 μl ALF stop buffer (Pharmacia). The mixture was heated to 95°C and the primer extension product was analyzed in conjunction with sequencing reaction products performed on the corresponding DNA. The analyses were carried out using the ALFexpress automated DNA sequencer (Amersham Biosciences) at the University of Cape Town, in the department of Molecular and Cell Biology, University of Cape Town.

3.3 RESULTS

3.3.1 Antibiotic susceptibility testing

Antibiotic disk susceptibility testing showed that *E. coli* AC3 and AC4 were resistant to amoxicillin-clavulanate, and AC1 and AC2 had intermediate susceptibility to this combination whilst all of the isolates were susceptible to cefuroxime, cefotaxime, ceftazidime, cefepime and cefoxitin. Antibiotic susceptibility testing using Etest evaluation [2.2.2] showed that *E. coli* strains AC1-AC4 were clinically resistant to the inhibitor combination amoxicillin-clavulanate (Table 3.2).

TABLE 3.2 Amoxicillin-clavulanate MICs

Strain	MIC (μg/ml)
AC1	24
AC2	16
AC3	96
AC4	128

Since all the isolates were susceptible to cefoxitin, the possibility of this resistance being due to overproduction of the AmpC enzyme was excluded.

3.3.2 Detection and characterization of bla_{TEM} genes

To determine the presence of TEM-related genes in the *E. coli* strains, PCR assays were carried out using primers targeted to the internal portion [3.2.2.1] of bla_{TEM}. A product of the expected size (517 bp) was obtained from each of the strains, indicating the presence of TEM related genes.

As a first step to ascertaining whether the TEM genes identified are hyperproduced or encode IRTs, PCR assays were performed using primers to amplify the functional gene

with its regulatory region [3.2.2.1]. A product of the expected size (1.232 kb) was obtained from each of the strains. PCR products were eluted from the gel [2.2.3.3] and either sequenced directly, or cloned into pBGS8 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Braunschweig, Germany) prior to sequencing. When sequencing data was obtained from cloned products, the inserts from three clones were sequenced on both strands.

An alignment of the nucleotide sequences from AC1, AC2, AC3 and AC4 with corresponding TEM gene sequences in the databank enabled identification of the genes. The bla_{TEM} structural gene from AC1 and AC2 are downstream of *Pa*/*Pb* promoters and are identical to $bla_{\mathrm{TEM\text{-}1B}}$ (Table 3.3). The bla_{TEM} structural gene from AC3 is identical to IRT $bla_{\mathrm{TEM\text{-}30A}}$ and is located downstream of promoters *Pa*/*Pb* (Table 3.3). Two TEM genes were identified in AC4: $bla_{\mathrm{TEM\text{-}1B}}$(*P3*) and $bla_{\mathrm{TEM\text{-}30A}}$(*Pa*/*Pb*).

TABLE 3.3 [a]Nucleotide mutations in TEM and IRT β-lactamase-encoding genes.

Strain or reference	*bla*$_{TEM}$ (promoter)	**32**	**141**	**162**	**175**	226 (6)	346 (48)	407 (69)	436 (78)	604 (134)	682 (160)	925 (242)	929 (244)
Leflon-Guibout *et al.*, 2000b	1A (*P3*)	C	C	G	A	C	A	A	C	G	T	G	C
Lartigue *et al.*, 2002	1B (*Pa/Pb*)	T				T			T	T			
Pomba-Féria and Caniça, 2003	1B (*P3*)				G	T			T	T			
Lartigue *et al.*, 2002	1B (*P4*)			T		T			T	T			
Lartigue *et al.*, 2002	1B (*P5*)		G			T			T	T			
Leflon-Guibout *et al.*, 2000b	1C (*P3*)								T				
Leflon-Guibout *et al.*, 2000b	1D (*P3*)								T				
Leflon-Guibout *et al.*, 2000b	1E (*P3*)				G	T	G		T	T			
Leflon-Guibout *et al.*, 2000b	1F (*P4*)			T			G	T			C		
Pomba-Féria and Caniça, 2003	1G (*P3*)								T	T			
Leflon-Guibout *et al.*, 2000b	2 (*Pa/Pb*)	T					G		T		C	A	
Leflon-Guibout *et al.*, 2000b	30A (*Pa/Pb*)	T											A
AC1	1B (*Pa/Pb*)	T			G	T			T	T			
AC2	1B (*Pa/Pb*)	T			G	T			T	T			
AC3	30A (*Pa/Pb*)	T											A
AC4	30A (*Pa/Pb*)	T											A
AC4	1B (*P3*)				G	T			T	T			

[a]Nucleotide numbering is according to the method of Sutcliffe, 1978
bla$_{TEM-1}$ framework is used as a reference , differences are indicated below the *bla*$_{TEM-1A}$ gene
The numbering in bold refers to the promoter region
Amino acid numbering is shown in brackets below the corresponding nucleotide number

3.3.3 Identification of the promoters and transcription start site of *bla*$_{TEM-30A}$

Using the RNA from AC3, containing *bla*$_{TEM-30A}$, three primer extension products were obtained (Figure 3.2). The major extension product was mapped to the dinucleotide TT located 171 bp upstream of the *bla*$_{TEM-30A}$ start codon. The -10 hexamer of promoter *Pa* was identified 4 bp upstream of the transcription site. Two smaller peaks (Figure 3.2) were observed. One mapped to a T 149 bp upstream of the *bla*$_{TEM-30A}$ start codon. The -10 hexamer of promoter *Pb* was identified 8 bp upstream of this transcription site. The third peak mapped to a T, 36 bp upstream of the *bla*$_{TEM-30A}$ start codon, and the *P3* -10 hexamer was identified 5 bp upstream of the transcription start site (Figure 3.1). Thus, *bla*$_{TEM-30A}$ is transcribed from *P3* and *Pa*/*Pb*.

3.3.4 Primer extension analysis

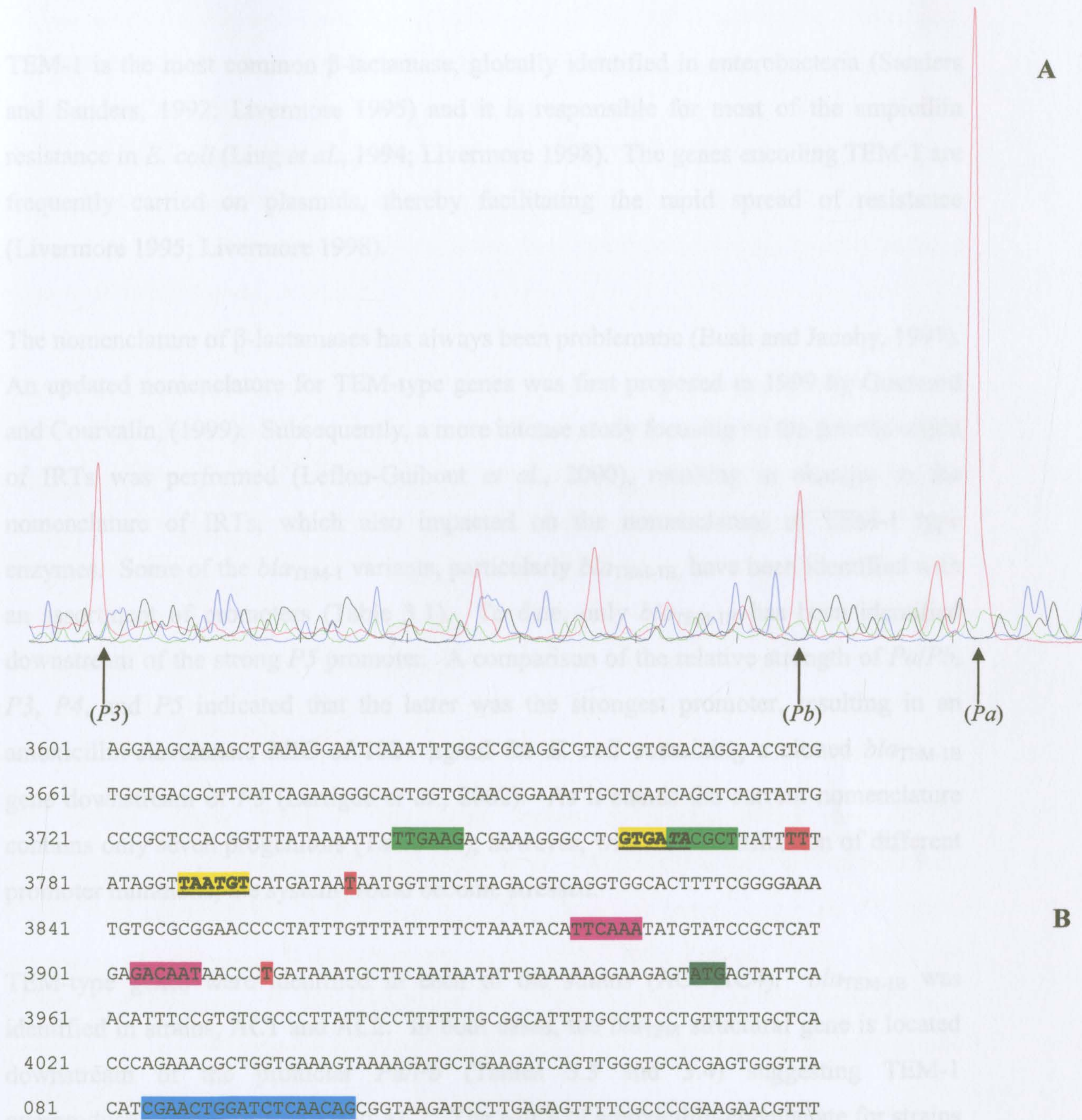

Figure 3.2 Mapping transcription start site of AC3 $bla_{\text{TEM-30}}$. **Panel A** The peaks in red represents the primer extension products labeled with (indodicarbocyanine) Cy5 primer electrophoresed along the appropriate sequencing reactions. The vertical arrows indicate the three major transcription start sites from the respective promoters indicated below the arrows. **Panel B** Nucleotide sequence of promoters *Pa*, *Pb*, and *P3* are highlighted in green, yellow and pink, respectively. The start codon is highlighted in dark green and the Cy5 labeled primer used in primer extension is indicated in turquoise. Transcriptional start sites are highlighted in red.

3.4 DISCUSSION

TEM-1 is the most common β-lactamase, globally identified in enterobacteria (Sanders and Sanders, 1992; Livermore 1995) and it is responsible for most of the ampicillin resistance in *E. coli* (Ling *et al.*, 1994; Livermore 1998). The genes encoding TEM-1 are frequently carried on plasmids, thereby facilitating the rapid spread of resistance (Livermore 1995; Livermore 1998).

The nomenclature of β-lactamases has always been problematic (Bush and Jacoby, 1997). An updated nomenclature for TEM-type genes was first proposed in 1999 by Goussard and Courvalin, (1999). Subsequently, a more intense study focusing on the genetic origin of IRTs was performed (Leflon-Guibout *et al.*, 2000), resulting in changes to the nomenclature of IRTs, which also impacted on the nomenclature of TEM-1 type enzymes. Some of the $bla_{\text{TEM-1}}$ variants, particularly $bla_{\text{TEM-1B}}$, have been identified with an assortment of promoters (Table 3.1). To date, only $bla_{\text{TEM-1B}}$ has been identified downstream of the strong *P5* promoter. A comparison of the relative strength of *Pa/Pb*, *P3*, *P4*, and *P5* indicated that the latter was the strongest promoter, resulting in an amoxicillin-clavulanate MIC of 1024 μg/ml for *E. coli* containing a cloned $bla_{\text{TEM-1B}}$ gene downstream of *P5* (Lartigue *et al.*, 2002). As it stands the current nomenclature contains only seven progenitors (Table 3.1); however, with the identification of different promoter mutations, the system would become stressed.

TEM-type genes were identified in each of the strains (AC1-AC4). $bla_{\text{TEM-1B}}$ was identified in strains, AC1 and AC2. In both cases, the bla_{TEM} structural gene is located downstream of the promoter *Pa/Pb* (Tables 3.3 and 3.4) suggesting TEM-1 overproduction in strains AC1 and AC2. The MICs of amoxicillin-clavulanate for strains AC1 and AC2 were 24 μg/ml and 16 μg/ml, respectively. These MICs are lower than the corresponding MIC (128 μg/ml) for an *E. coli* strain containing a cloned $bla_{\text{TEM-1B}}$ gene transcribed from *Pa/Pb* (Lartigue *et al.*, 2002). The other two strains, AC3 and AC4, contained $bla_{\text{TEM-30A}}$ encoding an IRT, situated downstream of *Pa/Pb*. Strain AC4 also contained $bla_{\text{TEM-1B}}$ adjacent to the weak promoter, *P3*. That the amoxicillin-clavulanate

MICs for the IRT containing strains were higher than the corresponding MICs for the *bla*$_{TEM-1B}$ strains was not surprising. Lartigue *et al.*, (2002) showed that the amoxicillin-clavulanate MIC for an *E. coli* with a *Pa*/*Pb* controlled *bla*$_{TEM-30B}$ gene was 2048 µg/ml as compared to 128 µg/ml for the same strain containing *bla*$_{TEM-1B,}$ transcribed from *Pa*/*Pb.* Regarding the four strains, the MIC of amoxicillin-clavulanate (128 µg/ml) was the highest for strain AC4 presumably because this strain contains two TEM-type genes (Table 3.4). *E. coli* isolates containing more than one type of *bla*$_{TEM}$ gene have been described by Miró *et al.*, 2002.

TABLE 3.4 Summary of the characteristics of *E. coli* strains AC1, AC2, AC3 and AC4

Strain	*bla*$_{TEM}$	Promoter	Amoxicillin-clavulanate MIC (µg/ml)
AC1	*bla*$_{TEM-1B}$	*Pa*/*Pb*	24
AC2	*bla*$_{TEM-1B}$	*Pa*/*Pb*	16
AC3	*bla*$_{TEM-30A}$	*Pa*/*Pb*	96
AC4	*bla*$_{TEM-30A}$ *bla*$_{TEM-1B}$	*Pa*/*Pb* *P3*	128

At various times, TEM-30 has been called E-GUER, TRI-1 and IRT-2 ((www.lahey.org/studies/temtable.stm; Vedel *et al.*, 1992). It was Leflon-Guibout *et al.*, (2000) who proposed the label *bla*$_{TEM-30A}$. Two *bla*$_{TEM-30}$ genes have been described; *bla*$_{TEM-30A}$ and *bla*$_{TEM-30B}$ (Leflon-Guibout *et al.*, 2000; Lartigue *et al.*, 2002). The latter differs from *bla*$_{TEM-30A}$ in that it contains three silent mutations, an A→G transition at position 175, a C→T transition at position 226 and a G→T transversion at position 604 (Leflon-Guibout *et al.*, 2000).

With respect to the mutation in TEM-30 resulting in resistance to amoxicillin-clavulanate, serine replaces arginine at amino acid 244 (R244S) (Bonomo and Rice, 1999; Yang *et al.*, 1999; www.lahey.org/studies/temtable.stm; Wang *et al.*, 2002; Knox, 1995). A conserved water molecule is anchored at arginine 244 and when this arginine is replaced with the serine residue, the water molecule is lost (Wang *et al.*, 2002). Loss of

the water molecule is thought to result in loss of the binding affinity for the inhibitor, allowing the enzyme to elude or avoid crosslinking with the inhibitor (Yang *et al.*, 1999; Wang *et al.*, 2002). Although the amino acid replacement results in reduced hydrolysis of ampicillin it increases resistance to inhibitors such as clavulanate (Yang *et al.*, 1999).

The transcription start of $bla_{TEM\text{-}30A}$, from *E. coli* isolate AC3, was determined. The fluorogram of the primer extension analysis indicates that mRNA transcription is initiated from at least three sites, which accords with S1-Nuclease studies (Chen and Clowes, 1984) performed to determine the transcription start site from a bla_{TEM}. With respect to $bla_{TEM\text{-}30A}$, transcription of the majority of transcripts proceeds from the *Pa* component of *Pa/Pb* indicating that, of the three promoters upstream of $bla_{TEM\text{-}30A}$, *Pa* is the most frequently recognised and therefore the most active promoter. Albeit to a lesser extent, as estimated by the peak size (Figure 3.2), transcription is also initiated from *Pb*. A similar level of transcription emanated from *P3*. Thus, transcription from all three promoters probably affects the level of resistance, although the mutation in *Pa/Pb* plays the major role in increased expression (Figure 3.2) of $bla_{TEM\text{-}30A}$.

In conclusion, resistance to amoxicillin-clavulanate in the four clinical isolates of *E. coli* was shown to be associated with increased expression of bla_{TEM} variants. However, experiments to show overexpression of the enzymes were not carried out.

CHAPTER 4

The genetic basis of cefoxitin, cefuroxime and amoxicillin-clavulanate resistance in two clinical isolates of *K. pneumoniae*

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4.3.7 Detection and characterization of OMPs

4.3.8 Molecular basis of porin loss in strain MAJ

4.4 DISCUSSION

4.1 INTRODUCTION

In the previous chapter the molecular nature of resistance to the inhibitor combination amoxicillin-clavulanate was investigated in a group of clinical *E. coli* isolates. This chapter describes the studies carried out to elucidate the genetic basis of β-lactam resistance in the two clinical *K. pneumoniae* strains designated strain ENG and strain MAJ, which displayed resistance to this inhibitor combination. Both strains were resistant to cefuroxime but only strain MAJ was resistant to cefoxitin.

Resistance to amoxicillin-clavulanate has been shown to be associated with IRTs (Lemozy *et al.*, 1995; Bermudes *et al.*, 1997; Girlich *et al.*, 2000) or the overproduction of TEM-1 (Wu *et al.*, 1994; Stapleton 1995) in *K. pneumoniae.* A plethora of ESBLs has been identified in *K. pneumoniae,* which account for resistance to the newer oxyiminocephalosporins in this organism (Gniadkowski *et al.*, 1998; Livermore *et al.*, 1998; Yang *et al.*, 1998; Laksai *et al.*, 2000; Essack *et al.*, 2001). Since strain ENG and strain MAJ were susceptible to ceftazidime and cefotaxime, which are substrates for these enzymes, it is unlikely that ESBLs play a role in the resistance phenotype. Further, ESBLs do not hydrolyze cephamycins, such as cefoxitin, and resistance to these antibiotics is associated with the production of cephamycinases (Bauernfeind *et al.*, 1996; Zhao *et al.*, 2001; Philippon *et al.*, 2002) or with outer membrane protein deficiency (Martínez-Martínez *et al.*, 1996).

The loss of porins in *K. pneumoniae* was previously implicated in resistance to cefotaxime, ceftazidime (Martínez-Martínez *et al.*, 1996; Crowley *et al.*, 2002) and cefoxitin (Martínez-Martínez *et al.*, 1996; Henández-Allés *et al.*, 2000; Crowley *et al.*, 2002).

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Bacterial strains and antibiotic susceptibility testing

K. pneumoniae designated strain MAJ was isolated from urine and identified in the diagnostic laboratory in Groote Schuur Hospital, Cape Town. *K. pneumoniae* designated strain ENG was isolated from blood and identified in the same laboratory. *K. pneumoniae* designated strain KAN, isolated from ascitic fluid, was used as a control when necessary. Antibiotic susceptibility testing was performed [2.2.2] except for cephalothin; the MIC of this antibiotic was determined using a two-fold agar dilution method on IsoSensitest agar (Oxoid, Basingstoke, UK) and an inoculum of 10^4 colony forming units/spot.

4.2.2 Methods used to detect β-lactamases

4.2.2.1 ESBL detection using double disk diffusion

ESBL activity was determined in the diagnostic laboratory in GSH using the double disk diffusion test. A disk containing amoxicillin-clavulanate was placed between a ceftazidime and ceftriaxone disk (20mm between adjacent edges of the disks). A dumbbell shaped extension of the zone of inhibition inferred ESBL activity. *E. coli* ATCC 25922 was used as a control.

4.2.2.2 Detection of β-lactamase activity using Isoelectric focusing

Isoelectric focusing was performed in the Department of Biochemistry at the University of the Western Cape, Cape Town, South Africa. Crude cell extracts were subjected to isoelectric focusing using a precast gel, Ampholine™ PAGplate for IEF(pH3.5-pH9.5), in the LKB 2117 Multiphor II Unit. After electrophoresis, bands of β-lactamases were visualised by treating the gel with nitrocefin (0.5 mg/ml). Crude cell extracts prepared from *E. coli* DH5α expressing TEM-1 from pUC19 was used as a control.

4.2.2.3 Detection of β-lactamase activity

To determine β-lactamase activity, a single colony was inoculated into 10 ml of Nutrient Broth (NB) containing ampicillin (50 μg/ml) and incubated O/N at 37°C with shaking. The cells were harvested by centrifugation and subsequently resuspended in 1 ml PBS for washing by centrifugation. The washed pellet was then resuspended in 1 ml PBS. To facilitate the release of the β-lactamases, the cells were inserted into liquid nitrogen until frozen, thawed at 37°C and then vortexed. The freeze-thawing was performed between 3-6 times, after which the cell debris was removed from the supernatant by centrifugation. β-lactamase activity in the supernatant fluid was determined spectrophotometrically (PharmaciaLKBUltrospecIII) using cephaloridine, cefuroxime, cefoxitin, cephalothin and ampicillin (Sigma-Aldrich, Poole, UK) as substrates in independent assays (Murphy *et al.*, 2003). A decrease in OD over a time period of 2 min was used to detect hydrolysis. Results were expressed as a change in OD/min.

4.2.2.4 Detection of bla_{TEM} and bla_{SHV} by PCR

Two primer sets complementary to the bla_{TEM} genes, [3.2.2.1] were used to detect the presence of bla_{TEM} genes. A 712 bp internal portion of the bla_{SHV} gene was amplified using the SHVA 5′-CTGGCGGTACACGCCAGC-3′ and SHVB 5′-TGCGCTCTGCTTTGTTA-3′ primers, (Whitelaw 1999) corresponding to nucleotides +49 to +66 and +744 to +760, of the structural gene. Strain *K. pneumoniae* K69 described and provided by Whitelaw 1999, was used as a positive control. Assays were carried out in a final volume of 50 μl containing 20 pmol of each primer, 200 μM of each dNTP and 2.5 units of Taq DNA polymerase in the buffer supplied. PCR was performed by initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extending at 72°C for 50 sec and a final extension at 72°C for 7 min.

4.2.3 Methods used to investigate outer membrane protein profiles

4.2.3.1 Extraction and SDS-PAGE analysis of outer membrane proteins

Cells were harvested from 100–250 ml O/N cell cultures grown with and without selection, as required, at 7000 × g for 10 min at 4°C. The pellets were washed by resuspending in 50-250 ml 50 mM Tris-HCl (pH 7.5) and centrifuged as before. After collecting the cells, the pellets were resuspended in 5 ml 50 mM Tris-HCl (pH 7.5) and lysed by subjecting to 3 rounds of French Pressing. Cell debris was removed by centrifugation at 1000 × g for 10 min and the pellet discarded. The supernatant was ultracentrifuged at 40000 rpm (100000 × g) for 1 hour at 25°C. The pellets were resuspended in 5 ml 0.5 % (w/v) Sodium N-laurylsarcosinate (sarkosyl) and incubated at 32°C for 30 min, to separate outer membrane from the inner membrane by selective solubilisation. This extraction was repeated and the OMPs collected by ultracentrifugation at 40000 rpm (100000 × g) at 25°C for 30 min. The pellets were then resuspended in the appropriate amount of 50 mM Tris-HCl, depending on the amount of protein. Before SDS-PAGE, the OMPs were denatured by the addition of the appropriate amount of 10 × resuspension buffer (10 % SDS, 10 % glycerol (v/v), 6.25 mM Tris-HCl (pH 6.8); 50 % β-mercaptoethanol, 10 % bromophenol blue). Protein concentrations were determined using a Bio-Rad *DC* protein assay. Polyacrylamide gel preparation and electrophoresis was performed as described (Ausabel *et al.*, 1987; Maniatis *et al.*, 1989) using 30 % acrylamide-0.8 % bis-acrylamide to prepare a 10 % polyacrylamide gel. Electrophoresis was performed using 30 mA constant current until the samples entered the resolving gel and then increasing the current to 40 mA. Gels were stained with Coomassie Blue and destained with 20 % ethanol and 10 % acetic acid.

4.2.3.2 MALDI TOF MS (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry) analysis

SDS-PAGE gels containing the OMPs of interest were submitted to the Department of Molecular and Cell Biology, University of Cape Town, for MALDI TOF analysis. The protein band of interest, a band from the marker lane (bovine carbonic anhydrase I) and a portion of gel containing no protein, were excised from SDS-PAGE gels and the protein in the gel was digested with trypsin. A list of peptide masses was obtained for each digest and the peptide mass fingerprint was analyzed using software at the following

websites to identify the proteins; PROFOUND, available at http://prowl.rockefeller.edu/; MS-FIT available at http://prospector.ucsf.edu/; EMBL Bioanalytical Research Group available at http://www.narrador.embl-heidelberg.de/GroupPages/Homepage.html.

4.2.3.3 Experiments to determine the effect of antibiotic selective pressure on the OMP expression of strain ENG and strain MAJ

4.2.3.3.1 Passage for five days in non-selective media

One hundred microlitres of an O/N culture of strain ENG and strain MAJ was inoculated into 10 ml of 2 × YT broth and incubated at 37°C O/N without shaking. An aliquot, (100 µl), of this culture was then transferred to 10 ml of fresh 2 × YT broth and incubated O/N without shaking. This procedure was repeated for a total of five days, after which the entire 10 ml was used to inoculate 200 ml of 2 × YT broth. At this point MICs were determined. Cells were cultured O/N at 37°C and OMPs were extracted and analyzed [4.2.3.1].

4.2.3.3.2 Growth of strain ENG in the presence of cefuroxime

An overnight culture of strain ENG was diluted 1:100 to a final volume of 300 ml, with 2 × YT broth containing cefuroxime (30 µg/ml), or no antibiotic. Aliquots (100 ml), were removed after 1 hour, 4 hour and O/N incubation. Antibiotic susceptibility was determined at each time point using the disk diffusion method [2.2.2]. The remainder of the cells was harvested by centrifugation, stored at -20°C and OMPs were extracted and analyzed [4.2.3.1].

4.2.3.4 Method used to determine the absence of OmpK36 expression in strain MAJ

Primers U228 and L1730 (Hernández-Allés *et al.*, 1999) were used to amplify the functional *ompK36* gene from genomic DNA [2.2.3.1]. PCR was carried out in a final volume of 50 µl containing 20 pmol of each primer, 200 µM of each dNTP and 2.5 units of Taq DNA polymerase in the buffer supplied. PCR was performed by initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 sec, annealing at

46°C for 60 sec and extending at 72°C for 45 sec and a final extension at 72°C for 7 min. PCR products were electrophoresed, gel purified [2.2.3.3] and cloned in pGEM-T Easy (Promega). Recombinant plasmids were introduced into *E. coli* JM109 cells which were made competent for DNA uptake using the $CaCl_2$ shock procedure (Dagert and Ehrlich, 1979). Recombinant plasmid DNA was extracted using the method previously described [2.2.3.1].

4.3 RESULTS

4.3.1 Antibiotic susceptibility testing

The MICs of β-lactams and β-lactam/inhibitor combinations for strain ENG and MAJ, and the susceptible isolate (strain KAN) are presented in Table 4.1. Strain ENG was resistant to amoxicillin, amoxicillin-clavulanate, cefuroxime, piperacillin and piperacillin-tazobactam. Compared with MICs of cefotaxime, ceftazidime and cefepime for strain KAN, strain ENG, although clinically sensitive had reduced susceptibility to these β-lactams.

Strain MAJ was resistant to amoxicillin, amoxicillin-clavulanate, cefuroxime, piperacillin and cefoxitin. Like strain ENG, strain MAJ had reduced susceptibility to cefotaxime, ceftazidime and cefepime although clinically sensitive. Strain MAJ had intermediate susceptibility to piperacillin-tazobactam.

TABLE 4.1 β-lactam MIC (μg/ml) determinations for *K. pneumoniae* strains

Antibiotic	KAN	ENG	MAJ	[a]Resistant	[b]Sensitive
Amoxicillin	64	>256	>256	≥32	≤8
Amoxicillin-clavulanate	1.5	64	32	≥16/8	≤8/4
Cefuroxime	2	32	64	≥32	≤8
Cefoxitin	3	3	64	≥32	≤8
Cefotaxime	0.032	2	0.38	≥64	≤8
Ceftazidime	0.19	4	1.5	≥32	≤8
Cefepime	0.064	1	1	≥32	≤8
Piperacillin	12	>256	>256	≥128	≤16
Piperacillin-tazobactam	2	>256	24	≥128/4	≤16/4

[a,b] NCCLS MIC breakpoints

4.3.2 ESBL detection using double disk diffusion

No dumbbell shaped zone of inhibition was observed around either of the ceftazidime or ceftriaxone disks extending towards the clavulanate disk with the ESBL determination [4.2.2.1] for strain ENG, strain MAJ and for strain KAN. This suggests that these strains do not contain ESBLs. This result is supported by their antibiograms [4.3.1] as all three of the strains were susceptible to ceftazidime and cefotaxime, which are substrates for ESBLs.

4.3.3 Detection of β-lactamase activity using Isoelectric focusing

Nitrocefin hydrolysis after isoelectric focusing revealed bands in both of the *K. pneumoniae* isolates strain ENG and strain MAJ co-migrating with TEM-1 (pI 5.4). No other bands were observed.

4.3.4 Hydrolysis of cefoxitin by strain MAJ

Strain MAJ was resistant to cefoxitin. Although IEF and the resistance phenotype suggested that this strain does not contain a cephamycinase, the hydrolyzing activity of β-lactamases, extracted from strain MAJ, against cefoxitin was determined. Based on decrease in activity over time, hydrolysis of ampicillin and cephaloridine was detected. No hydrolysis of cefoxitin was detected, suggesting that strain MAJ does not contain cephamycinases.

4.3.5 Molecular detection of TEM and SHV genes

Using the primers to detect bla_{TEM}-related genes [3.2.2.1] a product of the expected size (1.2 Kb) was obtained for both strain ENG and strain MAJ. The PCR products were cloned [3.2.2] and sequenced [3.2.2.2]. PCR products of the expected size (712 bp) were observed with the primer pair SHVA and SHVB for both strain ENG and strain MAJ.

4.3.6 Sequencing analysis and characterization of the *bla*$_{TEM}$ genes

Analysis [3.2.2.2] of the sequencing data obtained by direct sequencing of the PCR products generated with the primer pair, 3601TEM and DEB [3.2.2.1], revealed that the *bla*$_{TEM}$ gene in strain ENG was identical to *bla*$_{TEM-1E}$ except for a T→C transition at bp position 682. This *bla*$_{TEM}$ was located downstream of a *Pa*/*Pb* promoter. It is proposed that this *bla*$_{TEM}$ be called *bla*$_{TEM-1H}$. Strain MAJ contained a *bla*$_{TEM-1C}$ gene downstream of a *P3* type promoter.

[a]Nucleotide numbering according to Sutcliffe	*bla*$_{TEM-1C}$ Leflon-Guibout *et al.*, 2000	*bla*$_{TEM-1E}$ LeflonGuibout *et al.*, 2000	*bla*$_{TEM-1H}$ Strain ENG	*bla*$_{TEM-1C}$ Strain MAJ
32	C	C	C	C
141	C	C		
162	G	G	G	G
175	A	G	G	A
226(6)	C	T	T	C
317(39)	C	C	C	C
346(48)	A	G	G	A
407(69)	A	A	A	A
409(69)	G	G	G	G
436(78)	T	T	T	T
604(134)	G	T	T	G
682(160)	T	T	C	T
695(165)	T	T	T	T
913(237)	C	C	C	C
925(242)	G	G	G	G
929(244)	C	C	C	C
931(244)	C	C	C	C
1020(275)	G	G	G	G
1022(276)	A	A	A	A

TABLE 4.2 Nucleotide mutations in *bla*$_{TEM}$ encoding genes

[a]Nucleotide numbering is according to the method of Sutcliffe, 1978
The numbering in bold refers to the promoter region
Amino acid numbering is shown in brackets next to the corresponding nucleotide number

4.3.7 Detection and characterization of OMPs in strain ENG and strain MAJ

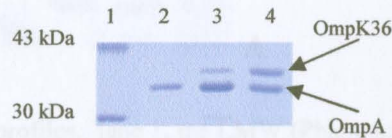

Figure 4.1 SDS-PAGE analysis of OMP profiles. lane 1, Low Molecular Weight (LMW) marker (Pharmacia Biotech); lane 2, 10 μg OMP from strain MAJ; lane 3, 10 μg OMP from strain ENG; lane 4, 10 μg OMP from strain KAN.

To determine whether the absence of one or more OMPs could account for the β-lactam resistance, OMPs were extracted from strain ENG and strain MAJ cultured in the presence of cefuroxime (30 μg/ml). A comparison of the OMP profile from strain ENG with the corresponding profile from the susceptible strain, KAN, identified a protein band of approximately 40 kDa migrating above the constitutively expressed OmpK34 (OmpA) (Nguyen *et al.*, 1998; Hernandéz-Allés *et al.*, 1999; Hernandéz-Allés *et al.*, 2000) in each of strains (Figure 4.1). Compared with the 40 kDa protein band in strain KAN, the level of expression of its counterpart in strain ENG is reduced. A protein band of 40 kDa was not observed in strain MAJ. The 40 kDa protein bands from strain ENG and strain KAN were excised from the gel. Analysis of the mass peptide fingerprints generated by MALDI-TOF mass spectrometry, revealed a match of eight out of 38 peptides of the 40 kDa protein for strain KAN to OmpK36, suggesting that this protein could be OmpK36. Similarly, a match of ten out of 57 peptides was obtained for strain ENG also suggesting that this protein was OmpK36.

4.3.7.1 Passaging of strain ENG and strain MAJ

To test whether the reduced expression, or absence, of OmpK36 was influenced by the presence of cefuroxime, strain ENG and strain MAJ were passaged through 5 successive overnight cultures in antibiotic-free broth [4.2.3.3.1]. As shown in figure 4.2 A, the level of expression of OmpK36 in the passaged strain ENG (strain ENG^P) was similar to that in the strain KAN, indicating restoration of OmpK36.

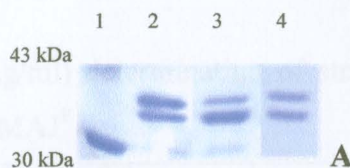

Figure 4.2 **A** SDS-PAGE analysis of OMP profiles. lane 1, the LMW (Pharmacia Biotech); lane 2, OMP from strain ENG^P (10 μg); lane 3, OMP from strain ENG grown in 30 μg/ml cefuroxime; lane 4, OMP (10 μg) from strain KAN

ENG^P had an identical antibiotic resistance profile to the parent strain ENG, except that it was susceptible to amoxicillin-clavulanate, and cefuroxime (Table 4.3). ENG^P had a reduced susceptibility to piperacillin-tazobactam, although not clinically sensitive. ENG^P was also found to still harbor the bla_{TEM} gene as detected by PCR [3.2.2.1].

However, unlike strain ENG, OmpK36 expression was not restored and the 40 kDa protein band that was not observed in strain MAJ remained absent (Figure 4.2 B) in the passaged strain MAJ (MAJ^P). MAJ^P had an identical resistance profile to the parent strain MAJ, except that the susceptibility to cefuroxime had increased and MAJ^P was clinically sensitive to piperacillin-tazobactam (Table 4.3). The increased susceptibility in MAJ^P could be attributed to the expression of two alternate OMPs (Omp1 and $OmpA^*$) (Figure 4.2 B). Analysis of the mass peptide fingerprints generated by MALDI-TOF mass spectrometry, revealed a match of four out of nine peptides for the protein band migrating above OmpA, to SHV-1, suggesting that this protein (Omp1) is SHV-1 related in terms of its trypsin digestion profile. Similarly, a match of seven out nine peptides related the protein band migrating below OmpA ($OmpA^*$), to OmpA precursor protein in *E. coli* and *Shigella flexneri* 2a. MAJ^P was also found to still harbor the bla_{TEM} gene as detected by PCR [3.2.2.1].

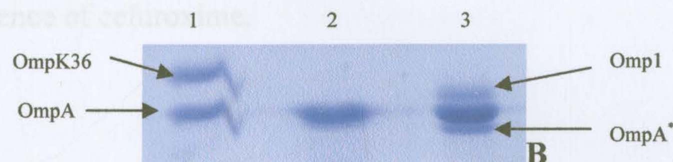

Figure 4.2 B SDS-PAGE analysis of OMP profiles. lane 1, OMP from strain KAN (10 μg);lane 2, OMP from strain MAJ (10 μg);lane 3, OMP from stain MAJ^P (10 μg).

TABLE 4.3 β-lactam MIC (μg/ml) determination of strain ENG and MAJ and their passaged derivatives ENG^{P} and MAJ^{P}

Antibiotic	**ENG**	**ENG^{P}**	**MAJ**	**MAJ^{P}**	**[a]Resistant**	**[b]Sensitive**
Amoxicillin	>256	>256	>256	>256	≥32	≤8
Amoxicillin-clavulanate	64	8	32	16	≥16/8	≤8/4
Cefuroxime	32	6	64	24	≥32	≤8
Cefoxitin	3	3	64	256	≥32	≤8
Cefotaxime	2	1.5	0.38	0.5	≥64	≤8
Ceftazidime	4	2	1.5	1.0	≥32	≤8
Cefepime	1	1	1	2.0	≥32	≤8
Piperacillin	>256	>256	>256	>256	≥128	≤16
Piperacillin-tazobactam	>256	32	24	16	≥128/4	≤16/4

ENG^{P}, strain ENG passaged in non-selective media

MAJ^{P}, strain MAJ passaged in non-selective media

[a,b] NCCLS MIC breakpoints

4.3.7.2 Growth of strain ENG in the presence of cefuroxime

To study OmpK36 expression over time in strain ENG, OMP profiles were obtained at various time points during growth in cefuroxime [4.2.3.3.2]. Incubation in the presence of cefuroxime decreased the level of OmpK36 and OmpA expression between 4 hours (Figure 4.3 Panel **A** lane 2) and the O/N time point (Figure 4.3 Panel **A** lane 3). OmpK36 expression in strain ENG when incubated in the absence of selection (Figure 4.3 Panel **B** lane 4,5,6) was identical to OmpK36 expression in the susceptible isolate, strain KAN (Panel **B** lane 1,2,3). Thus OmpK36 expression in strain ENG was affected when incubated in the presence of cefuroxime.

Figure 4.3 SDS-PAGE analysis of OMP profiles, each lane containing 10 µg of OMP. **Panel A** lanes 1-3, OMPs extracted from strains ENG at various time points (1 hour, 4 hours and O/N) grown in the presence of cefuroxime (30 µg/ml); lane 4, empty; lane 5, Rainbow Marker (*amersham pharmacia* biotech). **Panel B** lanes 1-3, OMPs extracted from strain KAN grown in the absence of selection at various time points (1 hour, 4 hours and O/N); lanes 4-6, OMP extracted from strain ENG at various time points (1 hour, 4 hours, and O/N) without any selection.

4.3.8 Molecular basis of porin loss in strain MAJ

Studies used to identify the mechanism responsible for the loss of OmpK36 in strain MAJ were carried out. Using primers designed to anneal to the functional *ompK36* sequences, products of 2.2 kb and 1.5 kb were obtained from strain MAJ and the control strain respectively. The size from strain MAJ suggested that the *ompK36* from this strain contained an insertion. The 2.2 kb PCR product was cloned into pGEM-T Easy (Promega), generating pJAM100 and sequenced. Analysis of the sequencing data (Figure 4.4) [3.2.2.2] identified IS*1* 15 bp upstream of the *ompK36* start codon. The element generated a 8 bp duplication of the target sequence in the form of a direct repeat and is bounded by imperfect terminal inverted repeats of 24 bp. The element contains two open reading frames (*insA* and *insB'*), typical of IS*1* elements (Mahillon and Chandler, 1998).

```
IS1             ------------------------------------------------------------
ompK36          ----------------------CGTGCTTAGAACTGGTAAACCAGGCCCAGTGCAACCAC
RC_ompK36C3     GGCGCCTTTTTCGTAACTTCAGCGTGCTTAGAACTGGTAAACCAGGCCCAGAGCAACCAC

IS1             ------------------------------------------------------------
ompK36          GTCGTCGGTAGAGATACCGGCGTTGCGGGTGAAGCTGTTGTCGTCCAGCAGGTTGATTTT
RC_ompK36C3     GTCGTCGGTAGAGATACCGGCGCTGCGGGTGAAGCTGTTGTCGTCCAGCAGGTTGATTTT

IS1             ------------------------------------------------------------
ompK36          GTAGTCAACATAGGTGGACATGTTTTTGTTGAAGTAGTAGGTCGCGCCAACGTCAACATA
RC_ompK36C3     GTAATCAACATAGGTAGACATGTTTTTGTTGAAGTAGTAGGTCGCGCCTACGTCAACATA

IS1             ------------------------------------------------------------
ompK36          TTTCAGGATGTCCTTGTCGCCGTAGCCGCGCTCCAGATCCTTACCTTTAGACTGCAGGTA
RC_ompK36C3     TTTCAGGATATCCTGGTCGCCGTAACCGTT---CAGGTCCTTACCTTTAGACTGCAGGTA

IS1             ------------------------------------------------------------
ompK36          AGCCACAGACGGACGCAGACCGAAGTCGAACTGGTACTGAGCAACCACTTCGAAGTTCTG
RC_ompK36C3     AGCAACGGACGGACGCAGACCGAAATCGAACTGGTACTGTGCAGCCACTTCGAAGTTCTG

IS1             ------------------------------------------------------------
ompK36          CGCTTTGTTTGCAAAGCCCAGGGAACCGGCGCGAGTTGCGTTGTAGGTCTGGGTGTACTG
RC_ompK36C3     TGCTTTGTTAGCAAAGCCCAGGCTACCAGCGCGAGTTGCGTTGTAGGTCTGAGTGTACTG

IS1             ------------------------------------------------------------
ompK36          AGAGGCCAGGTAGATGTTGTTGGCGTCGTATTTCAGACCACCGGTGTAGGTTTCAGCGTT
RC_ompK36C3     AGTTGCCAGGTAGATGTTGTTCGCGTCGTATTTCAGACCGCCGGTGTAGGTTTCAGCGTG

IS1             ------------------------------------------------------------
ompK36          GTCGCCACGACCCAGTGCCGGAACACTATTCTGCTCGTTGGTACGTTTGGAGTGAGAGTA
RC_ompK36C3     ATCGCCTTCGCCCAGCAGCAGCTGG---TTCTGATCGTCGGTACGCTTGGAGTTAGCATA

IS1             ------------------------------------------------------------
ompK36          CGCGAAACCAGCGCTGATACCATCCCAAATATCGTAGGTCAGAGAGGTGCCGAAGCCGTC
RC_ompK36C3     CGCGAAACCAGCGCTGATACCGTCGAAGATATCATAAGTTACGGAAGTACCGAAACCGTC

IS1             ------------------------------------------------------------
ompK36          GCCGTTCTGTTTGCTCCCACCACGACCGTTGTTGGTCGCGCCTTCGCCGCTGACGCTGCC
RC_ompK36C3     GCCGTTCTGTTTCAGAGCGCCACGACCGTTGTTGGTCGCGCCTTCGCCGCTGACGCTGCC

IS1             ------------------------------------------------------------
ompK36          GTTTTTACCCTGATACTGCAGAGCAAAGTTCAGGCCGTCAACCAGACCGAAGAAATCAGA
RC_ompK36C3     GTTTTTGCCCTGATACTGCAGAGCAAAGTTCAGGCCGTCAACCAGACCGAAGAAGTCAGA

IS1             ------------------------------------------------------------
ompK36          GTTACGGTAGGTTGCAACGCCGTTAGCACGGGACTGCAGGAAGTTGTCAGAACCGTAGGT
RC_ompK36C3     GTTACGGTAGGTTGCAACGCCGTTAGCACGGGACTGCAGGAAGTTGTCAGAACCGTAGGT

IS1             ------------------------------------------------------------
ompK36          GTCGCCGCCGAATTCCGGCAGAACGTCGGTCCAGGACGTTACGTCGTATACTACGCCGTA
RC_ompK36C3     GTCGCCGCCGAATTCCGGCAGAACGTCGGTCCAGGAAGTTACGTCGTAAACAACGCCGTA

IS1             ------------------------------------------------------------
ompK36          GTTACGACCGTAGTCGAAAGAGCCCGCGTCGCCAAATTTCAGGCCTGCGAATGCCAGACG
RC_ompK36C3     GTTACGACCGTAGTCGAAAGAGCCCGCGTCGCCGAATTTCAGACCTGCGAATGCCAGACG
```

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IS1              ------------------------------------------------------------
ompK36           AGTCCATGCCTGATCGCTGGAGCTTTCAGTGTTGTTCGCCTGAACGTTGTATTCCCACTG
RC_ompK36C3      AGTCCATGCCTGATCGCTGGAGCTTTCAGTGTTGTTCGCCTGAACGTTGTATTCCCACTG

IS1              ------------------------------------------------------------
ompK36           GCCGTAACCGGTCAGCTGGTCGTTGATCTGGGTTTCGCCTTTCACGCCTACACGCATGTA
RC_ompK36C3      GCCGTAACCGGTCAGCTGGTCGTTGATCTGGGTTTCGCCTTTCACGCCAAGACGCATGTA

IS1              -------------------------------------------CCTGGTTCACCACGCGG
ompK36           GGTCTGGTCGCCGTCGACGCTCTTGTCGTCAGAGAAGTAGTGCAGACCGTCAATTTTACC
RC_ompK36C3      GGTCTGGTCGCCGTCTACGTCCTTGTCATCAGAGAAATAGTGCAGACCGTCGATTTTGCC
                                                                  **

IS1              GAAACGGTCTGATAAGAGACACCGGCATA-------------------------------
ompK36           GTACAGGTCTAATTTGTTGCCGTCTTTGTTATAAATTTCAGCCGCATTTGCTGCGCCTGC
RC_ompK36C3      GTACAGGTCTAATTTGTTGCCATCTTTGTTATAAATTTCAGCCGCATTTGCTGCGCCTGC
                 * *  ***** **  *   *

IS1              -------------------------------------------------------CTCTG
ompK36           TACCAGCAGAGCCGGTACCAGGAGGGACAGTACTTTAACTTTCATGTTATTAACCCTCTG
RC_ompK36C3      TACCAGCAGAGCCGGTACCAGGAGGGACAGTACTTTAACTTTCATGTTATTA--------

IS1              CGAC---GGTGATGCTGCCAACTTACTGATTTAGTGTATGATGGTGTTTTTGAGGTGCTC
ompK36           ATTTGGGGGTGATGCTGCCAACTTACTGATTTAGTGTATGATGGTGTTTTTGAGGTGCTC
RC_ompK36C3      ------------------------------------------------------------

IS1              CAGTGGCTTCTGTTTCTATCAGCTGTCCCTCCTGTTCAGCTACTGACGGGGTGGTGCGTA
ompK36           CAGTGGCTTCTGTTTCTATCAGCTGTCCCTCCTGTTCAGCTACTGACGGGGTGGTGCGTA
RC_ompK36C3      ------------------------------------------------------------

IS1              ACGGCAAAAGCACCGCCGGACATCAGCGCTATCTCTGCTCTCACTGCCGTAAAACATGGC
ompK36           ACGGCAAAAGCACCGCCGGACATCAGCGCTATCTCTGCTCTCACTGCCGTAAAACATGGC
RC_ompK36C3      ------------------------------------------------------------

IS1              AACTGCAGTTCACTTACACCGCTTCTCAACCCGGTACGCACCAGAAAATCATTGATATGG
ompK36           AACTGCAGTTCACTTACACCGCTTCTCAACCCGGTACGCACCAGAAAATCATTGATATGG
RC_ompK36C3      ------------------------------------------------------------

IS1              CCATGAATGGCGTTGGATGCCGGGCAACAGCCCGCATTATGGGCGTTGGCCTCAACACGA
ompK36           CCATGAATGGCGTTGGATGCCGGGCAACAGCCCGCATTATGGGCGTTGGCCTCAACACGA
RC_ompK36C3      ------------------------------------------------------------

IS1              TTTTACGTCACTTAAAAAACTCAGGCCGCAGTCGGTAACCTCGCGCATACAGCCGGGCAG
ompK36           TTTTACGTCACTTAAAAAACTCAGGCCGCAGTCGGTAACCTCGCGCATACAGCCGGGCAG
RC_ompK36C3      ------------------------------------------------------------

IS1              TGACGTCATCGTCTGCGCGGAAATGGACGAACAGTGGGGCTATGTCGGGGCTAAATCGCG
ompK36           TGACGTCATCGTCTGCGCGGAAATGGACGAACAGTGGGGCTATGTCGGGGCTAAATCGCG
RC_ompK36C3      ------------------------------------------------------------

IS1              CCAGCGCTGGCTGTTTTACGCGTATGACAGTCTCCGGAAGACGGTTGTTGCGCACGTATT
ompK36           CCAGCGCTGGCTGTTTTACGCGTATGACAGTCTCCGGAAGACGGTTGTTGCGCACGTATT
RC_ompK36C3      ------------------------------------------------------------

IS1              CGGTGAACGCACTATGGCGACGCTGGGGCGTCTTATGAGCCTGCTGTCACCCTTTGACGT
ompK36           CGGTGAACGCACGATGGCGACGCTGGGGCGTCTTATGAGCCTGCTGTCACCCTTTGACGT
RC_ompK36C3      ------------------------------------------------------------
```

```
IS1                GGTGATATGGATGACGGATGGCTGGCCGCTGTATGAATCCCGCCTGAAGGGAAAGCTGCA
ompK36             GGTGATATGGATGACGGATGGCTGGCCGCTGTATGAATCCCGCCTGAAGGGAAAGCTGCA
RC_ompK36C3        ------------------------------------------------------------

IS1                CGTAATCAGCAAGCGATATACGCAGCGAATTGAGCGGCATAACCTGAATCTGAGGCAGCA
ompK36             CGTAATCAGCAAGCGATATACGCAGCGAATTGAGCGGCATAACCTGAATCTGAGGCAGCA
RC_ompK36C3        ------------------------------------------------------------

IS1                CCTGGCACGGCTGGGACGGAAGTCGCTGTCGTTCTCAAAATCGGTGGAGCTGCATGACAA
ompK36             CCTGGCACGGCTGGGACGGAAGTCGCTGTCGTTCTCAAAATCGGTGGAGCTGCATGACAA
RC_ompK36C3        ------------------------------------------------------------

IS1                AGTCATCGGGCATTATCTGAACATAAAACACTATCAATAAGTTGGAGTCATTACCCTCTG
ompK36             AGTCATCGGGCATTATCTGAACATAAAACACTATCAATAAGTTGGAGTCATTACCCTCTG
RC_ompK36C3        ----------------------------------------------------ACCCTCTG
                                                                       ********

IS1                CGACATCGTATAACGTTACTGGTTTCACATTCACC----ACCCTGAATTGACTCTCTTCC
ompK36             TTTGTTATATGCCTTTTATTATGCCACTGCTTACTGATTACCCTTCTAATCAGTCGGCTA
RC_ompK36C3        TTTGTTATATGCCTTTTATTATGCCACTGCTTACTGATTACCCTTCTAATCAGTCGGCTA
                        *         *** *          * **     *****

IS1                ------------------------------------------------------------
ompK36             T-TTCATTGTGCTGCAAAATGCAGAATAATCCAACAAGAATATGATACTAAAACTTCTAA
RC_ompK36C3        TATTCATTGTGCTGTAAAATGCAGAATAATCCAACAAGAATATGATACGAAAACTTCTAA

IS1                ------------------------------------------------------------
ompK36             GATGTTTCAAAGTTCCCACAAGATGTTTCAATTTGTAAATTCAGCGGAACTTTTTTCACC
RC_ompK36C3        GGTGTTTCAAAATGCGCACAAGATGTTTCAATTTGTAAATGCAAGGGAAC-TTTTCGCAA

IS1                ------------------------------------------------------------
ompK36             GCAAAATGGTCAGGGAAATACGCACTAATCATCAAAAAAATTATTTAAACTT-TTATAAT
RC_ompK36C3        CGATGAAAAATCGTAAAATAAGCACCAATAATCAAGAAAATAATTTTTTTCAATTACCAT

IS1                ------------------------------------------------------------
ompK36             CAAAGTGATAGACAACAACATATCTATAGCACTGAATGGTAAAACAAAATGATCATTGGT
RC_ompK36C3        CAGGTAGTTACACACCACGTCAGGTATAGCACTGAATGACAAAACAAAATGGTCGCCTGC

IS1                ------------------------------------------------------------
ompK36             GGCTAAAATAAGAATCGCTATCATCATTAACTTTATTTATTACCGTCATTCAGTTCTGAA
RC_ompK36C3        GACTAGAATAACTCCCGCTATCATCATTAACTTTATTTATTACCGTCATTCAGTTCTGAA

IS1                ----------------------------------------------------
ompK36             TGTCTGTTTACCCCTATTTCAACCGGATGCCTCGCATCCGGTTTTTTTTACC
RC_ompK36C3        TGTCTGTTTACCCCTATTTCGACCGGATGCTTCGCATCCGGTTTTTTTTACC
```

Figure 4.4 Alignment of the *ompK36* (ompK36) from strain MAJ with IS*1* nucleotide sequence (IS*1*) (Johnsrud, 1979) and the reverse complement (RC_ompK36C3) of *ompK36*C3 (Alberti *et al*., 1995). The direct repeat is highlighted in green. The imperfect terminal inverted repeats are underlined in blue. The start and stop codons of *ompK36* from strain MAJ are highlighted in red. The rest of *ompK36* open reading frame from strain MAJ is highlighted in yellow. The direction of the *ompK36* reading frame from strain MAJ is indicated by the horizontal red arrow. Areas of 100 % identity are indicated at the bottom of the aligned sequences by a star (*).

4.4 DISCUSSION

K. pneumoniae is an important cause of serious nosocomial infections (Wiener *et al.*, 1999; Wong-Beringer *et al.*, 2002). Most *K. pneumoniae* strains carry a chromosomal class SHV-1 β-lactamase gene; the enzyme has limited activity against ampicillin, but it does not hydrolyze the newer extended spectrum antibiotics (Livermore 1998; Chaves *et al.*, 2001). Over the last decade, *K. pneumoniae* has become increasingly resistant to frequently used β-lactams, including the extended spectrum β-lactams and amoxicillin-clavulanate. Overexpression of bla_{TEM} and production of IRTs are known to influence resistance to amoxicillin-clavulanate and to a lesser extent, piperacillin-tazobactam in *E. coli* (Chapter 3; Livermore 1998). The first report of an IRT found in *K. pneumoniae* was made in France (Lemozy *et al.*, 1995). However, not many IRTs have been identified in *K. pneumoniae* since 1995 (Bermudes *et al.*, 1997; Girlich *et al.*, 2000). Extensive nucleotide sequencing of bla_{TEM} has identified a variety of structural genes, transcribed from an assortment of promoters [Chapter 3]. The promoter region of the bla_{TEM} structural gene from strain ENG contains a C32T, generating the strong *Pa/Pb* promoter (Table 4.2), previously described for $bla_{\mathrm{TEM\text{-}2}}$ and $bla_{\mathrm{TEM\text{-}1B}}$ (Lartigue *et al.*, 2002; Leflon-Gebout *et al.*, 2000). The promoter region also contains an A175G transition. The coding sequence of the bla_{TEM} gene is identical to $bla_{\mathrm{TEM\text{-}1E}}$ except for the silent mutation T682C previously identified in $bla_{\mathrm{TEM\text{-}1F}}$ and $bla_{\mathrm{TEM\text{-}2}}$ (Table 3.3 and Table 4.2). Thus a novel bla_{TEM} sequence framework has been identified in strain ENG and was designated $bla_{\mathrm{TEM\text{-}1H}}$.

In both strain ENG and strain MAJ, isoelectric focusing identified only one β-lactamase corresponding to TEM-1. Cefotaxime and ceftazidime, which are ESBL substrates, were not compromised in either of the strains. Thus, supporting the absence of ESBLs. The resident chromosomal mediated β-lactamase gene, bla_{SHV}, (Babini and Livermore, 2000; Chaves *et al.*, 2001) was also found to present in both strain ENG and strain MAJ.

Cefotaxime, ceftazidime and cefoxitin resistance was previously implicated with the loss of porins (Martínez-Martínez *et al.*, 1996; Crowley *et al.*, 2002). More specifically, the loss of OmpK36 in combination with the expression of β-lactamases was shown to result in ceftazidime and cefotaxime resistance (Martínez-Martínez *et al.*, 1996; Crowley *et al.*, 2002). Furthermore, expression of OmpK36 in a strain, *K. pneumoniae* 103624, shown to produce reduced amounts of OmpK36 (Crowley *et al.*, 2002) and in a porin deficient strain, *K. pneumoniae* LB4 (Martínez-Martínez *et al.*, 1996), restored susceptibility to cefotaxime. Similarly, the MIC of cefotaxime was reduced to an intermediate susceptible level when OmpK36 was expressed in another 'porinless strain', *K. pneumoniae* CSUB10R (Crowley *et al.*, 2002). Resistance to ceftazidime seemingly was not affected by the expression of OmpK36 in a porin deficient strain, *K. pneumoniae* LB4 (Martínez-Martínez *et al.*, 1996). However, expression of OmpK36 in a strain, *K. pneumoniae* 103624, shown to produce reduced amounts of OmpK36 (Crowley *et al.*, 2002) restored the MIC of ceftazidime to clinically susceptible levels. Similarly, in the 'porinless strain', *K. pneumoniae* CSUB10R (Crowley *et al.*, 2002) the MIC of ceftazidime was reduced, although the strain still remained clinically resistant, when OmpK36 was expressed, indicating that ceftazidime resistance is notably influenced by OmpK36 expression only at very high MICs (>256 μg/ml) of ceftazidime. Contrary to the above findings for cefotaxime and ceftazidime, isogenically derived mutants lacking both OmpK35 and OmpK36 remained clinically susceptible to cefotaxime and ceftazidime (Henández-Allés *et al.*, 2000) although the MICs were higher than the parent strains expressing both OmpK35 and OmpK36.

Isogenically derived mutants lacking both OmpK35 and OmpK36 were clinically resistant to cefoxitin (Henández-Allés *et al.*, 2000), although cefoxitin resistance was also observed in the strain producing a reduced amount of OmpK36 (Crowley *et al.*, 2002). Cefoxitin resistance was shown to be abated to clinical sensitive levels when OmpK36 was expressed in the strain shown to produce reduced amounts of OmpK36 (Crowley *et al.*, 2002) or strains lacking OmpK36 (Martínez-Martínez *et al.*, 1996; Crowley *et al.*, 2002).

Surprisingly, in strain ENG the reduced expression of OmpK36 did not result in cefoxitin resistance as for strains previously reported by Crowley *et al.*, 2002. The restoration of OmpK36 expression plays a role in the β-lactam resistance albeit not solely responsible for all the resistance in strain ENG and strain ENG^P. The MICs of the inhibitor combinations, amoxicillin-clavulanate and piperacillin-tazobactam were affected by the restoration of OmpK36 resulting in an increased susceptibility of strain ENG^P to these compounds (Table.4.3). However, the MICs still remained above that of a clinically sensitive *K. pneumoniae* isolate indicating that overexpression of TEM-1 is sufficient to render strain ENG^P with a decreased susceptibility to amoxicillin-clavulanate and piperacillin-tazobactam. Thus, resistance to the inhibitor combinations in strain ENG is due to a combination of uptake of β-lactams and overexpression of TEM-1. However, Crowley *et al.*, 2002 demonstrated that after OmpK36 restoration, resistance to piperacillin-tazobactam was abolished suggesting that expression of OmpK36 is sufficient to confer susceptibility to their strain. Resistance to cefuroxime in strain ENG is attributed to the decreased expression of OmpK36.

After 4 hours of growth in selection (cefuroxime 30 μg/ml), the systems of *ompK36* has been down regulated in strain ENG. Growth without selection increased OmpK36 levels comparable to those in the susceptible strain KAN. (Figure 4.3 Panel A). Susceptibility testing at this point and at the O/N time point, indicated that strain ENG had become susceptible to cefuroxime. Thus, in the absence of selection, OmpK36 expression systems are increased with a concomitant decrease in resistance to cefuroxime.

In strain MAJ, as for strain ENG, the resistance to cefuroxime is attributed to decreased uptake of this β-lactam. OmpK35 and OmpK36 were not present in OMP profiles obtained from strain MAJ, making cefoxitin resistance the resultant, as previously reported (Martínez-Martínez *et al.*, 1996; Hernández-Allés *et al.*, 2000; Doménech-Sánchez *et al.*, 2000). In accordance with its resistance profile (Table 4.3) after 5 days of passage in antibiotic-free media, OmpK36 was still absent in strain MAJ^P. However, the expression of two alternate OMPs, (Figure 4.2 B), could account for the susceptibility to cefuroxime and to piperacillin-tazobactam. In strain MAJ, the $bla_{\text{TEM-1C}}$ gene was

associated with a weak *P3* promoter, and could thus not confer resistance to amoxicillin-clavulanate or piperacillin-tazobactam. In strain MAJ, IS*1* was found to be inserted 15 bp upstream of the *ompK36* ATG start codon. The absence of OmpK36 has previously been shown to be due to the insertion of the IS*1* element into the structural *ompK36* gene in laboratory derived mutants of *K. pneumoniae* (Hernández-Allés *et al.*, 1999). Other IS elements too were found to interrupt both the structural gene and the regulatory region of *ompK36* in clinical isolates of *K. pneumoniae* (Hernández-Allés *et al.*, 1999).

Interestingly, correlations have been shown to exist between ESBL producers and their porins in *K. pneumoniae*. A strong correlation has been made in strains that are ESBL negative and express both OmpK35 and OmpK36, or strains that express only one porin which are ESBL positive (Hernández-Allés *et al.*, 1999; Doménech-Sánchez *et al.*, 2000; Martínez-Martínez *et al.*, 2002). Contrarily neither porin profile of strain ENG nor strain MAJ conforms to the ESBL correlation.

CHAPTER 5

Cefoxitin, cefuroxime and amoxicillin-clavulanate resistance in *Acinetobacter baumannii* strain RAN, mediated by *ampC*

5.1 INTRODUCTION

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5.1 INTRODUCTION

A. baumannii is becoming an increasingly important nosocomial pathogen. This organism is associated with burn units (Bang *et al.*, 2002) and Intensive Care Units in hospitals. In Chapter 2 the chromosomal *ampC* gene from *E. coli* clinical isolates was investigated with respect to the cephalosporins. In this chapter an *A. baumannii* clinical isolate designated strain RAN is investigated for its resistance to cefuroxime, cefoxitin and amoxicillin-clavulanate. The regulation of its *ampC* gene and its genetic context is also investigated.

The chromosomal β-lactamases of *A. baumannii* have been biochemically characterized (Perilli *et al.*, 1996; Bou *et al.*, 2000; López-Hernández *et al.*, 2000). In all three of these studies it was shown that amoxicillin-clavulanate does not inhibit the chromosomal cephalosporinase of *A. baumannii*, now termed AmpC (Bou *et al.*, 2000). The *ampC* from *A. baumannii* was only recently cloned (Bou *et al.*, 2000). The upstream region of the OXA-23 gene has been termed the homologous region (Segal *et al.*, 2003) and was shown to provide a promoter for an aminoglycoside resistance gene in a strain of *A. baumannii*. Very recently Corvec *et al.*, 2003, identified this region upstream of the *ampC* gene in ceftazidime resistant *A. baumannii* strains. However, no information on the regulation of AmpC is available as of yet.

AmpC in combination with other mechanisms of resistance was suggested to contribute to various β-lactam resistance phenotypes (Danes *et al.*, 2002) in *A. baumannii*.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Bacterial strains and plasmids

A clinical *Acinetobacter baumannii* strain designated RAN was isolated from blood and identified in the diagnostic laboratory in Groote Schuur Hospital, Cape Town during 2001. *E. coli* JM109 was used as a recipient in transformation studies. Plasmid pBGS8 (Spratt *et al.*, 1986) was used as a vector in cloning experiments. All other *A. baumannii* strains were also obtained from GSH.

5.2.2 Antimicrobial susceptibility testing

MICs were performed using Etest strips (AB Biodisk, Solna, Sweden), according to the manufacturers' instructions. The MIC of cephalothin was performed using a doubling agar dilution method on IsoSensitest agar (Oxoid, Basingstoke, UK) and an inoculum of 10^4 colony forming units/spot.

5.2.3 Methods used to investigate β-lactamases and their genes in strain RAN

Various methods were used to determine possible mechanisms of β-lactam resistance in strain RAN. The presence of bla_{TEM} genes was determined as in [3.2.2.1]. Enzyme activity was determined as in [4.2.2.3]. Isoelectric focusing was used to detect β-lactamases via the hydrolysis of nitrocefin [4.2.2.4].

5.2.4 Methods used to investigate *ampC* and its expression in *A. baumannii* strain RAN

5.2.4.1 Cloning of the *ampC* gene

Approximately 24 μg of genomic DNA isolated [2.2.3.1] from strain RAN was digested with *Hin*dIII. This DNA was ligated to similarly restricted vector, pBGS8 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Braunschweig, Germany) and the recombinant plasmids transformed [2.2.4.2] into competent *E. coli*

JM109. *E. coli* JM109 recombinants were selected for on 2 × YT agar plates containing ampicillin 25 (μg/ml), IPTG (2.5 mg/L) and X-gal (0.5 %).

5.2.4.2 Induction of *ampC* expression

Overnight cultures were grown in 10 ml nutrient broth (NB) with ampicillin (50 μg/ml) as selection. One millilitre of this O/N culture was transferred to 10 ml fresh NB without antibiotic, and incubated at 37°C for 1 hour with shaking. The inducer was then added at sub-inhibitory concentrations of 10 μg/ml and 100 μg/ml of cefoxitin. The cultures were then incubated for 1 hour, 2 hours and 4 hours. Cultures incubated, for the same durations, in the absence of inducer were used as controls. After each time point the cells were collected in Falcon tubes by centrifugation at 10°C. The spent broth supernatant was discarded and the pellet resuspended in PBS. Crude cell extracts were obtained and hydrolysis assays were then performed [4.2.2.3]. Enzyme activity was detected using cephaloridine. Results were expressed as OD/min/mg of protein.

5.2.4.3 Determination of the location of the *A. baumannii ampC* gene

Five strains of *A. baumannii* from GSH were used to identify the location of *ampC*. Primers used to detect the presence of *ampC* were P1 and P2, previously described by Bou *et al.* (2000). The primer pair used to determine whether the *ampC* gene is adjoined to the homologous region (Segal *et al.*, 2003) (upstream of *ampC*) in all *A. baumannii* strains is ampC-R2 Rev, 5′-ATAGTTTCACCCGACCA-3′ and P1. The primer pair used in the detection of the location of the *ampC* gene with the gene immediately downstream of it, is comA-ampC For 5′-GGCTTCCCATCCTGCA-3′ and comA-ampC Rev 5′-GCGACGTATACAAGTGGTA-3′. To determine whether the *ampC* structural gene is always flanked by the homologous region and the downstream region (found in strain RAN) the primer pair ampC-R2 For 5′-TGGCGTCAAGCTTAGGA-3′ and ampC-R2 Rev 5′-ATAGTTTCACCCGACCA-3′ were used. PCR was performed using an initial denaturation step at 94°C for 4 min, and cycling using a denaturation step at 94°C for 1 min, an annealing step at 50°C for 1 min, and an extension step at 72°C for 1 min. A final extension step at was performed at 72°C for 4 min.

5.2.4.4 Primer extension to determine the transcriptional start site of *ampC*

PCR was performed on strain RAN and on *E. coli* pMERL100, in parallel with RNA extraction [2.2.5.1] to confirm that the *ampC* gene is still in the right location before primer extension. RNA was extracted from both these strains after 4 hours of incubation (1:100) in 10 ml 2 × YT either in the presence of cefoxitin (30 μg/ml) or in the presence of ampicillin (50 μg/ml). To determine the transcriptional start site the *ampC* gene, primer extension analysis was carried out as follows. One hundred micrograms of total RNA was precipitated and resuspended in 100 μl HP buffer (40 mM PIPES, pH 6.4: 1 mM EDTA, pH 8.0; 400 mM NaCl; 80 % formamide). The (indodicarbocyanine) Cy5 labeled primer 5′-TACCTGGCACATCATATT-3′ (2 to 5 pmol) was mixed with the RNA heated to 95°C and allowed to anneal overnight at 45°C in a PCR tube or in an Eppendorf tube. The RNA was precipitated, washed and dried. The pellet was resuspended in 20 μl RTB (4 μl 5× M-MLV Reverse Transcriptase buffer; Promega, 1 μl (10 mM) dNTP (TaKaRa), 1 μl (40 U) RNAse inhibitor, (Boehringer Mannheim), 2 μl Actinomycin D (1 mg/ml), 12 μl dH_2O). M-MLV Reverse Transcriptase (for longer transcripts and for less degradation by RNAse A than AMV) (200 U) (Promega Madison, USA) was added and incubated for two hours at 42°C. The reaction was stopped by addition of 1 μl 0.5 M EDTA (pH 8.0). Ribonuclease A (1 μl of 0.5 mg/ml) (Boehringer Mannheim) was added and left at 37°C for 30 minutes to remove the RNA. The primer extension product was precipitated with 150 μl TES (10 mM Tris; 1 mM EDTA; 100 mM NaCl) and 100 % ethanol, after which the pellet was reconstituted in 5 μl TE and 8 μl ALF stop buffer (Pharmacia). The mixture was heated to 95°C and run alongside the products of the appropriate sequencing reactions.

5.2.4.5 Sequencing analysis

Automated sequencing of the 5.2 kb insert was performed at the University of Dundee, Scotland and at the University of Stellenbosch, South Africa. Sequencing analysis was performed using DNAMAN software [2.2.3.4]. Protein sequence was obtained by translation of the *ampC*. Similarly, protein and DNA alignments were performed using DNAMAN software. Sequence homologies were determined using the GenBank DNA

and protein sequence databases at the National Centre for Biotechnology (NCBI). Basic Local Alignment Search Tool, BLAST searches for the identification of homologous DNA and proteins were performed using the website www.ncbi.nlm.nih.gov at the NCBI, National Institute of Health. Similarly, the predictions of ORFs were performed using the website www.ncbi.nlm.nih/gorf or using DNAMAN software.

5.3 RESULTS

5.3.1 Antimicrobial susceptibility testing of *A. baumannii* strain RAN

MICs indicated that strain RAN was resistant to the cephalosporins (Table 5.1), and also to the inhibitor combinations amoxicillin-clavulanate (>256 μg/ml) and piperacillin-tazobactam (>256 μg/ml).

The antibiotic susceptibility profile of *A. baumannii* strain RAN is shown in Table 5.1.

TABLE 5.1 MICs (μg/ml) for *A. baumannii* strain RAN

β-lactam	**MIC (μg/ml)**
Cefuroxime	>256
Cefoxitin	>256
Cephalothin	>128
Amoxicillin	>256
Amoxicillin-clavulanate	>256
Piperacillin	>256
Piperacillin-tazobactam	>256

5.3.2 Cloning of *ampC* from *A. baumannii* strain RAN

Three recombinants were obtained after shotgun cloning [5.2.4.1]. Restriction digests using *Hin*dIII released an insert of approximately 5-6 kb from one of the recombinants. The recombinant plasmid designated pMERL100, conferred resistance to cephalothin and cefuroxime, and diminished susceptibility to cefoxitin on its *E. coli* host. MICs are shown in Table 5.2.

TABLE 5.2 MICs (µg/ml) of β-lactams

Antibiotic	*A. baumannii* (RAN)	pMERL100 in *E. coli* JM109	*E. coli* JM109
Cefuroxime	>256	32	2
Cefoxitin	>256	24	3
Cephalothin	>128	>128	ND
Amoxicillin	>256	>256	2
Amoxicillin-clavulanate	>256	16	1
Piperacillin	>256	16	1
Piperacillin-tazobactam	>256	3	0.75

ND, not determined

5.3.3 Hydrolysis profiles of strain RAN and *E. coli* JM109 harboring pMERL100

Hydrolysis [4.2.2.3] of cephaloridine, cephalothin and cefuroxime but not of cefoxitin, was observed for *A. baumannii* strain RAN. Extracts of *Pseudomonas aeruginosa* PAO1 were used to control the hydrolysis of cefoxitin. Hydrolysis of only cephaloridine and cephalothin was observed for pMERL100 expressed in *E. coli* JM109.

5.3.4 The bla_{TEM} gene of strain RAN

A bla_{TEM} gene was detected by PCR [3.2.2.1]. Sequencing analysis [3.2.2.2] determined the bla_{TEM} gene to be bla_{TEM-1F} associated with a strong *P4* promoter. Direct sequencing of the PCR product obtained with primers 3601TEM and DEB revealed that the bla_{TEM} gene in strain RAN was identical to bla_{TEM-1F} except for a G→T transversion at bp position 604. It is thus proposed that this bla_{TEM} be named bla_{TEM-1I}.

5.3.5 Is the *ampC* from *A. baumannii* strain RAN inducible?

To determine whether the *ampC* was inducible by β-lactams, induction assays were performed on both strain RAN and on the *E. coli* JM109 harboring the recombinant plasmid, pMERL100. Initial assays [5.2.4.2] using cefoxitin as the inducing agent at sub-inhibitory concentrations of 10 μg/ml and 100 μg/ml, sampling crude enzyme extracts at time intervals of 1, 2 and 4 hours, showed no significant levels of enzyme activity above that of the same strain grown in the absence of cefoxitin, suggesting no induction by cefoxitin at both concentrations.

5.3.6 Sequencing analysis of *ampC* on pMERL100

The sequence between nucleotide bases 424 and 1367 (Figure 5.1) shows 99.47 % identity with the $phaB_{AC}$ gene previously described by Schembri *et al.*, 1995, and thus the upstream region of the *ampC* gene described by Corvec *et al.*, 2003. This region also shows 99.47 % identity with the upstream region (homologous region) of the aminoglycoside gene identified by Segal *et al.*, 2003 and 99.79 % identity with the upstream region of the OXA-23 gene identified by Afzal-Shah *et al.*, 2001. The nucleotide sequence bp 1 to 423 (Figure 5.1) is part of ORF2 (Segal *et al.*, 2003).

Alignments of the deduced amino acid sequence of the structural *ampC* gene with published *ampC* sequences (Figure 5.2 Panel B) showed that it has 98.4 %-99 % identity to the corresponding amino acid sequences from *A. baumannii* (Bou *et al.*, 2000; Mammeri *et al.*, 2003) and 99 % identity to the $bla_{ABA\text{-}1}$, thought to originate in *Acinetobacter*, from *Oligella urethralis* (Mammeri *et al.*, 2003). The open reading frame found immediately upstream of the *ampC* within the homologous region, show similarity to a transposase from *Deinococcus radiodurans* (Segal *et al.*, 2003). The *ampC* start

codon is found 9 bp downstream of a G residue which defines the boundary of the homologous region. This G also forms part of the ribosomal binding site (RBS) indicating that the ribosomal binding site is a composite, two nucleotides (AG) in the homologous region and two (GA) in the sequence that abuts this region. A putative promoter was identified in the homologous region upstream of *ampC* using primer extension analysis (Fig 5.5). The DNA sequence at the 3′ end of *ampC* contains one open reading frame (ORF3) (Fig 5.4). Fifty two nucleotides separate ORF3 and *ampC*. ORF3 is in the same transcriptional orientation as *ampC*. A putative RBS (GGA) was identified upstream of ORF3 albeit atypically spaced (20 bp), relative to the start codon. Two potential -10 hexamers (TCTCAT and GATAAT) separated by 23 and 18-bp, respectively from a putative -35 hexamer (TTGCCA) were identified upstream of the start codon. The translation product of ORF3 has 52 % identity with the C-terminal (amino acids 477-792) of ComA from *Acinetobacter calcoaceticus* BD413.

```
ampC-RAN   GAAATCAGTAGTGGTATACAAATTTTTAACCCACTTTTCAACCATTCTATTTGTATGATCTGACTCTTACTAAATACTAGTCCTTTCTTTAAAGCTAACC  100
OXA-23     ....................................................................................................    0
SKIF       ....................................................................................................    0
phaB       ....................................................................................................    0
Consensus

ampC-RAN   AACCTACAATCAAAGCAATCAAGTCAGAGACTATCCAAGCATCAAGAATACTATCGAGTTGATTTTCAACTAGTGCAAAAATCATACATCCAATAATAAG  200
OXA-23     ....................................................................................................    0
SKIF       ....................................................................................................    0
phaB       ....................................................................................................    0
Consensus

ampC-RAN   GTAACACCAAAGGCCACTTCTAATAAAAAGTAGGTTATTTGCCATAACTGTTTTTTTATCAATAATCAAAAGCCGCATGAGTTCCTGATTTAAATGTTCC  300
OXA-23     ....................................................................................................    0
SKIF       ....................................................................................................    0
phaB       ....................................................................................................    0
Consensus

ampC-RAN   AAAACTATCAGCAAACCCAAAAAAAATAACCACTTAGTTGGAATCGAGTAACTAGCAAGGAAAAGAATAATTGGTAATATAACAATATAAGCAAAAATTA  400
OXA-23     ....................................................................................................    0
SKIF       ....................................................................................................    0
phaB       ....................................................................................................    0
Consensus

ampC-RAN   TTAAAAAACTTTGACTTTTTAAAAAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGGGTCTGTCAAGCGCGTATTTTCAAGATTAAACCC  500
OXA-23     .......................AAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGGGTCTGTCAAGCGCGTATTTTCAAGATTAAACCC   77
SKIF       .......................AAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGGGTCTGTCAAGCGCGTATTTTCAAGATTAAACCC   77
phaB       .......................AAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGCGTCTGTCAAGCGCGTATTTTCAAGATTAAACCC   77
Consensus                         aagcttatagctaacaccgcaatcaatttttttcactcgtctag gtctgtcaagcgcgtattttcaagattaaaccc

ampC-RAN   GCGTCCTTTGAGACAACTGAATAAGGTTTCAATTTCCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACGACGAGTAAAAGC  600
OXA-23     GCGTCCTTTGAGACAACTGAATAAGGTTTCAATTTCCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACGACGAGTAAAAGC  177
SKIF       GCGTCCTTTGAGACAACTGAATAAGGTTTCAATTTCCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACGACGAGTAAAAGC  177
phaB       GCGTCCTTTGAGACAACTGAATAAGGTTTCAATTTCCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACGACGAGTAAAAGC  177
Consensus  gcgtcctttgagacaactgaataaggtttcaatttcccagcgtaatgcataatcctgaatagcattggcattaaactgaggagaaacgacgagtaaaagc

ampC-RAN   TCTCCATTTTCTAACTGTAGTGCACTTATATATAGTTTCACCCGACCAACCAAAATCCGTCGTTTACGACATTCAATTTGACCAACTTTAAGATGGCGAA  700
OXA-23     TCTCCATTTTCTAACTGTAGTGCACTTATATATAGTTTCACCCGACCAACCAAAATCCGTCGTTTACGACATTCAATTTGACCAACTTTAAGATGGCGAA  277
SKIF       TCTCCATTTTCTAACTGTAGTGCACTTATATATAGTTTCACCCGACCAACCAAAATCCGTCGTTTACGACATTCAATTTGACCAACTTTAAGATGGCGAA  277
phaB       TCTCCATTTTCTAACTGTAGTGCACTTATATATAGTTTCACCCGACCAACCAAAATCCGTCGTTTACGACATTCAATTTGACCAACTTTAAGATGGCGAA  277
Consensus  tctccattttctaactgtagtgcacttatatatagtttcacccgaccaaccaaaatccgtcgtttacgacattcaatttgaccaactttaagatggcgaa

ampC-RAN   ATAAATCACTAATTTTATGATTCTTTCCTAAATGATTGGTGACAATGAAGTTTTTTTAACACGAATGCAGAAGTTGATGTCTT.GTTCAATTAACCATGT  799
OXA-23     ATAAATCACTAATTTTATGATTCTTTCCTAAATGATTGGTGACAATGAAGTTTTTTTAACACGAATGCAGAAGTTGATGTCTT.GTTCAATTAACCATGT  376
SKIF       ATAAATCACTAATTTTATGATTCTTTCCTAAATGATTGGTGACAATGAAGTTTTTTTAACACGAATGCAGAAGTTGATGTCTTGGTTCAATTAACCATGT  377
phaB       ATAAATCACTAATTTTATGATTCTTTCCTAAATGATTGGTGACAATGAAGTTTTTTTAACACGAATGCAGAAGTTGATGTCTTGGTTCAATTAACCATGT  377
Consensus  ataaatcactaattttatgattctttcctaaatgattggtgacaatgaagtttttttaacacgaatgcagaagttgatgtctt gttcaattaaccatgt

ampC-RAN   AAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCAAAGCAATACGCTCT  899
OXA-23     AAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCAAAGCAATACGCTCT  476
SKIF       AAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGGGTTGAATCAAAGCAATGCGCTCT  477
phaB       AAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCAAAGCAATACGCTCT  477
Consensus  aaaccactgctcaccgataaactctctgtctgcgaacacattcacaatacggtctttaccaaaaatggctataaag gttgaatcaaagcaat cgctct

ampC-RAN   TTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTT  999
OXA-23     TTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTT  576
SKIF       TTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTT  577
phaB       TTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTT  577
Consensus  ttcgtatctgaatttccacgtttattaagcaatgtccaaaggataggtatcgctattccacgataaacgattgcgagcatcaggatattaatatttcgtt

ampC-RAN   TTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTG  1099
OXA-23     TTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTG   676
SKIF       TTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTG   677
phaB       TTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTG   677
Consensus  ttccccatttccaattggttctatctaaagtcagttgcacttggtcgaatgaaaacatattgaaaatcaactgagaaatttgacgataatcaaaatactg

ampC-RAN   ACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAACACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAA  1199
OXA-23     ACCTGCAAAGAAACGCTGCATACGTCGATAAAATGATTGTGGTAAACACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAA   776
SKIF       ACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAA   777
phaB       ACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAA   777
Consensus  acctgcaaagaa cgctgcatacgtcgataaaatgattgtggtaa cacttgatgggcaaggctttagatgcagaagaaagattacatgtttgctttaaa

ampC-RAN   ATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAGATATAACTCATTGAGATGTGTCATAG  1299
OXA-23     ATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAGATATAACTCATT.AGATGTGTCATAG   875
SKIF       ATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAGATATAACTCATTGAGATGTGTCATAG   877
phaB       ATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGATATTTGTTTAAGATAAGATATAACTCATTGAGATGTGTCATAG   877
Consensus  ataatcacaagcatgatgagcgcaaagcactttaaatgtgacttgttccattttaga atttgtttaagataagatataactcatt agatgtgtcatag

ampC-RAN   TATTCGTCGTTAGAAAACAATTATTGTGACATTATTTCAATGAGTTATCTATTTTTGTCGTGTACAGAGGAGCTAATCATG                   1380
OXA-23     TATTCGTCGTTAGAAAACAATTATTGTGACATTATTTCAATGAGTTATCTATTTTTGTCGTGTACAGAG                                944
SKIF       TATTCGTCGTTAGAAAACAATTATTATGACATTATTTCAATGAGTTATCTATTTTTGTCGTGTACAGAG                                946
phaB       TATTCGTCGTTAGAAAACAATTATTATGACATTATTTCAATGAGTTATCTATTTTTGTCGTGTACAGAG                                946
Consensus  tattcgtcgttagaaaacaattatt tgacattatttcaatgagttatctatttttgtcgtgtacagag
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Figure 5.1 Alignment of the *ampC* regulatory region of strain RAN with the homologous regions of bla_{OXA-23}, SKIF (GenBank Accession No. AY138987) and $phaB_{AC}$ genes. The start codon of *ampC* is underlined in dash. The putative promoter is underlined in pink. The transcriptional start site is indicated by the vertical arrow. The open reading frames ORF1 and ORF2 are underlined in black and the direction of their transcription is indicated by a blue and green arrow, respectively. The consensus sequence is indicated below the alignment. Nucleotides highlighted in pink share ≥ 75 %, in blue ≥ 50 % and yellow ≥ 33 %.

```
ABAC-1      ATGCGATTTAAAAAAATTTCTTGTCTACTTTTATCCCCGCTTTTTTTTTTTAGTACCTCAATTTATGCGGGCAATACACCAAAAGACCAAGAAATTAAAA   100
ABAC-2      ATGCGATTTAAAAAAATTTCTTGTCTACTTTTATCCCCGCTTTTTATTTTTAGTACCTCAATTTATGCGGGCAATACACCAAAAGACCAAGAAATTAAAA   100
ABA-1       ATGCGATTTAAAAAAATTTCTTGTCTACTTTTATCTCCGCTTTTTATTTTTAGTACCTCAATTTATGCGGGCAATACACCAAAAGACCAAGAAATTAAAA   100
ampC-RAN    ATGCGATTTAAAAAAATTTCTTGTCTACTTTTATCCCCGCTTTTTATTTTTAGTACCTCAATTTATGCGGGCAATACACCAAAAGACCAAGAAATTAAAA   100
RYC52763    ATGCGATTTAAAAAAATTTCTTGTCTACTTTTATCCCCGCTTTTTATTTTTAGTACCTCAATTTATGCGGGCAATACACCAAAAGACCAAGAAATTAAAA   100
Consensus   atgcgatttaaaaaaatttcttgtctacttttatc ccgcttttt tttttagtacctcaatttatgcgggcaatacaccaaaagaccaagaaattaaaa

ABAC-1      AACTGGTAGATCAAAACTTTAAACCGTTATTAGAAAAATATGATGTGCCGGGTATGGCTGTGGGTGTTATTCAAAATAATAAAAAGTATGAAATGTATTA   200
ABAC-2      AACTGGTAGATCAAAACTTTAAACCGTTATTAGAAAAATATGATGTGCCGGGTATGGCTGTGGGTGTTATTCAAAATAATAAAAAGTATGAAATGTATTA   200
ABA-1       AACTGGTAGATCAAAACTTTAAACCGTTATTAGAAAAATATGATGTGCCAGGTATGGCTGTGGGTGTTATTCAAAATAATAAAAAGTATGAAATGTATTA   200
ampC-RAN    AACTGGTAGATCAAAACTTTAAACCGTTATTAGAAAAATATGATGTGCCAGGTATGGCTGTGGGTGTTATTCAAAATAATAAAAAGTATGAAATGTATTA   200
RYC52763    AACTGGTAGATCAAAACTTTAAACCGTTATTAGAAAAATATGATGTGCCAGGTATGGCTGTGGGTGTTATTCAAAATAATAAAAAGTATGAAATGTATTA   200
Consensus   aactggtagatcaaaactttaaaccgttattagaaaaatatgatgtgcc ggtatggctgtgggtgttattcaaaataataaaaagtatgaaatgtatta

ABAC-1      TGGTCTTCAATCTGTTCAAGATAAAAAAGCCGTAAATAGCAGTACCATTTTTGAGCTAGGTTCTGTCAGTAAATTATTTACTGCGACAGCAGGTGGATAT   300
ABAC-2      TGGTCTTCAATCTGTTCAAGATAAAAAAGCCGTAAATAGCAGTACCATTTTTGAGCTAGGTTCTGTCAGTAAATTATTTACTGCGACAGCAGGTGGATAT   300
ABA-1       TGGTCTTCAATCTGTTCAAGATAAAAAAGCCGTAAATAGCAGTACTATTTTTGAGCTAGGTTCTGTCAGTAAATTATTTACCGCGACAGCAGGTGGATAT   300
ampC-RAN    TGGTCTTCAATCTGTTCAAGATAAAAAAGCCGTAAATAGCAGTACTATTTTTGAGCTAGGTTCTGTCAGTAAATTATTTACCGCGACAGCAGGTGGATAT   300
RYC52763    TGGTCTTCAATCTGTTCAAGATAAAAAAGCCGTAAATAGCAGTACTATTTTTGAGCTAGGTTCTGTCAGTAAATTATTTACCGCGACAGCAGGTGGATAT   300
Consensus   tggtcttcaatctgttcaagataaaaaagccgtaaatagcagtac atttttgagctaggttctgtcagtaaattatttac gcgacagcaggtggatat

ABAC-1      GCAAAAAATAAAGGAAAAATCTCTTTTGACGATACGCCTGGTAAATATTGGAAAGAACTAAAAAATACACCGATTGACCAAGTTAACTTACTTCAACTCG   400
ABAC-2      GCAAAAAATAAAGGAAAAATCTCTTTTGACGATACGCCTGGTAAATATTGGAAAGAACTAAAAAATACACCGATTGACCAAGTTAACTTACTTCAACTCG   400
ABA-1       GCAAAAAATAAAGGAAAAATCTCTTTTGACGATACGCCTGGTAAGTATTGGAAAGAACTAAAAAATACACCGATTGACCAAGTTAACTTACTTCAACTCG   400
ampC-RAN    GCAAAAAATAAAGGAAAAATCTCTTTTGACGATACGCCTGGTAAATATTGGAAAGAGCTAAAAAATACACCGATTGACCAAGTTAACTTACTTCAACTCG   400
RYC52763    GCAAAAAATAAAGGAAAAATCTCTTTTGACGATACGCCTGGTAAGTATTGGAAAGAACTAAAAAATACACCGATTGACCAAGTTAACTTACTTCAACTCG   400
Consensus   gcaaaaaataaaggaaaaatctcttttgacgatacgcctggtaa tattggaaaga ctaaaaaatacaccgattgaccaagttaacttacttcaactcg

ABAC-1      CGACGTATACAAGTGGTAACCTTGCCTTGCAGTTCCCAGATGAAGTACAAACAGACCAACAAGTTTTAACTTTTTTCAAAGACTGGAAACCTAAAAACCC   500
ABAC-2      CGACGTATACAAGTGGTAACCTTGCCTTGCAGTTCCCAGATGAAGTACAAACAGACCAACAAGTTTTAACTTTTTTCAAAGACTGGAAACCTAAAAACCC   500
ABA-1       CGACGTATACAAGTGGTAACCTTGCCTTGCAGTTTCCAGATGAAGTACAAACAGACCAACAAGTTTTAACTTTTTTCAAAGACTGGAAACCTAAAAACCC   500
ampC-RAN    CGACGTATACAAGTGGTAACCTTGCCTTGCAGTTCCCAGATGAAGTACAAACAGACCAACAAGTTTTAACTTTGTTCAAAGACTGGAAACCTAAAAACCC   500
RYC52763    CGACGTATACAAGTGGTAACCTTGCCTTGCAATTTCCAGATGAAGTAAAAACAGATCAGCAAGTTTTAACATTTTTTAAAGACTGGAAACCTAAAAACTC   500
Consensus   cgacgtatacaagtggtaaccttgccttgca tt ccagatgaagta aaacaga ca caagttttaac tt tt aaagactggaaacctaaaaac c

ABAC-1      AATCGGTGAATACAGACAATATTCAAATCCAAGTATTGGCCTATTTGGAAAGGTTGTAGCTTTGTCTATGAATAAACCCTTCGACCAAGTCTTAGAAAAA   600
ABAC-2      AATCGGTGAATACAGACAATATTCAAATCCAAGTATTGGCCTATTTGGAAAGGTTGTAGCTTTGTCTATGAATAAACCTTTCGACCAAGTCTTAGAAAAA   600
ABA-1       AATCGGTGAATACAGACAATATTCAAATCCAAGTATTGGCCTATTTGGAAAGGTTGTAGCTTTGTCTATGAATAAACCTTTCGACCAAGTCTTAGAAAAA   600
ampC-RAN    AATCGGTGAATACAGACAATATTCAAATCCAAGTATTGGCCTATTTGGAAAGGTTGTAGCTTTGTCTATGAATAAACCTTTCGACCAAGTCTTAGAAAAA   600
RYC52763    AATCGGTGAATATCGACAATATTCAAACCCAAGCATTGGTTTATTTGGAAAAGTTGTAGCTTTGTCTATGAATAAACCTTTCGACCAAGTCTTAGAAAAA   600
Consensus   aatcggtgaata  gacaatattcaaa ccaag attgg  tatttggaaa gttgtagctttgtctatgaataaacc ttcgaccaagtcttagaaaaa

ABAC-1      ACAATTTTTCCGGCCCTTGGCTTAAAACATAGCTATGTAAATGTACCTAAGACCCAAATGCAAAACTATGCTTTTGGCTATAACCAAGAAAATCAGCCGA   700
ABAC-2      ACAATTTTTCCGGCCCTTGGCTTAAAACATAGCTATGTAAATGTACCTAAGACCCAAATGCAAAACTATGCTTTTGGCTATAACCAAGAAAATCAGCCGA   700
ABA-1       ACAATTTTTCCGGCCCTTGGCTTAAAACATAGCTATGTAAATGTACCTAAGACCCAAATGCAAAACTATGCTTTTGGCTATAACCAAGAAAATCAGCCGA   700
ampC-RAN    ACAATTTTTCCGGCCCTTGGCTTAAAACATAGCTATGTAAATGTACCTAAGACCCAAATGCAAAACTATGCTTTTGGCTATAACCAAGAAAATCAGCCGA   700
RYC52763    ACAATTTTTCCGGCCCTTGGCTTAAAACATAGCTATGTAAATGTACCTAAGACCCAGATGCAAAACTATGCTTTTGGCTATAACCAAGAAAATCAGCCGA   700
Consensus   acaatttttccggcccttggcttaaaacatagctatgtaaatgtacctaagaccca atgcaaaactatgcttttggctataaccaagaaaatcagccga

ABAC-1      TTCGAGTTAACCCTGGCCCACTCGATGCCCCAGCATACGGCGTCAAATCCACCTTACCGGATATGTTGAGTTTTATTTATGCCAACCTTAACCCACAGAA   800
ABAC-2      TTCGAGTTAACCCTGGCCCACTCGATGCCCCAGCATACGGCGTCAAATCCACCCTTCCGGATATGTTGAGTTTTATTTATGCCAACCTTAACCCACAGAA   800
ABA-1       TTCGAGTTAACCCCGGCCCACTCGATGCCCCAGCATACGGCGTCAAATCCACCTTACCGGATATGTTGAGTTTTATTAATGCCAACCTTAACCCACAAAA   800
ampC-RAN    TTCGAGTTAACCCCGGCCCACTCGATGCCCCAGCATATGGCGTCAAATCAACACTACCCGACATGTTGAGTTTTATTCATGCCAACCTAAATCCACAAAA   800
RYC52763    TTCGAGTTAACCCCGGCCCACTCGGTGCCCCAGCATATGGCGTCAAATCGACACTACCCGACATGTTGAGTTTTATTCATGCCAACCTAAATCCACAAAA   800
Consensus   ttcgagttaaccc ggcccactcg tgccccagcata ggcgtcaaatc ac  t cc ga atgttgagttttatt atgccaacct aa ccaca aa

ABAC-1      ATATCCGGCTGATATTCAAAGGGCAATTAATGAAACACATCAAGGTCGCTATCAAGTAAATACCATGTATCAAGCGCTTGGTTGGGAAGAGTTTTCTTAT   900
ABAC-2      ATATCCGGCTGATATTCAAAGGGCAATTAATGAAACACATCAAGGTCGCTATCAAGTAAATACCATGTATCAGGCACTCGGTTGGGAAGAGTTTTCTTAT   900
ABA-1       ATATCCGACAGATATTCAACGGGCAATTAATGAAACACATCAAGGTCGCTATCAAGTAAATACCATGTATCAAGCGCTTGGTTGGGAAGAGTTTTCTTAT   900
ampC-RAN    ATATCCGGCAGATATTCAACGTGCAATTAATGAAACACATCAGGGTCGCTATCAAGTAAATACCATGTATCAGGCACTCGGTTGGGAAGAGTTTTCTTAT   900
RYC52763    ATATCCAGCAGATATTCAACGGGCAATTAATGAAACACATCAGGGTCGCTATCAAGTAAATACCATGTATCAGGCACTCGGTTGGGAAGAGTTTTCTTAT   900
Consensus   atatcc  c gatattcaa g gcaattaatgaaacacatca ggtcgctatcaagtaaataccatgtatca gc ct ggttgggaagagttttcttat

ABAC-1      CCGGCAACGTTACAAACTTTATTAGACAGTAATTCAGAACAGATTGTGATGAAACCTAATAAAGTGACTGCTATTTCAAAAGAGCCTTCAGTTAAGATGT   1000
ABAC-2      CCGGCAACGTTACAAACTTTATTAGACAGTAATTCAGAACAGATTGTGATGAAACCTAATAAAGTGACTGCTATTTCAAAGGAACCTTCAGTTAAGATGT   1000
ABA-1       CCGGCAACGTTACAAACTTTATTAGACAGTAATTCAGAACAGATTGTGATGAAGCCTAATAAAGTGACTGCTATTTCAAAAGAGCCTTCAGTTAAGATGT   1000
ampC-RAN    CCGGCAATGTTACAAACTTTACTAGACAGTAATTCAGAACAGATTGTGATGAAACCTAATAAAGTGACTGCTATTTCAAAAGAACCTTCAGTTAAGATGT   1000
RYC52763    CCGGCAACGTTACAAACTTTATTAGACAGTAATTCAGAACAGATTGTGATGAAACCTAATAAAGTGACTGCTATTTCAAAGGAACCTTCAGTTAAGATGT   1000
Consensus   ccggcaa gttacaaacttta tagacagtaattcagaacagattgtgatgaa cctaataaagtgactgctatttcaaa ga ccttcagttaagatgt

ABAC-1      ACCATAAAACTGGCTCAACCAACGGTTTCGGAACATATGTGGTGTTTATTCCTAAAGAAAATATTGGTTTAGTCATGTTAACCAATAAACGTATTCCAAA   1100
ABAC-2      ACCATAAAACTGGCTCAACTAACCGTTTCGGAACATATGTGGTGTTTATTCCTAAAGAAAATATTGGTTTAGTCATGTTAACCAATAAACGTATTCCAAA   1100
ABA-1       ACCATAAAACTGGCTCAACTAACGGTTTCGGAACATATGTAGTGTTTATTCCTAAAGAAAATATTGGTTTAGTCATGTTAACCAATAAACGTATTCCAAA   1100
ampC-RAN    ACCATAAAACTGGCTCAACCAACGGTTTCGGAACGTATGTGGTGTTTATTCCTAAAGAAAATATTGGCTTAGTCATGTTAACCAATAAACGTATTCCAAA   1100
RYC52763    ACCATAAAACTGGCTCAACTAACCGTTTCGGAACATATGTGGTGTTTATTCCTAAAGAAAATATTGGTTTAGTCATGTTAACCAATAAACGTATTCCAAA   1100
Consensus   accataaaactggctcaac aac gtttcggaac tatgt gtgtttattcctaaagaaaatattgg ttagtcatgttaaccaataaacgtattccaaa

ABAC-1      TGAAGAGCGCATTAAGGCAGCTTATGCTGTGCTGAGTGCAATAAAGAAATAA                                                 1152
ABAC-2      TGAAGAGCGCATTAAGGCAGCTTATGCTGTGCTGAATGCAATAAAGAAATAA                                                 1152
ABA-1       TGAAGAGCGCATTAAGGCAGCTTATGCTGTGCTGAATGCAATAAAGAAATAA                                                 1152
ampC-RAN    TGAAGAGCGCATTAAGGCAGCGTATGCAGTTTTAAATGCAATAAAGAAATAA                                                 1152
RYC52763    TGAAGAGCGCATTAAGGCAGCTTATGCTGTGCTGAATGCAATAAAGAAATAA                                                 1152
Consensus   tgaagagcgcattaaggcagc tatgc gt  t a tgcaataaagaaataa
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A

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ABAC-1      MRFKKISCLLLSPLFFFSTSIYAGNTPKDQEIKKLVDQNFKPLLEKYDVPGMAVGVIQNNKKYEMYYGLQSVQDKKAVNSSTIFELGSVSKLFTATAGGY   100
ABAC-2      MRFKKISCLLLSPLFIFSTSIYAGNTPKDQEIKKLVDQNFKPLLEKYDVPGMAVGVIQNNKKYEMYYGLQSVQDKKAVNSSTIFELGSVSKLFTATAGGY   100
ABA-1       MRFKKISCLLLSPLFIFSTSIYAGNTPKDQEIKKLVDQNFKPLLEKYDVPGMAVGVIQNNKKYEMYYGLQSVQDKKAVNSSTIFELGSVSKLFTATAGGY   100
ampC-RAN    MRFKKISCLLLSPLFIFSTSIYAGNTPKDQEIKKLVDQNFKPLLEKYDVPGMAVGVIQNNKKYEMYYGLQSVQDKKAVNSSTIFELGSVSKLFTATAGGY   100
RYC52763    MRFKKISCLLLSPLFIFSTSIYAGNTPKDQEIKKLVDQNFKPLLEKYDVPGMAVGVIQNNKKYEMYYGLQSVQDKKAVNSSTIFELGSVSKLFTATAGGY   100
Consensus   mrfkkisclllsplf fstsiyagntpkdqeikklvdqnfkpllekydvpgmavgviqnnkkyemyyglqsvqdkkavnsstifelgsvsklftataggy

ABAC-1      AKNKGKISFDDTPGKYWKELKNTPIDQVNLLQLATYTSGNLALQFPDEVQTDQQVLTFFKDWKPKNPIGEYRQYSNPSIGLFGKVVALSMNKPFDQVLEK   200
ABAC-2      AKNKGKISFDDTPGKYWKELKNTPIDQVNLLQLATYTSGNLALQFPDEVQTDQQVLTFFKDWKPKNPIGEYRQYSNPSIGLFGKVVALSMNKPFDQVLEK   200
ABA-1       AKNKGKISFDDTPGKYWKELKNTPIDQVNLLQLATYTSGNLALQFPDEVQTDQQVLTFFKDWKPKNPIGEYRQYSNPSIGLFGKVVALSMNKPFDQVLEK   200
ampC-RAN    AKNKGKISFDDTPGKYWKELKNTPIDQVNLLQLATYTSGNLALQFPDEVQTDQQVLTLFKDWKPKNPIGEYRQYSNPSIGLFGKVVALSMNKPFDQVLEK   200
RYC52763    AKNKGKISFDDTPGKYWKELKNTPIDQVNLLQLATYTSGNLALQFPDEVKTDQQVLTFFKDWKPKNSIGEYRQYSNPSIGLFGKVVALSMNKPFDQVLEK   200
Consensus   aknkgkisfddtpgkywkelkntpidqvnllqlatytsgnlalqfpdev tdqqvlt fkdwkpkn igeyrqysnpsiglfgkvvalsmnkpfdqvlek

ABAC-1      TIFPALGLKHSYVNVPKTQMQNYAFGYNQENQPIRVNPGPLDAPAYGVKSTLPDMLSFIYANLNPQKYPADIQRAINETHQGRYQVNTMYQALGWEEFSY   300
ABAC-2      TIFPALGLKHSYVNVPKTQMQNYAFGYNQENQPIRVNPGPLDAPAYGVKSTLPDMLSFIYANLNPQKYPADIQRAINETHQGRYQVNTMYQALGWEEFSY   300
ABA-1       TIFPALGLKHSYVNVPKTQMQNYAFGYNQENQPIRVNPGPLDAPAYGVKSTLPDMLSFINANLNPQKYPTDIQRAINETHQGRYQVNTMYQALGWEEFSY   300
ampC-RAN    TIFPALGLKHSYVNVPKTQMQNYAFGYNQENQPIRVNPGPLDAPAYGVKSTLPDMLSFIHANLNPQKYPADIQRAINETHQGRYQVNTMYQALGWEEFSY   300
RYC52763    TIFPALGLKHSYVNVPKTQMQNYAFGYNQENQPIRVNPGPLGAPAYGVKSTLPDMLSFIHANLNPQKYPADIQRAINETHQGRYQVNTMYQALGWEEFSY   300
Consensus   tifpalglkhsyvnvpktqmqnyafgynqenqpirvnpgpl apaygvkstlpdmlsfi anlnpqkyp diqrainethqgryqvntmyqalgweefsy

ABAC-1      PATLQTLLDSNSEQIVMKPNKVTAISKEPSVKMYHKTGSTNGFGTYVVFIPKENIGLVMLTNKRIPNEERIKAAYAVLSAIKK   383
ABAC-2      PATLQTLLDSNSEQIVMKPNKVTAISKEPSVKMYHKTGSTNRFGTYVVFIPKENIGLVMLTNKRIPNEERIKAAYAVLNAIKK   383
ABA-1       PATLQTLLDSNSEQIVMKPNKVTAISKEPSVKMYHKTGSTNGFGTYVVFIPKENIGLVMLTNKRIPNEERIKAAYAVLNAIKK   383
ampC-RAN    PAMLQTLLDSNSEQIVMKPNKVTAISKEPSVKMYHKTGSTNGFGTYVVFIPKENIGLVMLTNKRIPNEERIKAAYAVLNAIKK   383
RYC52763    PATLQTLLDSNSEQIVMKPNKVTAISKEPSVKMYHKTGSTNRFGTYVVFIPKENIGLVMLTNKRIPNEERIKAAYAVLNAIKK   383
Consensus   pa lqtlldsnseqivmkpnkvtaiskepsvkmyhktgstn fgtyvvfipkeniglvmltnkripneerikaayavl aikk
```

B

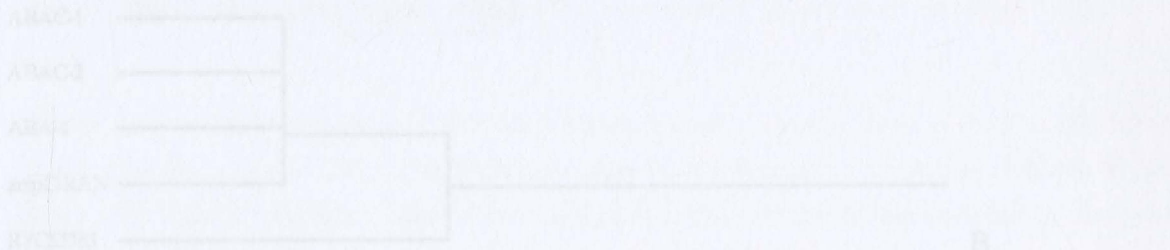
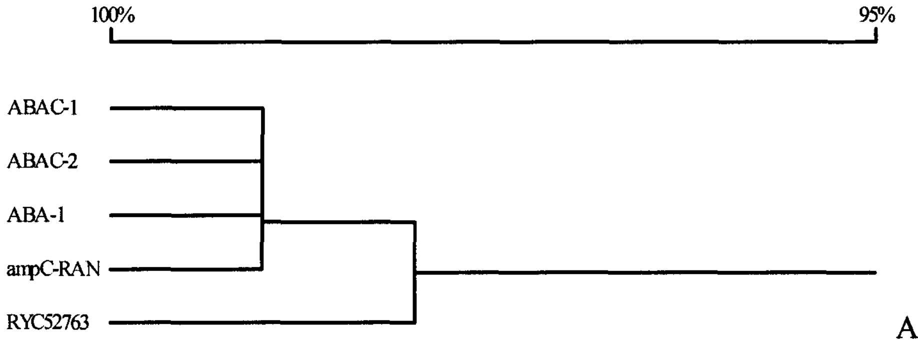


Figure 5.2 Panel A *ampC* alignments of ampC-RAN (*ampC* from *A. baumannii* strain RAN) with known nucleotide sequences. **Panel B** AmpC alignments of derived protein sequence of ampC–RAN (*ampC* from *A. baumannii* strain RAN) with known protein sequences. Sequences are from *A. baumannii* and *Oligella urithralis* (Mammeri *et al.*, 2003; Bou *et al.*, 2000). Nucleotides or amino acids highlighted in pink share homology ≥ 75 %, in blue ≥ 50 % and yellow ≥ 33 %.

Homology matrix of 5 DNA sequences

ABAC-1	100%				
ABAC-2	99.2%	100%			
ABA-1	99.0%	99.2%	100%		
ampC-RAN	98.7%	99.0%	99.0%	100%	
RYC52763	98.2%	99.0%	98.4%	98.4%	100%



Homology matrix of 5 protein sequences

ABAC-1	100%				
ABAC-2	99.2%	100%			
ABA-1	99.0%	99.2%	100%		
ampC-RAN	98.7%	99.0%	99.0%	100%	
RYC52763	98.2%	99.0%	98.4%	98.4%	100%

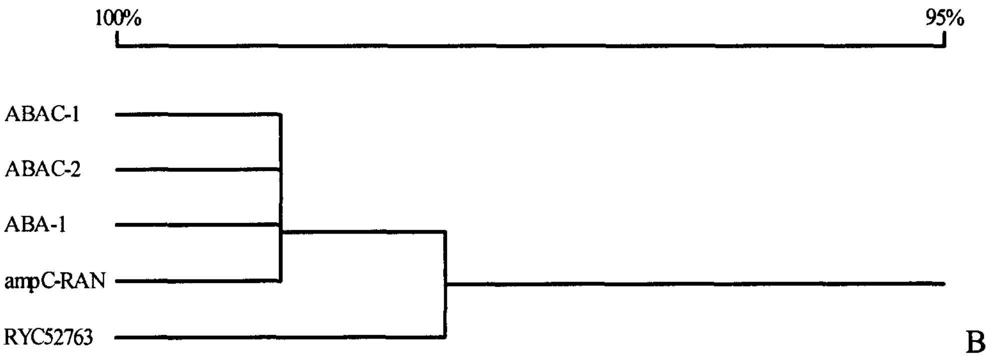


Figure 5.3 Homology matrices and homology trees. **Panel A** nucleotide sequences **Panel B** protein sequences; from *A. baumannii* strains and an *Oligella urithralis* source. ABA-1 from *Oligella urithralis*, ABAC-1 and ABAC-2 are from *A. baumannii* isolates, RYC52763 is also from an *A. baumannii* strain (Mammeri *et al.*, 2003; Bou *et al.*, 2000). ampC-RAN (*ampC* from *A. baumannii* strain RAN) is from this study.

```
1      CTTTAAAAAC CTCTACTCAA CCAGAGCCTA TCCAAACGTC GGTCATGTAA AACTATTTTG
61     TGATTTTCCA ACACAAAACT TAAGGTCCCT TGTTCCACAG TGCTTTTTAG CGGAATATGT
121    AGAGCTTTTA AACGTGCTAT AACTTGTTGG CTAGGATGGC CATAACGGTT ATCAAACCCT
181    GCCGATGCAA TGGCCAGTTT AGGTTTTAAG GTCGCCAAGA AATCATAAGC CGAACTGTGC
241    TTACTTCCAT GATGCCCTAG CACCAACACA TCTATCTTCA AGTTAGGATA ATCTTTTAAT
301    AACTCGTATT CGGCTTCCCA TCCTGCATCG CCCATAATAA GAAAATTTTG GTAACCACCA
361    ACTTTTTTAA ATTGAAGATA TACAACACAA GAATATTGAT TCTGGTTAGA AGCAACAAAA
421    GCCAGATCTT TTTCTTTAGG CCATAAAATT TGAATATCTA ACTCAGGATA ATGCCATTGT
481    TGCCCTTGAT GGCAATATTG GAATGGTTGC TTTAAATCAT TTGGTAATTG TTCATTCGAA
541    ATAAGCTGCT TTACAGGAAT CTCTTGTTGA ATAAGAGGAA ACGCGCCACT ATGGTCTTGA
601    TCAAGATGGG ATAGCACAAC ATGATCTAAT TGTCTTACGC CTTGCTGACG TAGAAAAGGC
661    ACTACAACAT TTTGTCCAAT ACTAAATATT TTTTCATCGT AAGAACCGCC TGTATCAATT
721    AACCAGTTTT GTTCGGAATG TTGTAAAAAA ATGGCTTGCC CCTGTCCAAC ATCTAAAATA
781    TTAAGCTGAA TTTGTTGACT CGTTTTGTTC ATAATAACTA AGGGTAAACA ACATAATATC
841    CCCCAAGTTT TGGGCAGAAT TCCTTTAGGT AAAAATAAAA TAATTATGGC AAAACTAATC
901    GCTAATAAAG ACAGTGGCGT CAAGCTTAGG ATATGTTTGG TTCTTTTAAA GAATAAGTGC
961    ATAAAAAAGA GAAATGAGAT TATCCATTTC TCTTTTTGTT TTTGGCAAAA ACGGTTA
```

Figure 5.4 Nucleotide sequence of ORF3, the 3′ region of *ampC*. The stop codon of *ampC* is highlighted in red. The start codon of ORF3 is highlighted in green and the direction of transcription indicated by the arrow. The putative ribosome binding site is highlighted in blue and the putative promoter hexamers are underlined.

5.3.7 Identification of the promoter and transcription start site of *ampC*

Using RNA from strain RAN, the primer extension product was mapped to a G located 35 nucleotides upstream of the *ampC* start codon (Fig. 5.5). The hexamers, TTAGAA (-35) and TTATTT (-10), separated by 16 bp, upstream of the transcription start site show similarity to the promoter consensus recognized by Eσ^{70}.

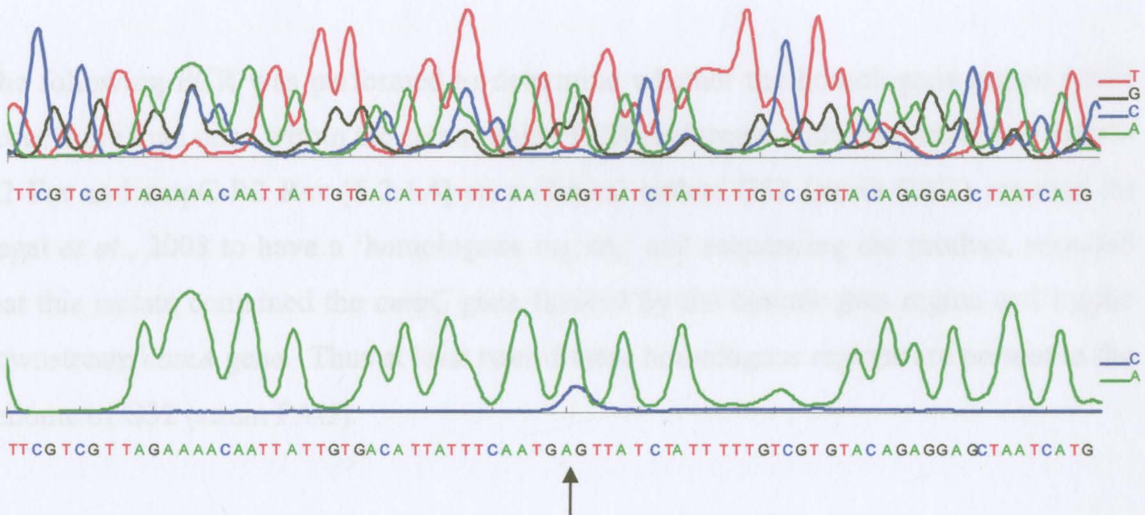


Figure 5.5 Mapping transcription start site of *ampC* in *A. baumannii* strain RAN. **Panel A** Sequencing reactions, obtained with the (indodicarbocyanine) Cy5 primer and pMERL100 as template, which were used to identify the location of the primer extension product on the sequencing gel. The nucleotide base key is given as T in red, G in black, C in blue and A in green **Panel B** The peak in blue represents the primer extension product labeled with (indodicarbocyanine) Cy5 primer which was electrophoresed along the appropriate sequencing reactions shown in Panel A. The peaks in green are A residues from Panel A which are superimposed onto the primer extension products' electrophoresis pattern. The vertical arrow indicate the transcription start site from the promoter.

5.3.8 The genetic location of *ampC* in other strains of *A. baumannii*

Analyses of five strains (including strain RAN) of *Acinetobacter baumannii* from GSH all containing *ampC* were used to investigate the genetic location of this gene. The

homologous region was found to be adjoined to the *ampC* in three of the five *A. baumannii* isolates. The same three isolates were positive when tested for the *comA* region downstream of the *ampC*. Using the primer pair flanking the *ampC* structural gene indicated that these three isolates contained the *ampC* gene, the upstream (homologous region) and the downstream gene (*comA*) in the same genetic context and orientation as the *ampC* in pMERL100. In addition to these three, another isolate had only the *comA* associated with the *ampC* without the homologous region being attached to it. The fifth isolate was only positive for the *ampC* gene and negative for the presence of both the homologous and *comA* region.

The following PCR was performed to determine whether the homologous region could occur more than once within the same strain. PCR performed with the primer pair ampC-R2 For and ampC-R2 Rev [5.2.4.4] on a clinical isolate G32 (strain PAU) reported by Segal *et al.*, 2003 to have a 'homologous region,' and sequencing the product, revealed that this isolate contained the *ampC* gene flanked by the homologous region and by the downstream *comA* gene. Thus at least two of these homologous regions are present in the genome of G32 (strain PAU).

DISCUSSION

The recombinant plasmid, pMERL100, containing the *ampC* from *A. baumannii* strain RAN was expressed in *E. coli* JM109. It conferred resistance to amoxicillin-clavulanate, cephalothin and cefuroxime, and a reduced susceptibility to cefoxitin and on its host. This resistance phenotype is different from that of *E. coli* containing the *A. baumannii ampC* gene in a similar vector (pBGS18) designated pGER1 (Bou *et al.*, 2000). The cefuroxime MIC (>256 µg/ml) was greater for *E. coli* (pGER1) than the corresponding MIC (32 µg/ml) for *E. coli* pMERL100 [Table 5.2]. In addition, based on the MIC determinations, AmpC in this study had activity against cefoxitin [Table 5.2], while activity against this antibiotic was not demonstrated for the AmpC previously described (Bou *et al.*, 2000). The MICs of the cephalosporins tested in this study were all higher for *E. coli* pMERL100, than for *E. coli* JM109 alone. However, none of the cephalosporin MICs, except for cephalothin, reached the MIC values originally determined for strain RAN. These differences probably reflect differences in gene expression between *A. baumannii* and *E. coli* JM109.

Although, clavulanate does not inhibit the chromosomal cephalosporinases of *A. baumannii* (Perilli *et al.*, 1996; Bou *et al.*, 2000; López-Hernández *et al.*, 2001) the MIC of amoxicillin-clavulanate was a only a meager 16 µg/ml for *E. coli* containing pMERL100.

Overexpression of TEM-1 enzymes are well known to confer resistance to the inhibitor combinations amoxicillin-clavulanate and piperacillin-tazobactam [Chapter 3]. In this study a bla_{TEM-1I} gene was found associated with a *P4* promoter. Together, AmpC and TEM-1 expression could account for amoxicillin-clavulanate resistance in *A. baumannii* strain RAN.

Alignment of the deduced amino acid sequence [Fig 5.2] of AmpC from pMERL100 and the corresponding amino acid sequence from pGER1, identified six amino acid changes. These amino acid substitutions are not in the vicinity of the active site and are therefore unlikely to play a role in the levels of β-lactam resistance. However, three dimensional

structural conformational changes arising from these amino acid substitutions cannot be excluded as a means of altering the substrate profile of AmpC.

Thus resistance to the cephalosporins and to amoxicillin-clavulanate in *A. baumannii* strain RAN is in part attributed to the expression of AmpC and TEM-1.

Sequencing data from pMERL100 showed that the 5'-end of *ampC* is adjacent to a sequence, which is similar to sequences found at the 5'-end of unrelated genes from *Acinetobacter* (Segal *et al.*, 2003). Data [5.3.8] from strain G32 (strain PAU) suggests that the homologous region can occur within the same strain (genome) more than once, and in all reported instances (Schembri *et al.*, 1995; Segal *et al.*, 2003) this region has been found upstream of structural genes, one of them being a β-lactamases (Donald *et al.*, 2000; Afzal-Shah *et al.*, .2001), thereby suggesting a possible role in gene expression. Recently Corvec *et al.*, (2003) identified an IS element providing a putative promoter upstream of *ampC* from *A. baumannii* conferring ceftazidme resistance. As reported previously, this homologous region contains outwardly directed promoters (Segal *et al.*, 2003). Primer extension analysis identified that the putative IS provides the promoter sequences TTAGAA (-35)N_{16}TTATTT (-10) for the transcription of *ampC*. There is good correlation between this promoter sequence and the consensus hexamers from *E. coli* recognized by $E\sigma^{70}$ polymerase, TTGACA (-35) and TATAAT (-10), and to the consensus length separating them (17 bp). Previous studies have shown that promoters in *A. baumannii* have poor similarity to $E\sigma^{70}$ consensus sequences (Segal and Elisha 1999). Whether the chromosomal *ampC* β-lactamases are inducible or constitutive has not been decided (Amyes and Young, 1996). In this study the strong inducer cefoxitin could not induce the expression of AmpC. Since *ampC* of *A. baumannii* was found downstream of different promoters (Mammeri *et al.*, 2003), it is possible that *ampC* expression may be increased above basal level in some *A. baumannii* strains. Corvec *et al.*, (2003) identified an insertion element upstream of *ampC* in *A. baumannii* strains that could provide the means for hyperproducing AmpC.

The first report of the DNA sequence of *ampC* from *A. baumannii* RYC 52763/97 did not include information on the regulation or environment of the gene (Bou *et al.*, 2000). Analysis of the DNA sequence upstream (100 bp) of this *ampC* gene showed that it is linked to the homologous region, which probably provides the promoter for its transcription. The *ampC* gene ($bla_{\text{ABA-1}}$) from *Oligella urethralis*, suggested to originate from *A. baumannii*, was identified downstream of a different IS element, *ISOur1* (Mammeri *et al.*, 2003). It was suggested that *ampC* and *ISOur1* was acquired by *O. urethralis* as a unit, implying that *ampC* may be regulated differently in unrelated *A. baumannii* strains.

Investigating the genetic surroundings of *ampC* in this study indicated that not all *ampC* genes are found downstream of the homologous region and thus do not use the same promoter. This finding is in accord with those of Corvec *et al.*, 2003. Not all the *ampC* genes investigated in this study were found upstream of the *comA* gene. Thus the location of *ampC* is not constant in all *A. baumannii* strains, suggesting fluidity in the genome of *A. baumannii* with respect to expression of the *ampC* gene.

CONCLUSIONS

Molecular techniques were employed to characterize resistance to selected β-lactams in *E. coli, K. pneumoniae* and *A. baumannii.*

Chapter 2, describes the genetic basis of β-lactam resistance in five clinical isolates of *E. coli*. The hyperproduction of *ampC* is due to mutations in the promoter region conferring resistance to amoxicillin-clavulanate, cefoxitin, cefuroxime and ceftazidime.

Chapter 3, Amoxicillin-clavulanate resistance in four *E. coli* clinical isolates is investigated. Identification of TEM-1 and IRT-30 genes associated with strong promoters is ascribed to this resistance.

Chapter 4, Two strains of *K. pneumoniae* were investigated for their resistance to the inhibitor combination amoxicillin-clavulanate and cefuroxime. In addition, strain MAJ was resistant to cefoxitin. The resistance in these two strains is attributed to changes in the outer membrane profiles and expression of TEM-1 β-lactamases.

Chapter 5, Describes the genetics of *ampC* in *A. baumannii* and its implication in the resistance to amoxicillin-clavulanate, cefuroxime and cefoxitin. The resistance is attributed to the AmpC and overproduction of TEM-1 β-lactamases.

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