# The role of β-lactamases in carbapenem-resistant Gram-negative bacteria

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**Submitted in satisfaction of the requirements for the degree of PhD**

**in the University of Edinburgh 1998**

# ABSTRACT

The carbapenems belong to the β-lactam antibiotics and are known to have an unrivalled antibacterial spectrum. An expanding number of different carbapenem-hydrolysing β-lactamases is increasingly being identified, especially in geographical areas where the carbapenems have been employed widely. These carbapenemases can be divided into two groups according to whether they are metallo-β-lactamases or serine β-lactamases.

Recently, there have been several reports from Japan of carbapenem-resistant isolates that produce a transferable metallo-β-lactamase designated IMP-1. In this thesis sixty-one clinical *Pseudomonas aeruginosa* isolates were acquired from hospitals within Japan and fifty-one of these strains were resistant to imipenem and/or meropenem (MIC >4mg/l). Neither IMP-1 nor a novel carbapenemase could be detected in any of these strains; instead synergism between a cephalosporinase and lowered outer membrane permeability was found to be the most prevalent mechanism of imipenem resistance.

The carbapenem-hydrolysing metallo-β-lactamases produced by members of the genus *Aeromonas* have in the past few years demanded attention from a clinical and enzymological point of view. Two imipenem-resistant *Aeromonas veronii* biovar *sobria* strains 13 and 99 were isolated from a water source in South India. An imipenem-based detection method applied after isoelectric focusing revealed that a

β-lactamase with a pI of 5.84 was responsible for carbapenem hydrolysis in strains 13 and 99 and unlike previously reported *Aeromonas* metallo-β-lactamases this enzyme could be detected with nitrocephin. Purification of this novel enzyme, nominated AVS-1, further demonstrated the unusual properties of this carbapenemase, most notably its insensitivity to EDTA. A metallo-β-lactamase gene was amplified from *A. veronii* bv. *sobria* strains 13 and 99 by PCR. Sequencing of the PCR product revealed that these two strains possess a metallo-β-lactamase gene that is closely related to the metallo-β-lactamase gene *imiS* previously identified in an isolate of *A. veronii* bv. *sobria*. Therefore, minor amino acid substitutions may account for the extended substrate specificity and unusual inhibitor profile of AVS-1. Two non-carbapenem-hydrolysing β-lactamases were also cloned from *A. veronii* bv. *sobria* strain 13. One of these β-lactamases a clavulanic acid sensitive β-lactamase was found to be an *ampS*-like penicillinase. The other cloned β-lactamase could unfortunately not be sequenced.

Biochemical studies have previously shown that the metallo-β-lactamases produced by *Stenotrophomonas maltophilia* are a heterogeneous group of enzymes. In this thesis heterogeneity was investigated at the molecular level by PCR. Seven *S. maltophilia* strains that produce biochemically different metallo-β-lactamases, were subjected to PCR with intragenic primers designed from the known nucleotide sequences of the *S. maltophilia* metallo-β-lactamase genes $bla_{L1}$ and *bla*S. A 561 base pair nucleotide sequence was determined from each of the PCR products amplified from the seven strains tested. The 187 amino acid residues deduced from the nucleotide sequence demonstrated that the metallo-β-lactamases from seven

different strains share ≥88.2 % homology with one another and ≥88.3 % homology with the metallo-β-lactamases encoded by *bla*$_{L1}$ and *bla*S. Therefore, the metallo-β-lactamases of *S. maltophilia* are a family of related enzymes differing by a few amino acids.

# DECLARATION

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

# ACKNOWLEDGEMENTS

I would firstly like to thank Sebastian Amyes for all his guidance and encouragement during my PhD. I would also like to thank David Payne and Chris Thomson for their help and advice. I am grateful to Tim Walsh and Peter Bennett at Bristol University for enabling me to work in their department and for their technical help in the cloning of the *Aeromonas* β-lactamase genes. I am also indebted to Wipa Suginta and Linda Gilmore in the Biochemistry department for allowing me to use their protein purification equipment and for useful discussion. I must also acknowledge Lisa Katz (SmithKline Beecham) for her help in determining DNA sequences and Brian Clarke (SmithKline Beecham) for carrying out sequence alignment studies.

I would sincerely like to thank all my friends inside and outside of Molecular Chemotherapy for making my time in the Medical Microbiology department both enjoyable and memorable. Finally, I would like to convey my thanks to my family, especially Simon and my Mum and Dad, for their constant support (as always) throughout this PhD.

This thesis is dedicated to my Grandad, William Horrill.

# PUBLICATIONS AND PRESENTATIONS

**R. A. Stunt (Walker), C. J. Thomson, D. J. Payne and S. G. B. Amyes** (1998). A study of the mechanisms involved in imipenem resistance in *Pseudomonas aeruginosa* isolates from Japan. *Journal of Antimicrobial Chemotherapy* 42:272-273.

**R. A. Stunt (Walker), A. K. B. Amyes, C. J. Thomson, D. J. Payne & S. G. B. Amyes** (1998). The production of a novel carbapenemase in *Aeromonas veronii* bv. *sobria*, and its association with imipenem resistance. *Journal of Antimicrobial Chemotherapy* 42: in press.

**R. A. Stunt (Walker), C. J. Thomson, D. J. Payne & S. G. B. Amyes** (1996). A rapid substrate based technique for the characterisation of carbapenemases after IEF. In: *Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy*, New Orleans, Abstract C-35 (poster presentation).

**R. A. Stunt (Walker), A. K. B. Amyes, C. J. Thomson, D. J. Payne & S. G. B. Amyes** (1997). Isolation of imipenem-resistant *Aeromonas veronii* bv. *sobria*, that possess a novel carbapenemase, from a water source in India. In: *Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto, Abstract C-96 (oral presentation).

**Sebastian G. B Amyes, Susan Brown, Thamarai Dorai-John, Rachel Stunt (Walker)** (1998). Carbapenemases in Gram-negative bacteria. Seventh β-lactamase workshop, Holy Island. Contribution of material for oral presentation given by S. G. B. Amyes.

# ABBREVIATIONS

| | |
|---|---|
| 7-ACA | 7-Aminocephalosporanic acid |
| AMP | Ampicillin |
| 6-APA | 6-Aminopenicillianic acid |
| bp | Base pair |
| bv. | Biovar |
| CARB | Carbenicillin |
| CEPH | Cephaloridine |
| CFOX | Cefoxitin |
| cfu | Colony forming unit |
| CIP | Calf intestine alkaline phosphatase |
| CIPRO | Ciprofloxacin |
| CPIME | Cefepime |
| CTAZ | Ceftazidime |
| CTAX | Cefotaxime |
| DAP | Diaminopimelic acid |
| DEAE | Diethylaminoethyl |
| DHP-I | Dehydropeptidase I |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| F1-dUTP | Fluorescein-11-dUTP |
| FPLC | Fast Protein Liquid Chromatography |
| $ID_{50}$ | Dose producing 50% inhibition |
| IEF | Isoelectric Focusing |
| IMP | Imipenem |
| IST | IsoSensitest |
| kb | Kilobases |
| Km | Michaelis constant |
| LB | Luria-Bertani |
| MPM | Meropenem |
| MIC(s) | Minimum inhibitory concentration(s) |

| | |
|---|---|
| MRSA | Methicillin-resistant *Staphylococccus aureus* |
| NAG | N-acetylglucosamine |
| NAM | N-acetylmuramic acid |
| ND | Not detected |
| NN | Not named |
| OMP(s) | Outer membrane protein(s) |
| PAGE | Polyacrylamide gel electrophoresis |
| PBP(s) | Penicillin binding protein(s) |
| PBS | Phosphate buffered saline |
| pCMB | p-Chloromercuribenzoate |
| PCR | Polymerase chain reaction |
| pI | Isoelectric point |
| PIPES | Piperazine-N,N'-bis(2-ethanesulphonic acid) |
| SDS | Sodium dodecyl sulphate |
| Spp. | Species |
| SSC | Sodium chloride/sodium citrate buffer |
| TAE | Tris acetate |
| TEMED | N,N,N',N'-tetramethyl-ethylenediamine |
| Vmax | Maximum rate of hydrolysis |
| v/v | Volume per volume |
| w/v | Weight per volume |

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## 10: GENERAL DISCUSSION

# 1: INTRODUCTION

## 1.1 Introduction to antibiotic resistance

The discovery of antibiotics in the earlier half of this century could be cited as one of the great landmarks in the history of medicine and the fight against infectious disease. However, seventy years after Alexander Fleming observed the antibacterial properties of penicillin (Fleming, 1929) we are now moving towards a situation where some bacteria are resistant to all commonly employed antibiotics (Spratt and Duerden, 1997).

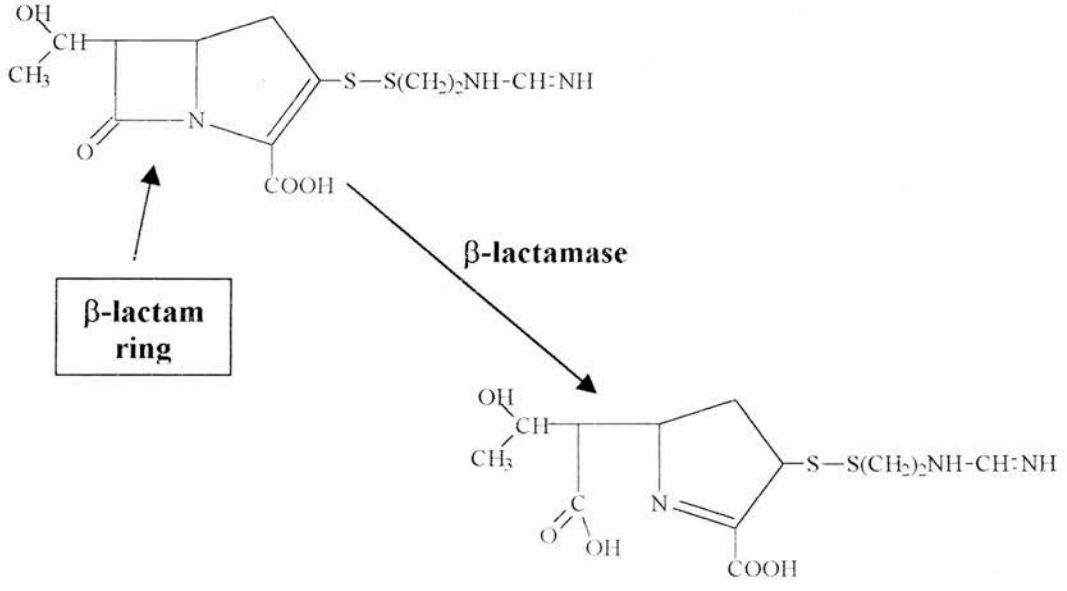
Bacteria have been subjected to intense selection pressures through the widespread use of antibiotics in medicine and farming, and as a consequence bacterial resistance mechanisms can now be identified against every clinically available agent. Advances in medical technology, the use and misuse of antibiotics and poor hospital infection control procedures are all factors contributing to the problem of antibiotic resistance which is currently most acute in hospital intensive care units (Amyes and Thomson, 1995).

## 1.2 β-Lactam antibiotics

The β-lactam family is the largest of all the antibiotic groups. β-Lactams have dominated the antibiotic arena since the general introduction of antibiotics into clinical use in the mid 1940s. The successful nature of the β-lactam antibiotics is

reflected by their selective toxicity, strong bactericidal activity and the ability to modify and improve their activity by chemically altering the β-lactam (Livermore, 1996). The four membered β-lactam ring is the structural feature that all β-lactams share in common and is also the point of attack for the β-lactamases (Figure 1.1). β-Lactamases hydrolyse the amide bond of the β-lactam ring to produce an antibacterially inactive acidic product (Figure1.1).

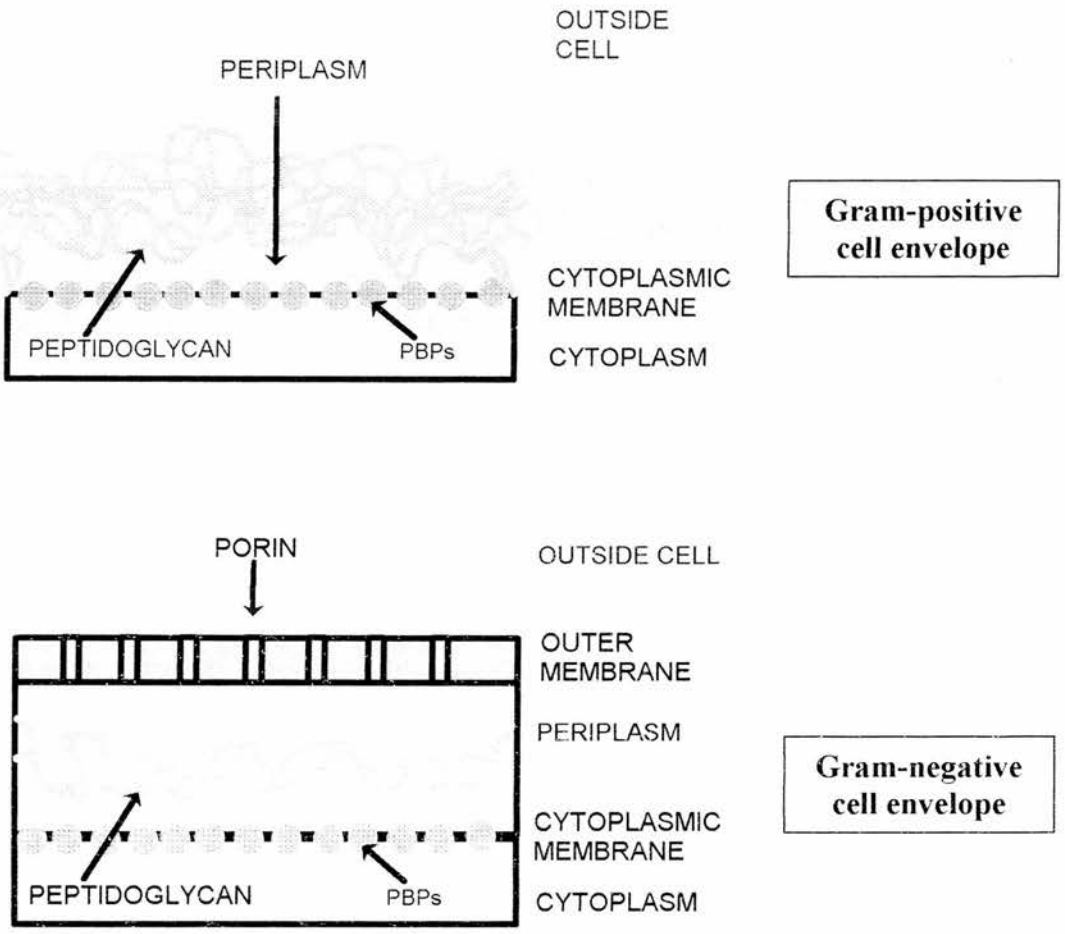
**Figure 1.1 β-Lactamase hydrolysis of a carbapenem**




## 1.3 Mode of β-lactam action

The mechanism of β-lactam action is known to involve the inhibition of peptidoglycan biosynthesis and the activation of endogenous autolytic enzymes leading to cell death (Tomasz, 1979). Both Gram-positive and Gram-negative bacteria possess a lattice of peptidoglycan in their cell envelope that maintains the cell shape and protects against osmotic forces (Figure 1.2).

**Figure 1.2 Simplified cross-section of Gram-positive and Gram-negative cell envelopes**



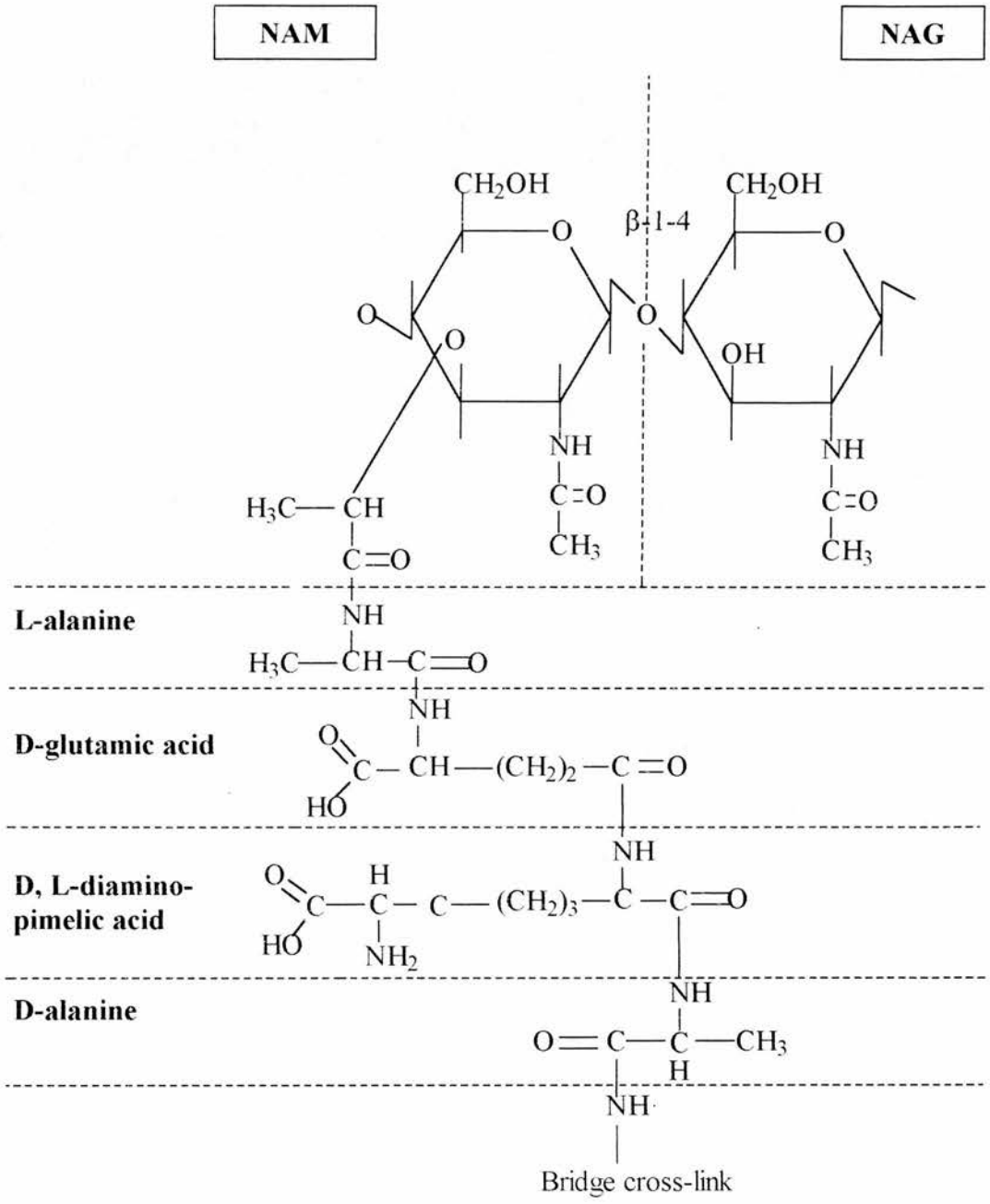

The peptidoglycan molecule consists of long polysaccharide chains made of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues (Figure 1.3). Pentapeptide chains are attached to the NAM groups and it is through the covalent cross-linking of pentapeptides that adjacent polysaccharide chains are connected to form the peptidoglycan matrix (Barnikel *et al.*, 1983). In *Enterobacteriaceae* the pentapeptide initially consists of L-alanine, D-glutamic acid, diaminopimelic acid (DAP) and two terminal D-alanine residues. During the cross-

linking reaction (transpeptidation) the D-alanyl-D-alanine bond is cleaved, providing energy for coupling of the carboxyl group of the penultimate D-alanine to the free amino group of DAP in the pentapeptide of an adjacent strand (Barnikel *et al.*, 1983).

In actively dividing bacteria β-lactams primarily inhibit the enzymes that catalyse transpeptidation reaction (D-alanyl-D-alanine transpeptidases). The amide group (O=C-N) in the β-lactam ring (Figure 1.1) is conformationally similar to the peptide link of the two terminal D-alanyl-D-alanine residues of the peptidoglycan pentapeptides, thereby, causing the transpeptidases to mistake the drug for their natural substrates (Tipper and Strominger, 1965; Waxman *et al.*, 1980). The enzymes lose their catalytic activity once the β-lactam has bound.

The β-lactam target enzymes occur on the cytoplasmic membrane and are collectively known as the penicillin binding proteins (PBPs). There are several distinct PBPs that demonstrate functional roles other than involvement in transpeptidation (Livermore and Williams, 1996). The ability to penetrate the cell wall and the degree of affinity to these PBPs determines the activity of the β-lactam on the bacterium. Although PBPs have been best studied in *Escherichia coli*, virtually all Gram-negative rods give identical patterns of seven PBPs, these are numbered 1a, 1b, 2, 3, 4, 5, and 6 in order of descending molecular weights (Georgopapadakou and Liu, 1980; Noguchi *et al.*, 1979). Some β-lactams bind almost exclusively to PBPs 2 or 3, although most bind to PBPs 1-3. PBPs 1-3 of Gram-negative bacteria are essential for peptidoglycan manufacture and inhibition of these components by β-lactams is lethal (Spratt, 1977).

**Figure 1.3 Structure of the repeating unit of peptidoglycan in *E. coli* (Taussig, 1984)**



## 1.4 Mechanisms of resistance to the β-lactams

Bacterial β-lactam resistance is often a multi-factorial event and thus can arise by one or more of the following mechanisms:

### 1.4.1 Modification of the penicillin binding protein targets

Modification of PBPs as a mechanism of β-lactam resistance is most important in Gram-positive cocci and the fastidious Gram-negatives *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Spratt, 1994). In other Gram-negative pathogens, PBP-mediated resistance is not such an important mechanism, although, it has been reported for example in *Pseudomonas aeruginosa* (Malouin and Bryan, 1986).

### 1.4.2 Impermeability

Impermeability of a β-lactam is a resistance mechanism associated with Gram-negative bacteria only, because Gram-negative bacteria, unlike Gram-positives, possess an outer membrane external to the cell wall that protects the PBPs (Figure 1.2). Certain hydrophilic semi-synthetic β-lactams can cross the outer membrane by passive diffusion through pores composed of porin proteins. The properties and numbers of the porins and the characteristics of the β-lactam (charge, size and hydrophobicity) determine the rate of uptake (Livermore, 1991). Reduced porin

expression has been associated with insusceptibility especially when the organism possesses a β-lactamase (see Section 1.10.1).

### 1.4.3 Efflux

There is increasing evidence that active efflux (active pumping out of an antibiotic entering the cell) plays a significant role in β-lactam resistance (Nikaido, 1994). In *P. aeruginosa*, for example, the over expression of an operon (*mexAmexBoprM*) that encodes an efflux system confers cross resistance to β-lactams, quinolones, tetracycline and chloramphenicol (see Section 1.10.1.1).

### 1.4.4 Production of a β-lactamase

The production of β-lactam inactivating enzymes is the most prevalent mechanism of resistance to the β-lactams amongst clinical bacteria (Medeiros, 1997). Gram-positive organisms release β-lactamases into the environment, whereas, the β-lactamases produced by Gram-negative bacteria are secreted into the periplasmic space between the cytoplasmic and outer membrane. The list of β-lactamases that have so far been identified from bacterial isolates is extensive and the number of uniquely described β-lactamases now exceeds more than 250 (Karen Bush - personal communication). The various β-lactamases will be discussed in more detail in Section 1.6.

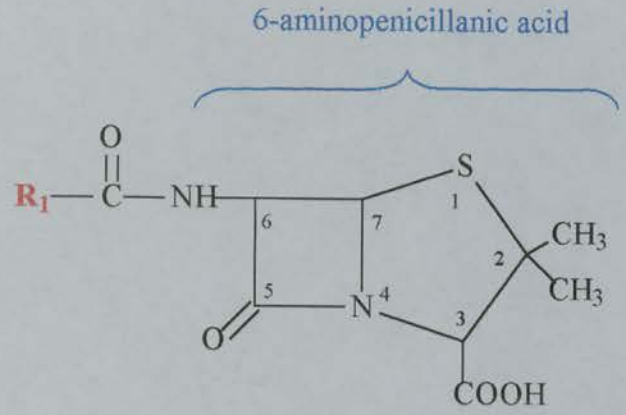
## 1.5 Classification of the β-lactams

The confusingly large group of β-lactam antibiotics is often divided into four different classes: the penicillins, cephalosporins, monobactams and carbapenems. Changing patterns of infection and resistance have driven the development of successive β-lactam generations.

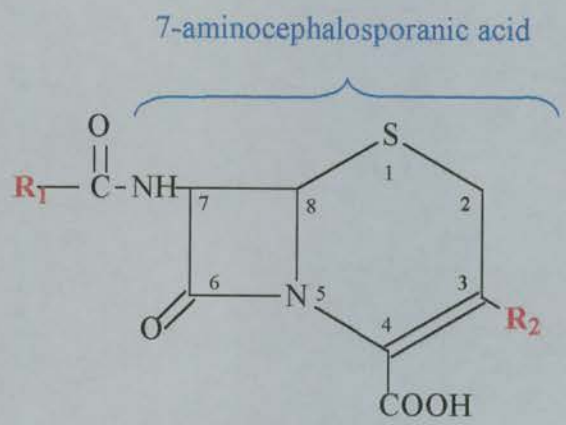
Figure 1.4 shows the chemical structures of the four β-lactam classes. The β-lactam ring of the penicillins and carbapenems is fused to a five-membered (thiazolidine) ring, whereas, the cephalosporins possess a β-lactam/six membered (dihydrothiazine) ring structure. The monobactams are monocyclic compounds, a feature that distinguishes them from the other β-lactams. With all of these compounds the β-lactam ring is essential for antimicrobial activity and it is the R-side chains that determine the spectrum of antibacterial activity and also the pharmacokinetic properties of the β-lactam.

A fifth group of important β-lactam agents are the β-lactamase inhibitors, which unlike the other four classes generally demonstrate little or no antibacterial activity. These β-lactamase inhibitors are able to bind to β-lactam hydrolysing enzymes and thus render them inactive. β-lactamase inhibitors are employed in a dual agent approach (Section 1.5.5).

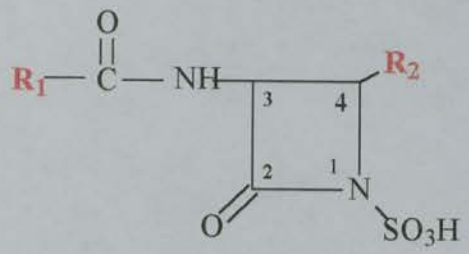
**Figure 1.4 Basic chemical structures of four β-lactam classes**



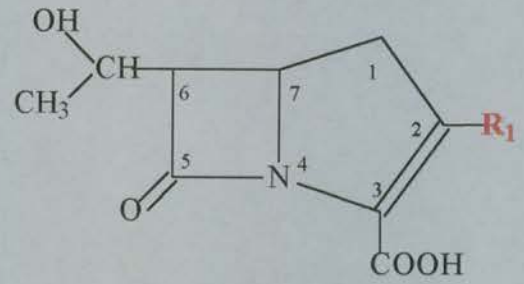

**Penicillin**




**Cephalosporin**




**Monobactam**




**Carbapenem**

### 1.5.1 Penicillins

The penicillin class covers a wide spectrum of antibacterial activity and can be divided into five groups according to a structure-activity relationship (adapted from Wright and Wilkowske, 1991). The five different penicillin subclasses are listed in Table 1.1 along with representatives from each subclass. Examples of some of the $R_1$ penicillin side chains are shown in Figure 1.5.

## Table 1.1 Classification of the penicillins

| **Natural narrow-spectrum penicillins** | **Narrow-spectrum penicillins resistant to staphylococcal penicillinase** | **Broad-spectrum penicillins** | **Penicillins active against *P. aeruginosa*** | **β-lactamase-resistant penicillins** |
|---|---|---|---|---|
| Benzylpenicillin | Methicillin | Ampicillin | Carbenicillin | Temocillin |
| Phenoxymethylpenicillin | Oxacillin | Amoxycillin | Ticarcillin | |
| | Dicloxacillin | | Azlocillin | |
| | Flucloxacillin | | Mezlocillin | |
| | | | Piperacillin | |

Natural narrow spectrum penicillins were the first β-lactams to be made clinically available. Benzylpenicillin, isolated as a product of *Penicillium notatum* (Fleming, 1929), was initially manufactured by adding maize extract rich in phenylacetic acid to the fermentation process (Raper *et al.*, 1944; Moyer and Coghill, 1946). In a similar fashion phenoxyacetic acid could be added to the procedure to yield phenoxymethylpenicillin. The main difference between these two penicillins resides in their stability to gastric acid. Phenoxymethylpenicillin is acid stable, unlike

benzylpenicillin and can therefore can be administered orally (Wright and Wilkowske, 1991).

In 1959 it was found that benzylpenicillin could be treated with an acylase to yield 6-aminopenicillanic acid (6-APA), this was an important discovery because 6-APA could then be substituted with various side chains to produce derivatives with improved oral absorption and a broader spectrum of activity (Rolinson, 1988). A further aim at this time was to develop penicillins active against penicillinase producing *Staphylococcus aureus*, which had emerged as a serious clinical problem (Barber and Whitehead, 1949). Methicillin was the first penicillin stable to the staphylococcal penicillinase and was followed by the appearance of the isoxazolyl penicillins (oxacillin, cloxacillin, dicloxacillin and flucloxacillin). These penicillins were stable to the staphylococcal penicillinase because they carry a bulky $R_1$ side chain which sterically inhibits the enzyme action (Figure 1.5) (Doyle *et al.*, 1961; Knudsen *et al.*, 1962; Sutherland *et al.*, 1970).

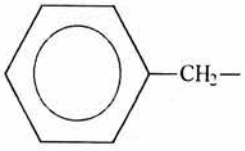
Ampicillin was the first penicillin to be active against many common Gram-positive and Gram-negative pathogens and involved adding an amino group as a side chain to the basic benzylpenicillin molecule (Figure 1.5). Amoxycillin is closely related to ampicillin in structure and function but demonstrates improved oral absorption.

Carbenicillin was the first penicillin to show significant activity against *P. aeruginosa*. Replacing the amino side chain group of ampicillin with a carboxyl group (Figure 1.5) produced Carbenicillin. Carbenicillin and ticarcillin are both

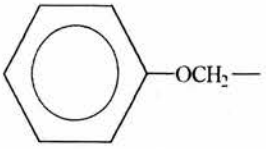
examples of carboxypenicillins. The ureidopenicillins (azlocillin, mezlocillin and piperacillin) are *P. aeruginosa* active ampicillin derivatives and have somewhat better activity than the carboxypenicillins against *P. aeruginosa* (Nathwani and Wood, 1993). Both the carboxypenicillins and ureidopenicillins can only be administered parenterally.

Temocillin is a derivative of ticarcillin and is the only penicillin to show general β-lactamase stability (Slocombe *et al.*, 1981). It is a 6-α-methoxy ($OCH_3$) group of Temocillin that makes it resistant to bacterial β-lactamases. The disadvantage of temocillin is its narrow-spectrum of activity, which is restricted to the *Enterobacteriaceae* (Wright and Wilkowske, 1991).

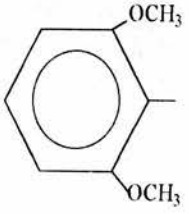
## Figure 1.5 Examples of $R_1$ penicillin side chains



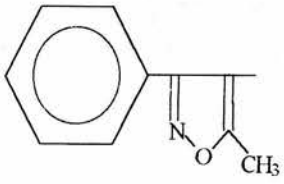
**Benzylpenicillin**



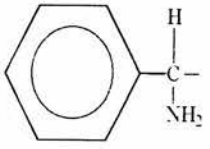
**Phenoxymethylpenicillin**



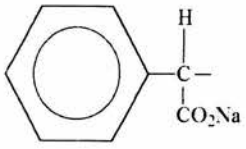
**Methicillin**



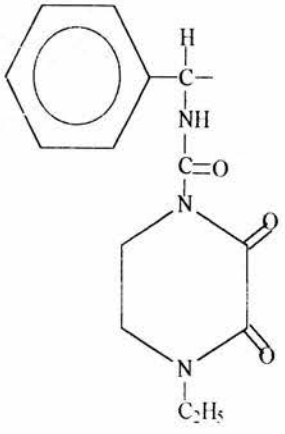
**Oxacillin**



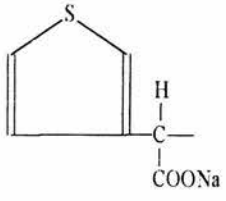
**Ampicillin**



**Carbenicillin**



**Piperacillin**



**Temocillin**

### 1.5.2 Cephalosporins

The successful discovery of penicillin promoted the search for other antibiotic-producing microorganisms. The fungus *Cephalosporium acremonium* was found to produce an antibacterial substance Cephalosporin C (Abraham and Newton, 1961). 7-Aminocephalosporanic acid (7-ACA) can be derived from cephalosporin C by chemical means and it is from this molecule that the various cephalosporins originate (Morin *et al.*, 1962).

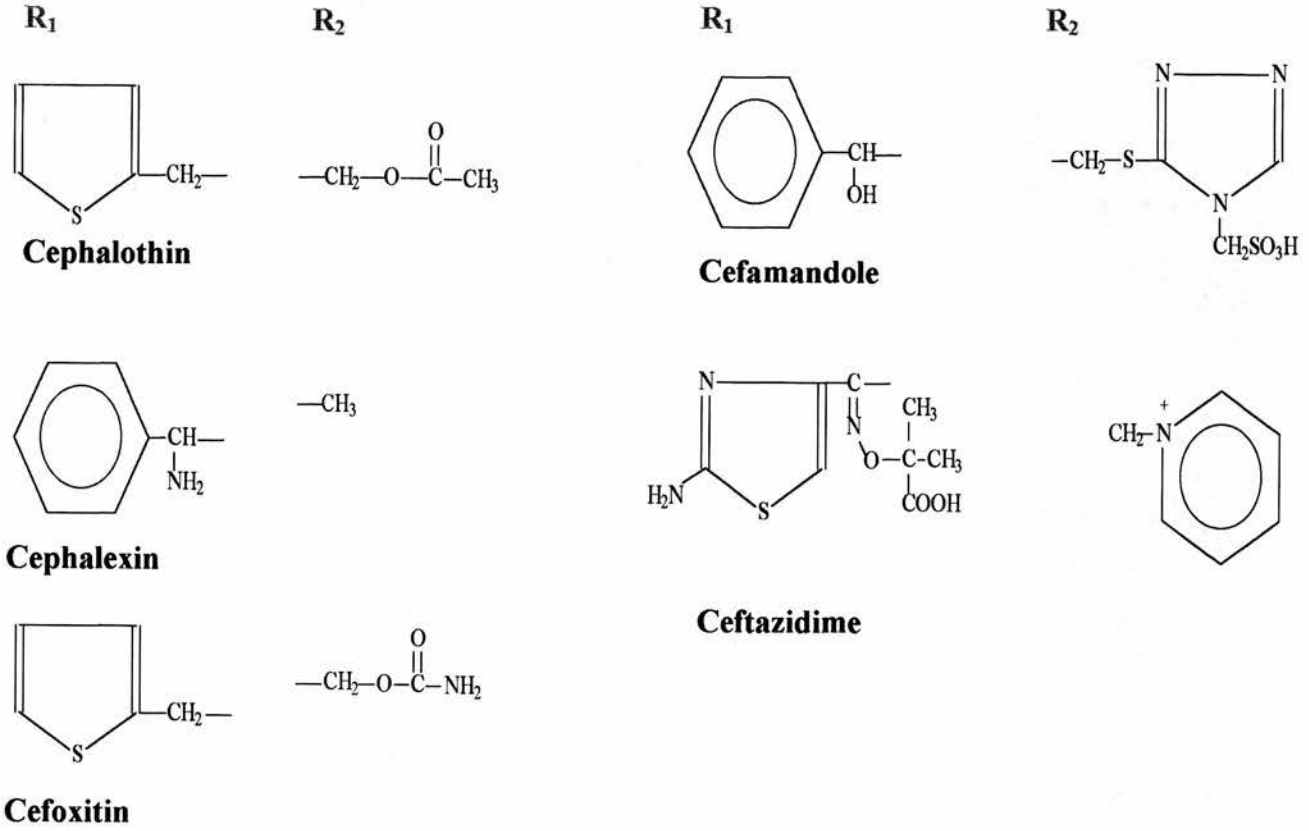
Unlike the 6-APA core of penicillins the 7-ACA nucleus of cephalosporins can be modified with two different side chains $R_1$ and $R_2$ (Figure 1.4). As with the penicillins modification of the basic structure has produced a variety of compounds with differences in spectrum of activity and pharmacokinetics. The cephamycins such as cefoxitin, which was initially derived from *Streptomyces lactamdurams*, are also generally considered along with the 'true' cephalosporins (Oniski *et al.*, 1974). Cephamycins are similar in structure to the cephalosporins except they are substituted at C-7 with a 7-α-methoxy group, which enhances β-lactamase stability. Figure 1.6 gives examples of the structural formulas of the $R_1$ and $R_2$ cephalosporin side chains. The substituent at the $R_1$ position generally affects antibacterial activity and β-lactamase stability whereas, position $R_2$ affects pharmacokinetic behaviour and metabolic stability.

Cephalosporins are traditionally classified according to generations that are based on the date they were introduced clinically and on their antibacterial properties. The

first generation agents have a narrower spectrum and are more active against Gram-positive bacteria than subsequent cephalosporin generations. The second-generation cephalosporins demonstrate an improved anti-Gram-negative spectrum of activity, which in some cases includes activity against anaerobes (for example the cephamycins cefoxitin, cefotetan and moxalactam). The greatest anti-Gram-negative activity has, however, been achieved with the third and newly described fourth generation agents. Representatives of the cephalosporin generations along with routes of administration are listed in Table 1.2.

The oxyaminothiazolyl group, that can be present at the $R_1$ position, enhances Gram-negative activity it is incorporated into most third and fourth generation cephalosporins (see $R_1$ side chain of ceftazidime – Figure 1.6). The oxyaminothiazolyl group blocks the access of β-lactamases to the β-lactam ring. Attaching an acidic moiety on the $R_1$ side chain (as in ceftazidime and moxalactam) increases cephalosporin activity against *P. aeruginosa* (O'Callaghan, 1979). The presence of a bulky side chain at the $R_2$ position markedly impairs oral absorption; orally administered cephalosporins (such as cephalexin) generally have a simple methyl group at this position (Figure 1.6).

**Figure 1.6 Examples of $R_1$ and $R_2$ cephalosporin side chains**



## Table 1.2 Classification of the cephalosporins

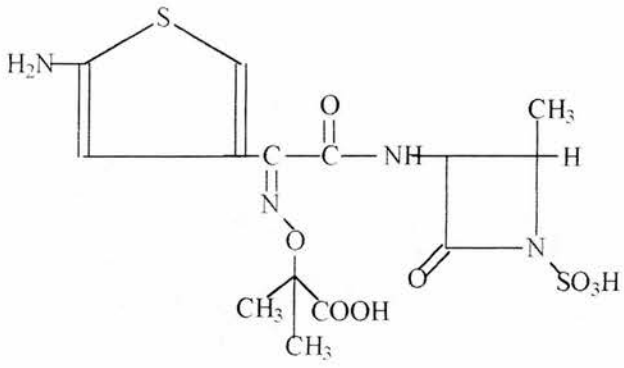
| First generation | Second generation | Third generation | Fourth generation |
|---|---|---|---|
| Cephalothin (P) | Cefamandole (P) | Cefoperazone (P) | Cefepime (P) |
| Cephaloridine (P) | Cefotetan (P) | Cefotaxime (P) | Cefpirome (P) |
| Cefazolin (P) | Cefoxitin (P) | Ceftazidime (P) | |
| Cephalexin (O) | | Ceftriaxone (P) | |
| Cefaclor (O) | | Moxalactam (P) | |

O – oral administration, P – parenteral administration.

### 1.5.3 Monobactams

Monobactams were discovered as naturally occurring compounds in Gram-negative soil bacteria (Sykes and Bonner, 1985). The weak antibacterial activity of these natural single-ringed substances led to the development of synthetic monobactams. Aztreonam was subsequently made available for clinical use in the 1980s (Figure 1.7). Aztreonam has no affinity for the PBPs of Gram-positive bacteria or anaerobic organisms and is considered a narrow-spectrum antibiotic, with its activity confined to Gram-negative bacteria including *P. aeruginosa* (Brewer and Hellinger, 1991). Aztreonam has the oxyaminothiazolyl side chain (at $R_1$) which is present in many third and fourth generation cephalosporins. Aztreonam is administered parenterally, oral absorption being poor (Brewer and Hellinger, 1991).

**Figure 1.7 Structure of aztreonam**




### 1.5.4 Carbapenems

The carbapenems have the broadest antibacterial spectrum of any currently available β-lactam and are active against Gram-positive and Gram-negative pathogens, aerobes and anaerobes. Thienamycin, produced by *Streptomyces cattleya*, was the first natural carbapenem to be discovered in the mid 1970s, however, it was found to be chemically unstable (Figure 1.8) (Barza, 1985; Moellering *et al.*, 1989). Imipenem (N-formimidoyl-thienamycin) is a semi-synthetic derivative of thienamycin and was introduced into the clinical setting in the 1980s (Figure 1.8). Finding new carbapenems has been relatively difficult because of their metabolic and chemical instability, together with poor semi-synthetic yields (Barza, 1985). The carbapenem choice has been extended by the addition of meropenem (Figure 1.8), and panipenem, the latter is available only in Japan (Edwards, 1995). These agents are all parenterally administered.

The carbapenems have high affinity for PBPs in the majority of bacteria the exceptions are methicillin-resistant *S. aureus* (MRSA) and *Enterococcus faecium*. PBP2 is the primary target of both meropenem and imipenem. Binding of the carbapenems to PBP2 is an advantage because there are relatively fewer molecules of PBP2 compared with other essential PBPs; therefore, inhibition of PBP2 can be achieved at lower antibiotic concentration (Williams *et al.*, 1986). Although both imipenem and meropenem bind to PBP2, there are some differences between imipenem and meropenem with regard to the additional PBPs that they can bind to; for example, meropenem is thought to have greater potency against *P. aeruginosa* because unlike imipenem it has affinity for both PBP2 and PBP3 (Edwards and Turner 1995).

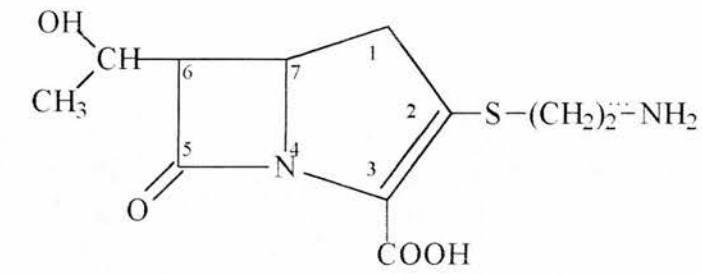
Both imipenem and meropenem have low molecular weights, are zwitterions, and have a hydrophilic structure; these properties account for their ability to penetrate bacteria readily (Moellering *et al.*, 1989). At position C-6 the carbapenems have a side chain in the *trans* conformation, this conformation is different from all other β-lactams, which have a substituent at the C-6 or C-7 that is in the *cis* position (Figure 1.8). The conformation of this side chain is associated with the stability of the carbapenems to many of the clinically relevant β-lactamases (Livermore and Williams, 1996).

The major differences between imipenem and meropenem are pharmacological rather than microbiological. Imipenem (and panipenem) unlike meropenem is destroyed by a renal enzyme dehydropeptidase I (DHP-I) to an inactive but

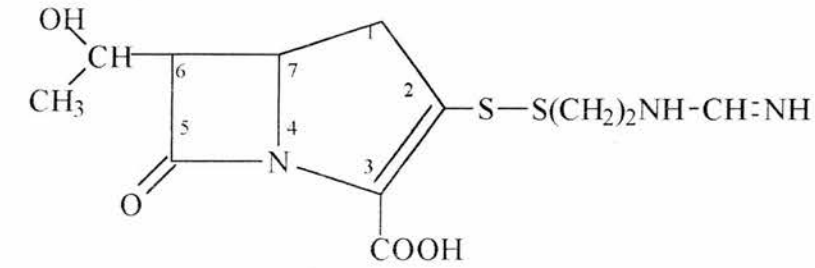
nephrotoxic metabolite. Imipenem is administered with cilastatin which inhibits DHP-1, preserving imipenem and preventing renal toxicity (Moellering *et al.*, 1989). The imipenem C-2 side chain is responsible for susceptibility to hydrolysis by DHP-1, and a methyl group at C-1 has been associated with the enhanced resistance of meropenem to DHP-1 (Moellering *et al.*, 1989). Imipenem has the potential for CNS disturbances, meropenem appears not to cause seizures and is suitable for the treatment of meningitis (Wiseman *et al.*, 1995).

Although, the first carbapenem was clinically introduced at a similar time to the 3GCs and aztreonam, carbapenem resistance has been developing much more slowly compared with these agents. Carbapenem-resistant bacteria are however beginning to be described with increasing frequency, especially in geographical areas where carbapenems have been widely employed. Moreover, an expanding number of different carbapenem hydrolysing β-lactamases can be identified from these carbapenem resistant isolates (discussed in Sections 1.11 - 1.12), especially in geographical areas were carbapenems have been employed widely. Therefore, as with all other β-lactam classes, the β-lactamases look set to seriously damage the therapeutic reliability of these agents.

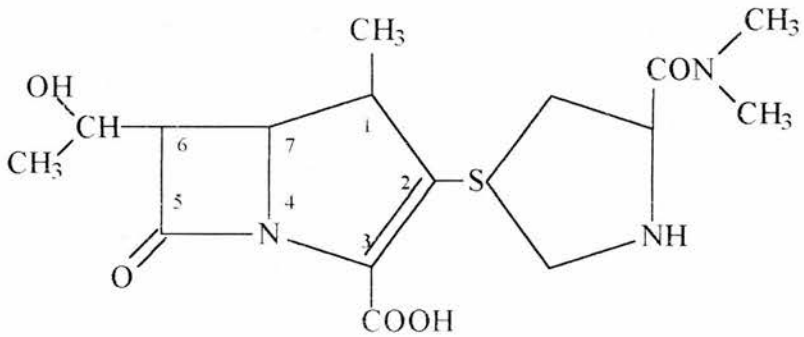
**Figure 1.8 Chemical structure of thienamycin, imipenem and meropenem**




**Thienamycin**




**Imipenem**




**Meropenem**

### 1.5.5 β-Lactamase inhibitors

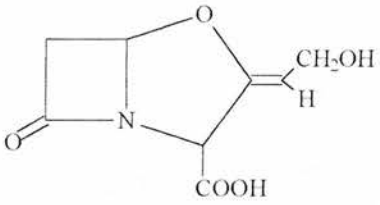
Two strategies have been adopted in the battle to overcome β-lactamase-mediated resistance: (i) development of β-lactam antibiotics stable to β-lactamase hydrolysis; (ii) combining a broad-spectrum β-lactamase labile β-lactam with a protective β-lactamase inhibitor. Clavulanic acid (produced by *Streptomyces clavuligerus*) was the first β-lactamase inhibitor to be described in the literature and to be applied

clinically (Brown *et al.*, 1976). The discovery of clavulanic acid stimulated further work on β-lactamase inhibitors and led to the clinical introduction of sulbactam and tazobactam which are penicillanic acid sulphones (English *et al.*, 1978; Arnoff *et al.*, 1984) (see Figure 1.9 for chemical structures). All three compounds generally have weak antibacterial activity and their advantage resides in their ability to inhibit certain β-lactamases (see Section 1.6).

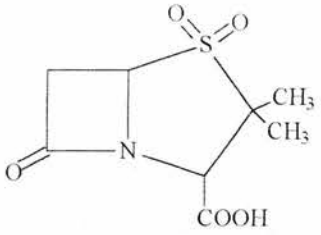
The three clinically available β-lactamase inhibitors are all classed as irreversible "suicide" inhibitors, that is they are molecules that bind initially at the β-lactamase active site where they are then, through the catalytic action of the β-lactamase itself, converted into an activator that renders the β-lactamase inactive (Bush, 1988). Amoxycillin/clavulanate, ticarcillin/clavulanate, ampicillin/sulbactam and piperacillin/ tazobactam are examples of the commercially available β-lactam/β-lactamase inhibitor preparations.

The main limit to the β-lactam/inhibitor combinations is that they are only effective against certain β-lactamases, namely molecular class A and some molecular class D β-lactamases that are described below (Livermore, 1993a).

**Figure 1.9 Structure of two suicide β-lactamase inhibitors**




**Clavulanic acid**




**Sulbactam**

## 1.6 Classification of the β-lactamases

### 1.6.1 The Ambler classification scheme

There are two commonly cited β-lactamase classification systems. The classification scheme initiated by Ambler (1980) is based on the amino acid sequence around the β-lactamase active site and now consists of four classes A, B, C and D (Jaurin and Grundström, 1991; Huovinen *et al.*, 1988). Ambler classes A, C, D are the serine β-lactamases whereas class B enzymes are metallo-β-lactamases. The primary structure and catalytic mechanisms of metallo-β-lactamases and serine β-lactamases are completely different and are discussed below (Section 1.7 – 1.8). A fifth molecular class (E) has been proposed for the metallo-β-lactamases from *Stenotrophomonas maltophilia* (Bicknall *et al.*, 1985), however, these enzymes are generally referred to as class B β-lactamases.

### 1.6.2 The Bush classification scheme

The Bush classification scheme is the most complete system so far to be devised for β-lactamases and correlates substrate and inhibitor profiles with molecular class (Bush *et al.*, 1995). This classification system does not use the location of the gene encoding the β-lactamase as a classification factor, and this reflects the appreciation of the mobility of β-lactamase genes, with chromosomal genes finding their way to plasmids and *vice-versa*. This classification scheme has divided β-lactamases into four main functional groups (Table 1.3).

**Table 1.3. The four main functional groups of Bush's classification scheme (adapted from Bush *et al.*, 1995)**

| Group | Enzyme type | Molecular class | Inhibition by clavulanic acid | Inhibition by EDTA | Examples |
|---|---|---|---|---|---|
| 1 | Cephalosporinase | C | No | No | AmpC, MIR-1 |
| 2a | Penicillinase | A | Yes | No | *S. aureus* PC1, LEN-1 |
| 2b | Broad-spectrum | A | Yes | No | TEM-1, TEM-2, SHV-1 |
| 2be | Extended-spectrum | A | Yes | No | TEM-3, SHV-2 |
| 2br | Inhibitor resistant | A | Diminished | No | TEM-30, TRC-1 |
| 2c | Carbenicillinase | A | Yes | No | PSE-1, CARB-3, BRO-1 |
| 2d | Cloxacillinase | D or A | Yes | No | OXA-1 |
| 2e | Cephalosporinase | A | Yes | No | L2, *B. fragilis* CepA |
| 2f | Carbapenemase | A | Yes | No | Sme-1, Nmc-A, IMI-1 |
| 3* | Metalloenzyme | B | No | Yes | L1, IMP-1, CphA |
| 4 | Penicillinase | | No | No | *B. cepacia* LCR-1 |

* Rasmussen and Bush (1997) have proposed a subgroup of group 3 metallo-β-lactamases based on functional characteristics (Section 1.11)

### 1.6.2.1 Bush group 1

The majority of Gram-negatives produce chromosomally encoded group 1 cephalosporin-hydrolysing β-lactamases, including most enterobacteria, *P. aeruginosa*, *Acinetobacter* spp. and *Aeromonas* spp. (Bush *et al.*, 1995). Group 1 enzymes from different species resemble one another and they are often referred to as AmpC-type β-lactamases, however, the expression of these enzyme is known to vary between species (Livermore, 1987). Clavulanic acid, tazobactam and sulbactam do not demonstrate good activity against this group of enzymes and therefore, they cannot be employed clinically to overcome this class of β-lactamases (Zhou *et al.*, 1993). In vitro, a compound known as BRL42715 has been shown to be a much more effective inhibitor of the Bush group 1, molecular class C cephalosporinases when compared to clavulanic acid, tazobactam and sulbactam; it additionally has the ability to inhibit molecular class A and class D β-lactamases (Coleman *et al.*, 1989).

### 1.6.2.2 Bush group 2

Members of the group 2 β-lactamases have a diverse substrate profile and fall into six subgroups based on their preferential hydrolysis of penicillins, cephalosporins, oxyamino-cephalosporins, cloxacillin, carbenicillin or carbapenems. With the exception of the 2br subgroup these enzymes are sensitive to clavulanic acid, sulbactam and tazobactam. This group contains some of the most common β-lactamases including the staphylococcal penicillinase, TEM-1 and SHV-1 and as with many of the group 2 β-lactamases these are found on plasmids. The group 2f

carbapenemases (Section 1.12.1) and group 2br TEM-derived β-lactamases with diminished sensitivity to clavulanate are the most recent subgroups.

### 1.6.2.3 Bush group 3

This functional group contains the zinc requiring metallo-β-lactamases; in vitro these enzymes are inhibited by metal-ion chelating agents such as EDTA and also the amino acid modifier *p*-chloromercuribenzoate (pCMB), but not by serine–directed inhibitors. Payne *et al.*, (1997) have recently reported the inhibition of some metallo-β-lactamases by mercaptoacetic acid thiol ester derivatives. The metallo-β-lactamases hydrolyse a broad-spectrum of substrates, most notably the carbapenems. This group of enzymes will be discussed later in detail (Section 1.11).

### 1.6.2.4 Bush group 4

This group consists of a small number of penicillinases not well inhibited by clavulanic acid. (Bush *et al.*, 1995).

## 1.7 Mechanisms of β-lactam hydrolysis by serine β-lactamases

Most β-lactamases are serine-based and belong to a superfamily of serine-proteases, that includes the PBPs. The reaction catalysed by β-lactamases and PBPs is essentially the same:

$$E + S \overset{1}{\leftrightarrow} ES \overset{2}{\rightarrow} E\text{-}S \overset{3}{\rightarrow} E + P$$

*E* represents the enzyme (either β-lactamase or PBP), *S* represents the β-lactam substrate, *ES* is a non-covalent intermediate and *E-S* represents the formation of a covalent acyl-enzyme intermediate. The main difference between the reaction catalysed by β-lactamases and the reaction catalysed by PBPs is the rate of the deacylation step (reaction 3). The deacylation step proceeds rapidly with β-lactamases but with PBPs this reaction is very slow or does not happen at all. β-Lactamases, therefore, can hydrolyse the β-lactam to form an inactive antibacterial product whereas the PBPs are inactivated by the β-lactam as result of the long-lived acyl-enzyme intermediate (Sanders and Sanders, 1992).

The serine β-lactamases (Ambler classes- A, C, and D) possess at least three highly conserved functional and structural elements at their active site, that supply most of the groups involved in catalysis. These elements are commonly referred to as the serine-X-X-lysine (SXXK –X is any amino acid), serine-aspartic acid-asparagine (SDN) and lysine-threonine-glycine (KTG) motifs (Bush, 1997). The serine residue

of the SXXK element is responsible for the attack on the carbonyl C of the β-lactam ring and for class A (and class D) enzymes this residue is designated serine-70, this is based on a standard numbering scheme proposed by Ambler *et al.*, (1991). In class C β-lactamases the active site serine is at position 64 (Medeiros, 1997).

The SXXK, SDN and KTG motifs are also also conserved in PBPs (Table 1.4), thus the serine β-lactamases and PBPs are thought to share a common evolutionary ancestor (Frére, 1995). Additionally, x-ray crystallography has shown that the class A and class C β-lactamases closely resemble the R61 D,D-peptidase from a *Streptomyces* species (Frére, 1995).

**Table 1.4 Conserved elements of serine β-lactamases involved in catalysis (adapted from Bush, 1997).**

| β-lactam-interactive protein | Element 1 | Element 2 | Element 3 |
|---|---|---|---|
| Class A β-lactamase | 70[a]<br>S-X-X-K[b] | 130<br>S-D-N<br>S-D-S | 234<br>K-T-G<br>K-S-G<br>R-S-G<br>R-T-G |
| Class C β-lactamase | 64<br>S-X-X-K | 150<br>Y-A-N | 314<br>K-T-G |
| Class D β-lactamase | 70<br>S-X-X-K | 144<br>Y-G-N | 214<br>K-T-G |
| R61 D.D-peptidase[c] | 62<br>S-X-X-K | 159<br>Y-S-N | 298<br>H-T-G |

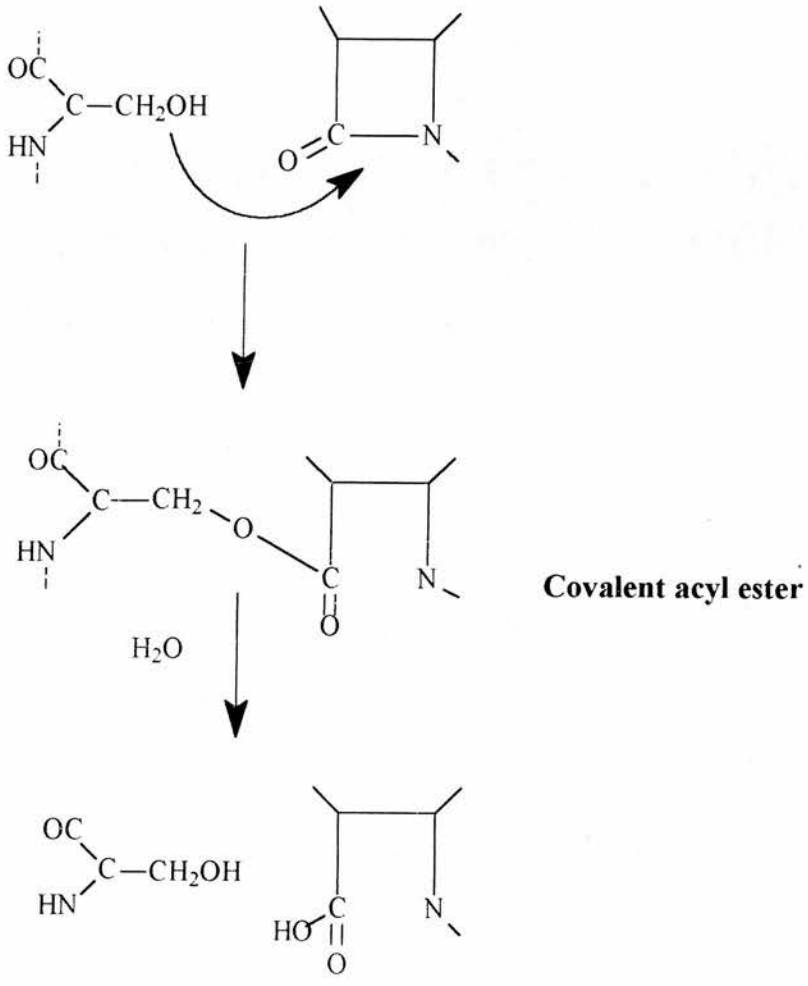
[a] Amino acid numbering according to Ambler *et al.*, (1991).

[b] X - any amino acid

[c] PBP of *Streptomyces* species R61 (Kelly and Kuzin, 1995)

In the class A β-lactamase mechanism of action lysine (K) 73 (Table 1.4) acts as a general base and deprotonates the hydroxyl group of serine (S) 70 (Table 1.4). This proton is then donated to serine 130 (Table 1.4) which in turn donates the proton to the nitrogen atom of the β–lactam amide bond (Stryndka *et al.*, 1992), this results in the fission of the amide bond (Figure 1.10). Class C β–lactamases have a tyrosine residue at position 150 which corresponds to serine 130 in class A β–lactamases and has been shown to be important for the hydrolysis of substrates but is not essential for the activity of the enzyme (Dubus *et al.*, 1994). The acyl-enzymes complex is bulky and only a small molecule such as water can gain access to the carbon atom to release the enzyme (Figure 1.10). In class A β–lactamases a glutamate residue at position 166 has been proposed as a possible residue to act as a general base and deprotonate the water molecule and donate the proton to the oxygen atom causing the bond between the enzyme and inactive β–lactam to break (Figure1.10).

**Figure 1.10 Action of a serine β-lactamase**



## 1.8 Mechanism of β-lactam hydrolysis by metallo-β-lactamases

The mechanism of β-lactam hydrolysis by class B metallo-β-lactamases is completely different from that of the serine β-lactamases and is known to involve a nucleophilic attack on the carbonyl carbon of the β-lactam ring by a water molecule polarized by a zinc ion. Conserved catalytic structural motifs are also shared amongst the small number of metallo-β-lactamases, although there is considerable sequence divergence among different members (Figure 1.11).

The crystal structures of three metallo-β-lactamases have been resolved these are β-lactamase II from *Bacillus cereus* 569/H, CcrA3 from *Bacteroides fragilis* QMCN3 and L1 *from S. maltophilia* IID1275 (Carfi *et al.*, 1995; Concha *et al.*, 1996; Carfi *et al.*, 1998*a*; Carfi *et al.*, 1998*b*; Ullah *et al.*, 1998). These enzymes all exhibit an αβ/βα fold that is unique to the metallo-β-lactamases (Ullah *et al*, 1998). The metallo-β-lactamase polypeptide chain is divided into two domains with the central β-sheets obeying an approximate two-fold symmetry relationship. The metallo-β-lactamase site is located in a groove between the two β-sheets which is associated with a number of loops which bear the residues linked with zinc binding (Ullah *et al.*, 1998). All protein zinc ligands are strictly conserved in all class B enzymes with the exceptions of two mutations –a histidine to asparagine change in the *Aeromonas* metallo-β-lactamases and a cysteine to aspartate change in the *S. maltophilia* metallo-β-lactamase (Ullah *et al.*, 1998).

**Figure 1.11 Sequence alignment for some molecular class B metallo-β-lactamases (adapted from Carfi *et al.*, 1998a)**

```
CcrA3    1 ----AQKSVKISD--DISITQLSDKVYTYVSLAEIEGWGMVPSNGMIVINNHQA  48
BlII     1 SQKVEKTVIK-NETGTISISQLNKNVWVHTELGSFNGEA-VPSNGLVLNTSKGL  52
IMP-1    1 ----------AESLPDLKIEKLDEGVYVHTSFEEVNGWGVVPKHGLVVLVNAEA  44
CphA     1 ------------------AAGMSLTQVSGPVYVVEDNYY-VQENSMVYFGAKGV  35
L1       1 ----------------VDASWLQPMAPLQIADHTWQIGT-EDLTALLVQTPDGA  37

CcrA3   49 ALLDTPINDAQTEMLVNWVTDSLHA-KVTTFIPNHWHGDCIGGLGYLQRK-GVQ 100
BlII    53 VLVDSSWDDKLTKELIEMVEKKFQK-RVTDVIITHAHADRIGGIKTLKER-GIK 104
IMP-1   45 YLIDTPFTAKDTEKLVTWFVERGY--KIKGSISSHFHSDSTGGIEWLNSR-SIP  95
CphA    36 TVVGATWTPDTARELHKLIKRVSRK-PVLEVINTNYHTDRAGGNAYWKSI-GAK  87
L1      38 VLLDGGMPQMASHLLDNMKARGVTPRDLRLILLSHAHADHAGPVAELKRRTGAK  91

CcrA3  101 SYANQMTIDLAKEK----------------GLPVPEHGFTDSLTVSLDGMPLQC 138
BlII   105 AHSTALTAELAKKN----------------GYEEPLGDLQTVTNLKFGNMKVET 142
IMP-1   96 TYASELTNELLKKD----------------GKVQATNSFS-GVNYWLVKNKIEV 132
CphA    88 VVSTRQTRDLMKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHDGDFTLQEGKVRA 141
L1      92 VAANAESAVLLARG----GSDDLHFGDGITYPPANADRIVMDGEVITVGGIVFT 141

CcrA3  139 YYLGGGHATDNIVVWLPTE------NILFGGCMLKDNQATSIGNISD-ADVTAW 185
BlII   143 FYPGKGHTEDNIVVWLPQY------NILVGGCLVKSTSAKDLGNVAD-AYVNEW 189
IMP-1  133 FYPGPGHTPDNVVVWLPER------KILFGGCFIKP---YGLGNLGD-ANIEAW 176
CphA   142 FYAGPAHTPDGIFVYFPDE------QVLYGNCILK----EKLGNLSF-ADVKAY 184
L1     142 AHFMAGHTPGSTAWTWTDTRNGKPV-RIAYADSLSAPGYQLQGNPRYPHLIEDY 194

CcrA3  185 PKTLDKVKAKFPSARYVVPGHGD-YGGTELIEHTKQIVNQYIESTSKP       232
BlII   190 STSIENVLKRYRNINAVVPGHGE-VGDKGLLLHTLDLLK                 227
IMP-1  177 PKSAKLLKSKYGKAKLVVPSHSE-VGDASLLKLTLEQAVKGLNESKKPSKPSN  228
CphA   185 PQTLERLKAMKLPIKTVIGGHDSPLHGPELIDHYEALIKAAPQS           228
L1     195 RRSFATVRAL--PCDVLLTPHPG-ASNWDYAAGARAGAKALTCKAYADAAEQKF 246

CcrA3
BlII
IMP-1
CphA
L1     247 DGQLAKETAGAR                                          257
```

CcrA3 – *B. fragilis* metallo-β-lactamase, BlII - β-lactamase II from *B. cereus*, IMP-1 found in *S. marcescens*, CphA – *Aeromonas hydrophila* metallo-β-lactamase, L1 – *S. maltophilia* metallo-β-lactamase.

Amino acids in red type indicate zinc-binding ligands.

Amino acids in blue type indicate zinc-binding ligand mutations in the *A. hydrophila* CphA metallo-β-lactamase and the *S. maltophilia* L1 metallo-β-lactamase.

Two zinc binding sites are identified in the metallo-β-lactamases although their requirement for zinc has been shown to differ from enzyme to enzyme. For example the CphA metallo-β-lactamase from *A. hydrophila* AE036 is known to function optimally in a mono zinc form, when a second zinc site is occupied enzyme activity was found to be inhibited (Valladares *et al.*, 1997). Conversely, the metallo-β-lactamases from *B. fragilis* and *S. maltophilia* require two $Zn^{2}$+ ions for optimal activity. The β-lactamase II enzyme from *B. cereus* was thought to be active in a mono zinc form (Carfi *et al.*, 1995), however, more recently it has been found bind a second zinc (highly pH dependent) which increases its activity (Carfi *et al.*, 1998*b*).

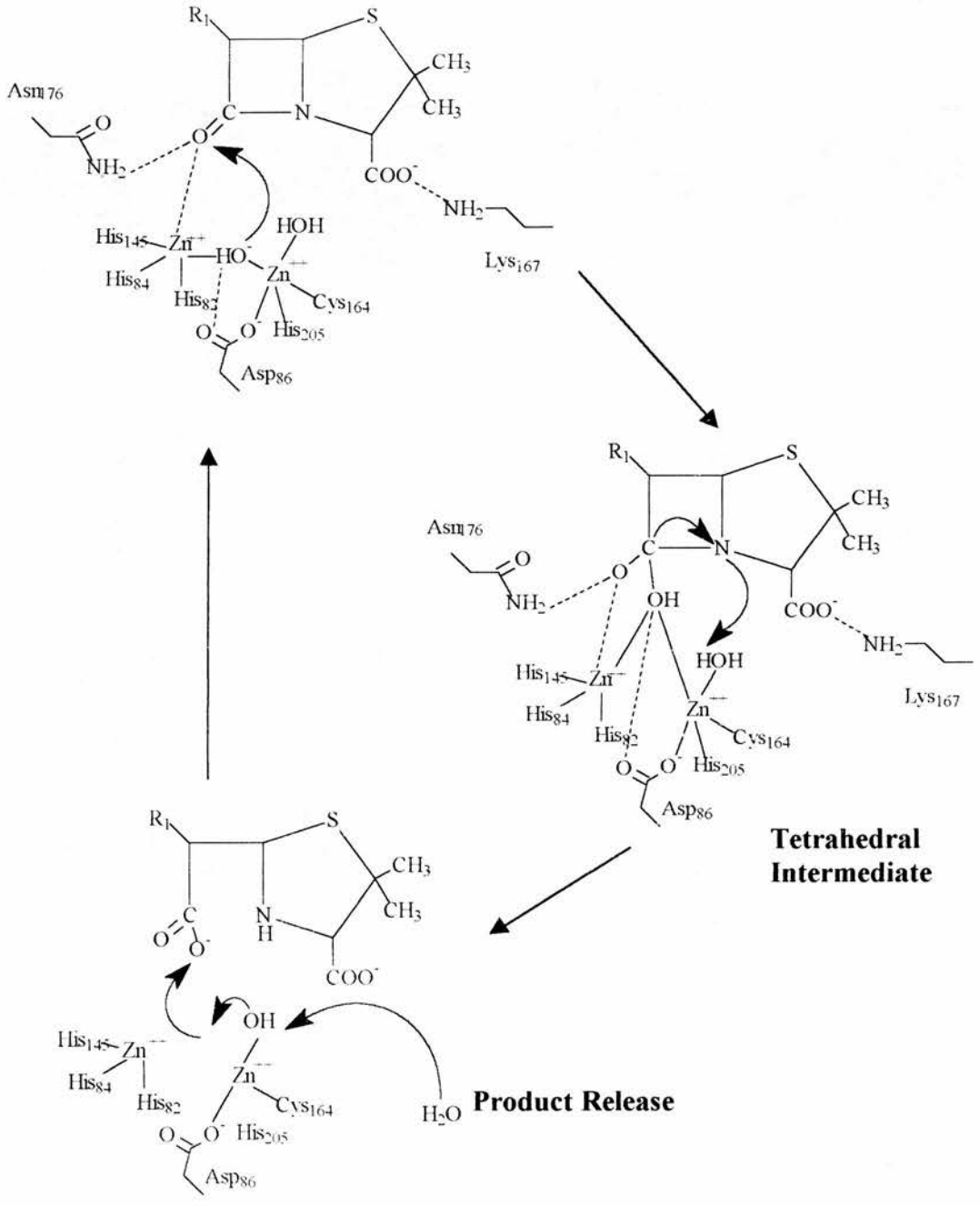
In the *B. fragilis* CcrA3 model (proposed by Concha *et al.*, 1996) a zinc ion (Zn1) is coordinated by three histidines (H82, H84 and H145 – CcrA3 numbering, Figure 1.11 and 1.12) and to a hydroxide that is also shared by the second zinc ion (Zn2). The second zinc (Zn2) is coordinated in a trigonal bipyramidal geometry to an aspartate, cysteine, histidine triad (D86, C164 and H205), the shared hydroxide and to an apical water molecule. Substrate binding involves the interaction of the β-lactam carbonyl oxygen with Zn1 and the β-lactam carboxylate group with a lysine residue (K167). The shared hydroxide makes a nucleophilic attack on the carbonyl carbon to form a tetrahedral intermediate. The apical water is then thought to transfer a proton to the nitrogen of the cleaved β-lactam (Concha *et al.*, 1996; 1997).

A mechanism of action has been proposed for the single zinc form of the *B. cereus* β-lactamase II enzyme (described by Carfi *et al.*, 1995), however, as mentioned above, this enzyme is now known to function optimally when a second zinc is bound (Carfi

*et al.*, 1998b). The first zinc (Zn1) is bound in a similar fashion to the CcrA3 enzyme by the equivalent three histidines (H86, H88, H149 - β-lactamase II numbering, Figure 1.11) and one water molecule, however, the second zinc is coordinated by one histidine, one cysteine, one aspartate (H210, C168, D90) and one unknown molecule (possibly a carbonate ion).

In the *S. maltophilia* L1 metallo-β-lactamase model there are also some differences in the residues involved in catalysis compared with the CcrA3 mechanism (Ullah *et al.*, 1998). Zn1 is coordinated by the conserved histidine triad at positions 72, 74 and 148 (L1 numbering Figure1.11) and a shared hydroxide. The pentavalent Zn2 is ligated to an aspartate, histidine, histidine triad (D76, H77 and H213, L1 numbering-Figure 1.11) instead of the aspartate, cysteine, histidine triad in CcrA3 (the cysteine residue is absent in the L1 sequence), the shared hydroxide and apical water are, however, equivalent to those found in CcrA3. As in the *B. fragilis* model the shared hydroxide functions as a nucleophile. The carboxylate moiety of the β-lactam is however hydrogen-bonded to a serine residue (S175) as opposed to the lysine (K167, CcrA3 numbering) residue of CcrA3, it should be noted that lysine is conserved in this position in all metallo-β-lactamases except for L1 (Ullah *et al.*, 1998).

**Figure 1.12 Proposed catalytic mechanism of the CcrA3 metallo-β-lactamase (Concha *et al.*, 1996)**



## 1.9 The regulation of β-lactamase expression

The expression of a β-lactamase is either inducible or constitutive. Induction can be defined as the transient switching-on of β-lactamase synthesis and occurs when bacterial cells are exposed to certain β-lactams, otherwise known as inducing agents. Conversely, constitutive β-lactam production is independent of an inducer being present and is the mode of expression of many of the Gram-negative plasmid-mediated β-lactamases. Changes in the level of β-lactamase expression can arise from an increase in the number of gene copies encoding β-lactamase, alteration in the promoter or attenuator regions affecting gene transcription, or from changes in the regulation of inducible β-lactamase expression (Sanders and Sanders, 1992).

### 1.9.1 Model for the regulation of AmpC β-lactamases in *Citrobacter freundii* and *Enterobacter cloacae*

Various models have been proposed to explain how the presence of a β-lactam antibiotic may lead to increased transcription of genes encoding β-lactamases. One widely accepted model of β-lactamase induction is based on the regulation of AmpC β-lactamases in *E. cloacae* and *C. freundii* and appears to be interlinked with the process of bacterial cell wall assembly and disassembly (Jacobs *et al.*, 1994).

In the proposed model cell wall fragments are released as a result of peptidoglycan breakdown triggered by β-lactam inhibition of PBP activities. A protein known as

AmpG serves as a permease and transports a large cell wall muropeptide into the cytoplasm and this contains anhydro muramic acid (Normark *et al.*, 1990). An AmpD protein (a muramyl-peptide amidase) specifically recognises substrates that contain anhydro muramic acid and can cleave the large muropeptide for the recycling of the cell wall (Höltje *et al.*, 1994). The increased breakdown of peptidoglycan that occurs as a result of β-lactam action leads to the AmpD enzyme becoming overloaded and thus large muropeptides are able to escape the action of AmpD and instead activate AmpR. AmpR is a DNA binding regulatory protein that can bind to *ampC* and increase its transcription and results in β-lactamase synthesis. Mutation of AmpD can lead to the modification of the muramyl-peptide amidase or to its complete inactivation. Complete loss of AmpD results in constant hyperproduction of β-lactamase and strains that have lost AmpD function are known as stably derepressed mutants. Derepressed mutants occur naturally in inducible populations usually at a frequency of $10^{-7}$ (Weidmann, 1986).

## 1.10 Mechanisms of carbapenem resistance

Soon after the introduction of the first carbapenem, imipenem, it became apparent that resistance could develop to this β-lactam. As with all other β-lactam classes carbapenem resistance can result from one or a combination of mechanisms including the association of outer membrane impermeability with a slow carbapenem hydrolysing β-lactamase, a PBP alteration, or the production of an efficient carbapenem-hydrolysing β-lactamase.

### 1.10.1 Association of outer membrane impermeability and the production of a β-lactamase

#### 1.10.1.1 Association of outer membrane permeability change and a Bush group 1 cephalosporinase in *P. aeruginosa*

*P. aeruginosa* strains were amongst the earliest imipenem-resistant bacteria to be isolated, often from patients suffering from either cystic fibrosis or nosocomial respiratory tract infections (Acar, 1985; Pedersen *et al.*, 1985). In the 1970s *P. aeruginosa* infections were identified as a serious problem resulting from the microorganisms high intrinsic resistance to a number of antibiotics and the production of antibiotic modifying enzymes (Bryan, 1979). It has been assumed that poor antibiotic permeability across the outer membrane is attributable to a limited number of small-sized diffusion pores that exclude many antibiotics; imipenem overcame this difficulty by penetrating through the D2 porin (Yoshihara and Nakae, 1989). However, imipenem resistance was subsequently found to be associated with the loss of this outer membrane protein (OMP) D2 (Buscher *et al.*, 1987; Lynch *et al.*, 1987). D2 is a trimer that forms a selective channel for low molecular weight zwitterionic compounds such as imipenem (Yoshihara *et al.*, 1991; Gotoh and Nishino, 1990).

Although impermeability has a major role to play in imipenem resistance in *P. aeruginosa* it has been recognised that it is the combination of the *P. aeruginosa* chromosomal cephalosporinase and a change in permeability that results in a

resistant phenotype (Livermore, 1992a). Imipenem hydrolysis has been detected with several Bush group 1 cephalosporinases, however, imipenem hydrolysis occurs at a very slow rate (Livermore and Yang, 1987). The importance of these enzymes in mediating resistance to the carbapenems is only apparent in a less permeable background. The slowed rate of entry of imipenem through the outer membrane, permits the large amount of slow acting β-lactamase in the periplasmic space to inactivate imipenem before it reaches its target site on the cytoplasmic membrane (Lindberg *et al.*, 1987). Laboratory mutants of *P. aeruginosa* that lack D2 and β-lactamase expression have been shown to be almost as susceptible to imipenem as mutants which expressed their full complement of D2 but no β-lactamase and this indicates the need for both enzyme and permeability to produce resistance (Livermore, 1992a). High level production of the cephalosporinase is not an essential requirement and it has been shown that the cephalosporinase of *P. aeruginosa* is part of the imipenem resistance mechanism whether it is inducible or derepressed (Zhou *et al.*, 1993).

Meropenem resistance appears to be little affected by the type of β-lactamase expression in *P. aeruginosa* mutants with or without D2 (Livermore, 1992a) and is instead associated with a MexA-MexB-OprM mediated efflux system (Masuda and Ohya, 1992, Chen *et al.*, 1995). This efflux system confers broad-spectrum cross-resistance to β-lactams, quinolones, tetracyclines and chloramphenicol in *P. aeruginosa* when it is over expressed, conversely inactivation of the efflux pump results in a hypersensitive phenotype (Li *et al.*, 1995). Imipenem is not affected by the MexA-MexB-OprM efflux mechanism and it has been suggested that a possible

reason for this is that either the efflux pump does not recognise imipenem, or imipenem enters the cell so rapidly that the pump is overwhelmed (Livermore, 1996).

### 1.10.1.2 Association of an outer membrane permeability change and a Bush group 1 cephalosporinase in enterobacteria

To date, carbapenem resistance has been rarely described in members of the family *Enterobacteriaceae*. One mechanism of carbapenem resistance that is described in enterobacteria is the production of a plasmid or chromosomally encoded β-lactamase that efficiently hydrolyses the carbapenems (see Section 1.11). Another mechanism of carbapenem resistance in the *Enterobacteriaceae* is a reduction in outer membrane permeability that is generally coupled with high-level cephalosporinase production. This has been reported in *Enterobacter aerogenes* (Chow and Shlaes, 1991; De Champs *et al.*, 1993; Erhard *et al.*, 1993; Hopkins and Towner, 1990; Tzouvelekis *et al.*, 1992), *E. cloacae* (Bush *et al.*, 1985; Lee *et al.*, 1991; Raimondi *et al.*, 1991), *Providencia* (*Proteus*) *rettgerii* (Raimondi *et al.*, 1991), *Proteus mirabilis* (Mentar *et al.*, 1991), *C. freundii* (Marinardi *et al.*, 1991) and *Klebsiella pneumoniae* (Bradford *et al.*, 1997). MacKenzie *et al.* (1997) recently described a carbapenem resistant *K. pneumoniae* isolate, identified during an outbreak in Scotland, however, the resistance mechanism was different from the one described by Bradford *et al.* (1997) and involved the loss of an OMP and increased production of an SHV-type extended spectrum β-lactamase (MacKenzie *et al.*, 1997).

The emergence of carbapenem resistant species that belong to the *Enterobacteriaceae* is disturbing as these organisms are commonly encountered hospital pathogens, particularly in intensive care units, and the carbapenems are one of the few antibiotic-options left available for their treatment (Bradford *et al.*, 1997; de Gheldre *et al.*, 1997). It has been suggested that the selection of cephalosporinase overproducing strains by broad-spectrum cephalosporins might subsequently facilitate the selection of carbapenem resistant strains. Fortunately, however, at present carbapenem resistant *Enterobacteriaceae*, with decreased permeability and hyperproduction of the chromosomal cephalosporinase remain rare, and it is interesting that, in the absence of imipenem porin deficient mutants are rapidly overgrown by revertants that have normal amounts of porin (Raimondi *et al.*, 1991). Therefore, in the absence of imipenem, porin deficient mutants appear to be at a disadvantage, which may be due to the inability to accumulate certain nutrients (Livermore, 1991).

### 1.10.2 Carbapenem-resistance and PBP modification

Alterations in PBPs leading to carbapenem resistance appear to be of little importance in Gram-negative bacteria. There are, however, examples of where PBP modification has been associated with resistance, such as in imipenem-resistant *Acinetobacter baumanii* and *P. aeruginosa* (Gehrlein *et al.*, 1991; Bellido *et al.*, 1990). PBP modification and carbapenem resistance is much more important in Gram-positive species. MRSA, *E. faecium* and *Listeria monocytogenes* are three

examples of Gram-positive organisms with PBP alterations leading to imipenem resistance (Livermore, 1996; Pierre *et al.*, 1990).

### 1.10.3 Carbapenem hydrolysing β-lactamases

Carbapenem hydrolysing enzymes include some of the most recently described β-lactamases. The molecular class C, Bush group1 cephalosporinases could also be included in this group. However, the carbapenems do not represent a major substrate in their hydrolytic profile and their carbapenemase activity is only of significance when coupled with impermeability. In contrast, several other β-lactamases efficiently hydrolyse the carbapenems and can be the primary cause of resistance. The carbapenem-hydrolysing β-lactamases are often referred to as 'carbapenemases', although it should be stressed that they do not exclusively hydrolyse carbapenems nor do they confer resistance to only imipenem and or meropenem. Carbapenemases can confer resistance to other β-lactams and may hydrolyse penicillins or cephalosporins more efficiently than the carbapenems. Carbapenemases can be divided into two groups according to whether they are metallo-β-lactamases or serine β-lactamases.

## 1.11 Carbapenem-hydrolysing metallo-β-lactamases

The chromosomally encoded *B. cereus* II enzyme was the first metallo-β-lactamase to be described back in 1966. It was shown that the cephalosporinase activity of the metalloβ-lactamase II enzyme could be inhibited by EDTA, and for many years the

*B. cereus* metallo-β-lactamases were considered to be biochemical curiosities (Sabath and Abraham, 1966). Following the identification of the *B. cereus* II β-lactamase, further metallo-β-lactamases were reported in *S. maltophilia*, *Myroides odoratus* (formerly *Flavobacterium odoratum*) and *Legionella gormanii*. The clinical significance of these enzymes was debatable because these organisms were considered to be rare pathogens (Saino *et al.*, 1982; Sato *et al.*, 1986; Fujii *et al.*, 1986). Although the clinical importance of some these enzymes was under question it was, however, speculated that carbapenemases belonging to the metallo-β-lactamase category would become the major threat to the reliability of the carbapenems (Livermore, 1993b; Payne, 1993). Indeed, in the past five or six years the clinical importance of metallo-β-lactamases has begun to be realised, as an increasing number of established pathogens have been reported to produce metallo-β-lactamases that are encoded by transferable plasmids (specifically the IMP-1 and CcrA enzymes – see Sections 1.11.1.3 and 1.11.1.5) (Bandoh *et al.*, 1992; Ito *et al.*, 1995; Minami *et al.*, 1996).

Rasmussen and Bush (1997) recently proposed a new classification of the Bush group 3 metallo-β-lactamases and on the basis of functional characteristics placed these enzymes into three subgroups (Table 1.5).

## Table1.5 Functional classification of metallo-β-lactamases

| Subgroup | Enzyme | Host organism |
|---|---|---|
| 3a | β-lactamase II | *Bacillus cereus* |
| 3a | BlaB | *Chryseobacterium meningosepticum* |
| 3a | CcrA | *Bacteroides fragilis* |
| 3a | L1 | *Stenotrophomonas maltophilia* |
| 3a | IMP-1 | *Serratia marcescens** |
| 3b | CphA | *Aeromonas hydrophila* |
| 3b | PCM-1 | *Burkholderia cepacia* |
| 3b | NN | *Myroides odoratus* |
| 3c | NN | *Legionella gormanii* |

NN-not named

* IMP-1 first reported in *S. marcescens*

### 1.11.1 Bacteria that produce subgroup 3a metallo-β-lactamases

Subgroup 3a includes the metallo-β-lactamases produced by *B. cereus*, *Chryseobacterium meningosepticum*, *B. fragilis*, *S. maltophilia* and the IMP-1 metallo-β-lactamase identified in a variety of Gram-negative bacteria (Rasmussen and Bush, 1997). These metallo-β-lactamases recognise a wide range of substrates and hydrolyse penicillins at a rate comparable to or better than of imipenem, although they usually hydrolyse cephalosporins more slowly than imipenem. The broad spectrum activity of the subgroup 3a metallo-β-lactamases is thought to be related to the direct interaction of β-lactams with the two zinc centres of these enzymes (Ullah *et al.*, 1998).

#### 1.11.1.1 *Bacillus cereus* β-lactamase II metallo-β-lactamase

The zinc dependent *B. cereus* II enzyme is chromosomally encoded and inducibly expressed (Rasmussen and Bush, 1997). The sequences of three β-lactamase II enzymes have been determined, two from *B. cereus* isolates and one from an alkalophilic *Bacillus* spp. and these sequences are very similar to one another (Ambler *et al.*, 1985; Hussain *et al.*, 1985; Kato *et al.*, 1985, Lim *et al.*, 1988).

#### 1.11.1.2 Metallo-β-lactamases produced by former *Flavobacterium* spp.

Bacterial species that were formerly found in the genus *Flavobacterium* are known to produce carbapenemases. Blahová *et al.* (1994) initially described a carbapenemase in *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* and more recently a metallo-β-lactamase known as BlaB that is produced by *C. meningosepticum* has been characterised in detail. BlaB has a broad substrate profile and therefore fits into Bush subgroup 3a, this enzyme appears to be universally produced by this species (Rossolini *et al.*, 1998).

Sato *et al.* (1986) have also detected a carbapenemase in *M. odoratus* (formerly *F. odoratum*) isolates, although this carbapenemase has been placed in subgroup 3b by Rasmussen and Bush (1997).

### 1.11.1.3 Metallo-β-lactamases produced by *Bacteroides fragilis*

Between 1-3% of *B. fragilis* isolates are known to produce a metallo-β-lactamase that has been named CcrA and CfiA (Bandoh *et al.*, 1993; Rasmussen *et al.*, 1994b; Rasmussen *et al.*, 1997). Sequence analysis has revealed that CcrA and CfiA are essentially the same enzymes and will be collectively referred to here as CcrA (Rasmussen *et al.*, 1990; Thompson and Malamy, 1990). On a single occasion the CcrA enzyme has been found on a small self-transmissible 13.6 kb plasmid (Bandoh *et al.*, 1992). The sequence of this plasmid-encoded β-lactamase is identical to the chromosomal CcrA β-lactamase (Rasmussen and Bush, 1997).

The majority of *B. fragilis* isolates that harbour CcrA express this enzyme at low levels which does not confer clinical resistance (Rasmussen and Bush, 1997). The expression of CcrA is non-inducible, although increased expression of CcrA, leading to high level resistance, can occur and this most commonly involves insertion sequence (IS) element insertions within the promoter region upstream of the Shine Dalgarno box (Podglajen *et al.*, 1992; Podglajen *et al.*, 1994; Rasmussen and Kovacs, 1991). The *ccrA* genes are transcribed from transcriptional initiation signals provided by an IS element integrated within the promoter rather than from their normal promoters (Podglajen *et al.*, 1994; Rasmussen and Kovacs, 1991). It is interesting that IS elements are rare among *B. fragilis* isolates that do not harbour the metallo-β-lactamase gene (Podglajen *et al.*, 1995). Therefore, metallo-β-lactamase positive imipenem susceptible isolates can easily convert to being imipenem resistant

in a single step by the insertion of an IS element into the promoter (Podglajen *et al.*, 1992; Podglajen *et al.*, 1994).

Other than *B. fragilis* the only other *Bacteroides* spp. known to produce a carbapenemase is *B. distasonis* and this enzyme appears to be distinct from CcrA (Hurlburt *et al.*, 1990).

#### 1.11.1.4 Metallo-β-lactamases produced by *Stenotrophomonas maltophilia*

High-level carbapenem resistance in *S. maltophilia* was first shown to be attributed to a metallo-β-lactamase, named L1, by Saino *et al.*, (1982). The *S. maltophilia* metallo-β-lactamases are inducible, chromosomally encoded enzymes that are found universally within the species and can hydrolyse a range of clinically important β-lactams (Akova *et al.*, 1991; Felici and Amicosante, 1995). The broad-spectrum L1 enzyme, initially studied in *S. maltophilia* strain GN12873, was found to function as a tetramer composed of four equal subunits and have an isoelectric point (pI) of 6.9 (Saino *et al.*, 1982). Subsequent reports have shown that *S. maltophilia* isolates produce metallo-β-lactamases with pI values different from that of the original L1 enzyme (Cullman and Dick, 1990; Payne *et al.*, 1994*a*; Paton *et al.*, 1994). Thus L1 from *S. maltophilia* GN12873 is not solely representative of the metallo-β-lactamases produced by this species.

An investigation by Payne *et al.*, (1994a) showed that 16 clinical isolates produced seven different metallo-β-lactamases (m-β-ls) all differentiated by pI and designated

m-β-l types 1-7. Biochemical characterisation of m-β-l types 1-6 further illustrated subtle differences within this group of enzymes (Payne *et al.*, 1994b). The differences in substrate profiles between this group of enzymes was most evident with the chromogenic cephalosporin nitrocephin where hydrolysis rates ranged from 12-87% relative to imipenem (Payne *et al.*, 1994b).

To date, the metallo-β-lactamases from only two *S. maltophilia* strains (IID1275 and GN12873) have been investigated and compared at the molecular level (Walsh *et al.*, 1994; Sanschagrin *et al.*, 1998). These enzymes are known to share 88.6% amino acid identity with one another, which further suggests that within the L1 type metallo-β-lactamase family there is a degree of heterogeneity (Sanschagrin *et al.*, 1998). Heterogeneity of these metallo-β-lactamases has important implications particularly in terms of future drug design.

### 1.11.1.5 Producers of the IMP-1 metallo-β-lactamase

The molecular characterisation of a metallo-β-lactamase designated IMP-1 was first described by Osano *et al.* (1994). IMP-1 was identified in a clinical strain of *Serratia marcescens* and was isolated in Japan, the metallo-β-lactamase was chromosomally encoded and it conferred resistance to imipenem and broad-spectrum β-lactams (Osano *et al.*, 1994). Prior to Osano's report, two other papers from Japan, one written by Watanabe *et al.* (1991) and the other by Minami *et al.* (1993), described unnamed plasmid mediated metallo-β-lactamase in *P. aeruginosa* and it is

now known that Watanabe's metallo-β-lactamase is identical in sequence to IMP-1 (Iyobe *et al.*, 1996).

Since these discoveries it is now recognised that the $bla_{IMP}$ gene has been dispersing widely among Gram-negative bacteria in hospitals throughout Japan. However, as yet there have been no published reports of the IMP-1 enzyme outside Japan. IMP-1 has been identified in *P. aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *S. marcescens*, *Alcaligenes xylosoxidans*, *K. pneumoniae*, *Acinetobacter* spp., *P. rettgeri* (Ito *et al.*, 1995; Senda *et al.*, 1996a; Senda *et al.*, 1996b; Ito *et al.*, 1997). The $bla_{IMP}$ cassette is found on a novel integron-like element where $bla_{IMP}$ is located between the integrase gene (*intI3*) and the aminoglycoside acetyltransferase gene (*acc*(6')-1b) and it is transposed into plasmids or the chromosomes of Gram-negative bacteria by this integron element (Arakawa *et al.*, 1995). Dispersion of IMP-1 amongst Gram-negative bacteria is mediated by large transferable plasmids with wide host ranges (Ito *et al.*, 1995). It has speculated that because of the transferable nature of this β-lactamase, carbapenem resistant Gram-negative organisms which produce IMP-1 may be prevalent in the near future (Senda *et al.*, 1996b).

It is interesting that the levels of carbapenem resistance found in IMP-1-positive strains are diverse, although resistance to other β-lactams such as ceftazidime is consistent (Senda *et al.*, 1996b). It is known that the acquisition of the IMP-1 gene alone does not necessarily confer high-level carbapenem resistance (Senda *et al.*, 1996a). In *P. aeruginosa* high levels of carbapenem resistance have been shown to

be associated with the diminished production of OMP D2 and the production of a metallo-β-lactamase with similar properties to IMP-1 (Minami *et al.*, 1993; Minami *et al.*, 1996). The combination of IMP-1 and decreased outer membrane permeability has also been shown to be major factors in high-level carbapenem resistance in *S. marcescens* (Marumo *et al.*, 1996). It has also been suggested that additional factors such as secondary changes in regulatory systems of metallo-β-lactamase gene expression, efflux system, and/or multiplication of the structural gene may be implicated in conveying high level carbapenem resistance (Senda *et al.*, 1996a). The involvement of these factors, unlike impermeability, is however, less likely because strains carrying IMP-1 are consistently highly resistant to other β-lactams.

In Japan the carbapenems are the market leaders among parenteral β-lactams, whereas in many other areas of the world their use is much more restricted (Rasmussen and Bush, 1997). The widespread use of the carbapenems, imipenem and also panipenem, has been suggested as a reason why IMP-1 has emerged in Japan (Livermore, 1997). The heavy prescription of 1-oxa-cephamycins moxalactam (latamoxef) and flomoxef has also been implicated in the selection of these IMP-1 producing strains (Livermore, 1997).

In recent reports from Japan great emphasis is often placed on the identification of IMP-1 producing bacteria. However, some of these papers have identified additional non-IMP-1 carbapenem resistant isolates but these have been somewhat ignored. For example one report identified twenty carbapenem-resistant *S. marcescens* strains and

only four of these isolates tested positive for IMP-1, therefore the mechanism of resistance in the remaining sixteen isolates remained undetermined (Ito *et al.*, 1995). In a further report one hundred and thirty two carbapenem resistant *P. aeruginosa* strains were probed for IMP-1 and fifteen strains were found to carry the $bla_{IMP}$ gene and again the resistance mechanisms in the majority of the strains were not studied (Senda *et al.*, 1996a). These points suggest that more thorough investigations are required to assess the true importance of different carbapenem resistance mechanisms.

### 1.11.2 Bacteria that produce subgroup 3b metallo-β-lactamases

Subgroup 3b is composed of enzymes that have a strong preference for the hydrolysis of carbapenems. The metallo-β-lactamases from *Aeromonas* species, *B. cepacia* and *M. odoratus* have been placed in this subgroup.

#### 1.11.2.1 Metallo-β-lactamases produced by *Aeromonas* spp.

Individual *Aeromonas* isolates have been found to produce up to three different chromosomally encoded β-lactamases, including a molecular class C Bush group 1 cephalosporinase, a molecular class A or D Bush group 2d penicillinase, and a molecular class B Bush group 3b metallo-β-lactamase (Hayes *et al.*, 1994; Walsh *et al.*, 1995a; Hayes *et al.*, 1996). The *Aeromonas* metallo-β-lactamases have a narrow substrate profile-they display high catalytic efficiencies for imipenem, but poor hydrolysis of either benzylpenicillin or cephaloridine (Rasmussen and Bush, 1997).



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It has been suggested that the narrow spectrum of activity of the *Aeromonas* metallo-β-lactamases may be a consequence of their mono-zinc nature which results in replacing direct metal substrate contacts with specific protein: substrate interactions leading to the preservation of catalytic efficiency at the expense of a greatly reduced spectrum of activity (Ullah *et al.*, 1998).

The narrow substrate spectrum of the *Aeromonas* metallo-β-lactamases has also been a stumbling block in their identification as they poorly hydrolyse nitrocephin, the standard substrate used for β-lactamase detection (Segatore *et al.*, 1993). This is illustrated by the work of Iaconis and Sanders (1990) who initially reported the identification of two β-lactamases in a strain of *A. jandaei* AER14 (formerly *A. sobria*), one of which was responsible for imipenem hydrolysis. Later, a Bush group 1, molecular class C cephalosporinase (AsbA1), and a Bush group 2d cloxacillin-hydrolysing β-lactamase (AsbB1) were characterised genetically and biochemically by Rasmussen *et al.* (1994a). These two β-lactamases corresponded to the β-lactamases from *A. jandaei* AER14 that were visualised on an IEF gel, however, neither of these β-lactamases hydrolysed imipenem. More recently it has been established that *A. jandaei* AER14 produces a third metallo-β-lactamase (AsbM1) not detectable with nitrocephin and this had been co-purified with the AsbB1 β-lactamase in the original report by Iaconis and Sanders (1990). Felici *et al.*, (1993), Hayes *et al.* (1994) and Walsh *et al.* (1995a) have also demonstrated that *Aeromonas* metallo-β-lactamases display a very low activity against nitrocephin. The *Aeromonas* metallo-β-lactamases are inhibited by EDTA and the addition of zinc has been shown to restore activity after EDTA inhibition (Rasmussen and Bush, 1997).

The sequences of three *Aeromonas* metallocarbapenemases have been previously determined; these include two *A. hydrophila* genes, *cphA* and *cphA2*, and the *A. veronii* bv. *sobria* (formerly *A. sobria*) *imiS* gene (Massidda *et al.*, 1991; Rasmussen and Bush, 1997; Walsh *et al.*, 1998). The amino acid sequences of CphA, CphA2 and ImiS share a high level of identity with one another (≥96%). The production of a metallo-β-lactamase related to CphA from *A. hydrophila* has been shown to be a common feature amongst several different *Aeromonas* species including *A. veronii* bv. *sobria* (Rossolini *et al.*, 1996). The N-terminus of an *A. jandaei* AsbM1 metallo-β-lactamase has also been sequenced but was shown to be only 26% similar to both CphA and ImiS over the first 27 amino acids (Yang and Bush, 1996; Walsh *et al.*, 1998). It has been speculated that if this low level of identity is maintained throughout the protein it would indicate a major divergence of the metallo-β-lactamases found in *Aeromonas* species (Walsh *et al.*, 1998).

Production of a metallo-β-lactamase is normally regulated in *Aeromonas* strains. The enzyme is produced at negligible levels in the absence of an inducer, while its production increases several hundred-fold in the presence of an inducer. Induction leads to the expression of all three β-lactamases that have been shown to be present in individual *Aeromonas* strains. Derepressed mutants that constitutively produce all three β-lactamases can also be obtained and it appears that these enzymes are all controlled by a common regulatory system (Walsh *et al.*, 1995a). Recent studies have shown that the expression of inducible chromosomally encoded β-lactamases in *Aeromonas* species is controlled by a two component system that differs radically

from the *C. freundii* paradigm for inducible β-lactamases (Alksne and Rasmussen, 1997; Niumsup *et al.*, 1997).

### 1.11.2.2 *Burkholderia cepacia* metallo-β-lactamase

A metallo-β-lactamase named PCM-1 that is produced by *B. cepacia* has also been assigned to subgroup 3b (Rasmussen and Bush, 1997). PCM-1 has an unusual inhibition profile in that it is inhibited by both metal-ion chelators and the inhibitors clavulanate and tazobactam (Baxter and Lambert, 1994). PCM-1 is inducibly expressed and appears to be ubiquitous amongst *B. cepacia* (Baxter and Lambert, 1994; Simpson *et al.*, 1993). No sequence is yet available for PCM-1.

### 1.11.3 Bacteria that produce subgroup 3c metallo-β-lactamases

Subgroup 3c consists of the metallo-β-lactamase from *Legionella gormanii*, which is distinguishable from the other metallo-β-lactamases because of its strong hydrolysis of cephalosporins, including expanded-spectrum cephalosporins (Fujii *et al.*, 1986; Rasmussen and Bush, 1997). The *L. gormanii* enzyme has not been studied in detail.

## 1.12 Serine-based carbapenemases

### 1.12.1 Molecular class A Bush group 2f carbapenemases

Three molecular class A Bush group 2f carbapenemases have been characterised in detail, these related enzymes have been designated IMI-1, NMC-A and Sme-1. IMI-1 was identified from two *E. cloacae* strains in California in 1984, NMC-A was found to be produced by a single *E. cloacae* strain isolated in Paris in 1990 and Sme-1 was reported from two *S. marcescens* strains isolated in London back in 1982 (Nordmann *et al.*, 1993; Rasmussen *et al.*, 1996; Yang *et al.*, 1990). Recently, there have been further reports of carbapenem resistant *S. marcescens* isolates producing a non-metallo imipenem-hydrolyzing -β-lactamase and it is presumed that these are Sme-1 homologues (Quinn *et al.*, 1997; Carmeli *et al.*, 1997).

The *E. cloacae* IMI-1 and NMC-A enzymes are highly homologous and share 95% amino acid identity, whereas Sme-1 shares about 70% amino acid identity with these two enzymes (Livermore, 1997). Although these enzymes have been found to be chromosomally encoded, they have been referred to as 'acquired carbapenemases', because they are supplementary to the normal chromosomal AmpC-type cephalosporinase of *E. cloacae* and *S. marcescens* (Livermore, 1997). IMI-1, NMC-A and Sme-1 are inducible β-lactamases that are under the control of a LysR-like regulator protein analogous to the AmpR regulator of Amp-C (Rasmussen *et al.*, 1996; Naas and Nordmann, 1994; Naas *et al.*, 1995). Some of the features that separate these serine carbapenemases from the metallo-β-lactamases include: greater

resistance to imipenem than meropenem, with meropenem MICs often below the breakpoints for susceptibility; hydrolysis of aztreonam (none of the metallo-β-lactamases so far described can hydrolyse this substrate) and inhibition by clavulanic acid (Livermore, 1997).

### 1.12.2 *Acinetobacter* carbapenemases

Carbapenem resistance is increasingly being reported in *Acinetobacter* spp. from different geographical locations (Livermore, 1997). ARI-1 was the first reported *Acinetobacter* carbapenem-hydrolysing β-lactamase, and was produced by a strain of *A. baumanii* that was originally isolated in Scotland in 1985 (Paton *et al.*, 1993). ARI-1 was subsequently found to be encoded by a 45kb plasmid-mediated (Scaife *et al.*, 1995).

More recently imipenem-hydrolysing β-lactamases have been identified in carbapenem resistant strains isolated from South America, Europe, South East Asia and the Middle East (Brown *et al.*, 1998; Hornstein *et al.*, 1997; Weinbren *et al.*, 1998; Afzal-Shah and Livermore, 1998). Hornstein *et al.* (1997) detected an oxacillin-hydrolysing β-lactamase that appears to be involved in the imipenem resistance mechanism of a single *Acinetobacter* isolate and Brown *et al.* (1998) have demonstrated the involvement of a plasmid-mediated molecular class A carbapenemase, ARI-2, in clinical isolates from S. America, Europe and S. E. Asia.

It should also be noted that a metallo-β-lactamase has also been identified in an *Acinetobacter* spp. although, there has been no molecular characterisation of this enzyme (Pérez *et al.*, 1996).

## 1.13 Clinical significance of carbapenemase-producing bacteria

Several different bacterial species have been found to produce carbapenemases. Many of these organisms have in the past not been considered as classical hospital pathogens, but have emerged as important causes of nosocomial infections in recent years.

### 1.13.1. *Acinetobacter* spp.

For many years *Acinetobacter* spp. were not considered as clinically important pathogens, however, they now account for 10% of nosocomial infections in intensive care units in Europe alone (Brown *et al.*, 1998). Moreover, the nosocomial *Acinetobacter* isolates are increasingly multi-drug resistant with the carbapenems often being the treatment of choice (Amyes and Thomson, 1995; Hornstein *et al.*, 1997). Therefore, the recent reports of carbapenem resistance in *Acinetobacter* are of serious concern to the medical community. The ARI-2 plasmid-encoded carbapenemase appears to be strongly associated with carbapenem resistance as it has been demonstrated that a strain cured of an ARI-2 bearing plasmid is susceptible to the carbapenems, unlike the parent strain (Brown *et al.*, 1998).

### 1.13.2 *Aeromonas* spp.

Species of the genus *Aeromonas* are widely dispersed in aquatic environments and are increasingly found to be causative agents in clinical infections. There are currently fourteen species in the genus that have been defined using biochemical typing methods, DNA-DNA hybridisation and 16S rRNA sequence data (Martinez-Murcia and Esteve, 1992; Jones and Wilcox, 1995). The major pathogenic species are *A. veronii* bv. *sobria*, *A. hydrophila* and *A. caviae* (Janda *et al.*, 1995).

Gastroenteritis is one of the frequent *Aeromonas* associated infections and appears to be common in countries with warmer climates. In some geographical regions isolation rates of up to 50% have been recorded for symptomatic patients (usually children under 5), although antibiotic treatment is usually unnecessary due to the self-limiting nature (San Joaquin and Pickett, 1988; Thornley *et al.*, 1997). Aeromonads additionally cause skin and soft tissue infections, although these occur more frequently in immunocompromised patients (Gold and Salit, 1992). *Aeromonas* bacteraemia is most commonly seen in individuals with haematological malignancies, solid tumours and hepatobiliary diseases, it is rare in immunocompetent patients (Janda and Duffy, 1988; Golik *et al.*, 1990). Additionally reported *Aeromonas* infections include meningitis, endocarditis, ocular infections and osteomyelitits (Frejj, 1984, Janda and Duffey, 1988).

Although most *Aeromonas* spp. produce an inducible carbapenemase, paradoxically, when standard in vitro susceptibility is performed these bacteria remain sensitive to

the carbapenems. Only a handful of naturally occurring carbapenem resistant *Aeromonas* isolates have been recorded when testing with a conventional inoculum size, these strains were derepressed for β-lactamase production (Morita *et al.*, 1994). It is however, known that the carbapenem minimum inhibitory concentrations (MICs) for *Aeromonas* strains that produce a carbapenemase are subject to an inoculum effect. Employing a large inoculum of $10^8$ colony forming units (cfu) results in the carbapenem MICs becoming higher than the breakpoint for susceptibility (Rossolini *et al.*, 1996). However, it is highly improbable that the bacterial population would be this large in vivo. Therefore, in its induced state the metallo-β-lactamase is probably not of clinical importance.

### 1.13.3 *Burkholderia cepacia*

*B. cepacia* is traditionally thought of as a pathogen of onions (Burkholder, 1950), however, more recently this organism has been recognised as an opportunistic pathogen in humans, particularly cystic fibrosis (CF) patients where it is associated with a rapidly fatal pneumonia and septicaemia (Pitt *et al.*, 1996). Acquisition of *B. cepacia* by CF patients is a problem because this organism is resistant to most antibiotics, including carbapenems (Simpson *et al.*, 1993). Carbapenem resistance in *B. cepacia* has been linked to the production of the PCM-1 carbapenemase (Baxter and Lambert, 1994).

### 1.13.4 *Bacillus* spp.

*Bacillus* spp. are aerobic Gram-positive bacilli that are usually found in soil, water, air and on vegetation. *B. cereus* is primarily associated with toxin-mediated food poisoning, and it is *B. anthracis* that is principal *Bacillus* spp. pathogen. Isolates of *Bacillus* spp. from clinical specimens are generally dismissed as contaminants, although non-anthrax *Bacillus* spp are occasionally reported to cause infections in immunocompromised patients. These infections in immunocompromised individuals remain extremely rare (Blue *et al.*, 1995; Strittmatter *et al.*, 1996; Berner *et al.*, 1997). The prevalence of metallo-β-lactamase II in *Bacillus* spp. has not been studied in any detail, it is thought, however, that this enzyme is universal in *B. cereus* (Livermore, 1992).

### 1.13.5 *Bacteroides* spp.

*Bacteroides* spp. are among the most clinically important anaerobic bacteria for two reasons. Firstly, they are the bacteria most often isolated from patients with post surgery anaerobic infections, and secondly they are resistant to a broad range of antimicrobials. β-Lactams are one of the agents most widely used to treat anaerobic infections (Rasmussen *et al.*, 1997). Cefoxitin, an agent formerly highly active against anaerobes now displays decreased potency and imipenem is often the main hope in terms of β-lactam therapy in conquering anaerobic infections. The identification of the CcrA carbapenemase in *B. fragilis* jeopardises the effectiveness of imipenem (Rasmussen *et al.*, 1997), however it should remembered that *ccrA*-like

gene is carried by only 1-3% and only a fraction of these strains are carbapenem resistant.

### 1.13.6 *Enterobacter cloacae*

*E. cloacae* is the species of the genus *Enterobacter* that is most commonly isolated from nosocomial infections (de Gheldre *et al.*, 1997). *E. cloacae* is the cause of bacteraemia, respiratory and urinary tract infections (Pitout *et al.*, 1997). Carbapenemase-producing strains appear at present to be extremely rare, whereas, carbapenem resistance involving the overproduction of a cephalosporinase and the loss of an OMP have been reported on a few occasions (Sections 1.10.2, 1.10.5.1). Multidrug resistant *E. cloacae* (including imipenem resistance) are a particular threat because there is virtually no therapeutic options left for such strains (de Gheldre *et al.*, 1997).

### 1.13.7 Former *Flavobacterium* spp.

The natural habitats of the ex-flavobacterial spp. are soil and aquatic environments. Both *M. odoratum* and *C. meningosepticum* can cause nosocomial infections. *C. meningosepticum*, as the name suggests is associated with meningitis in neonates and is considered to be the ex-flavobacterial spp. of greatest clinical significance (von Graévenitz, 1995). The ex-flavobacterial spp. have wide patterns of resistance to antibiotics, including the carbapenems.

### 1.13.8 *Serratia marcescens*

*S. marcescens* in common with *E. cloacae* is a common cause of hospital acquired infections in immunocompromised patients. *S. marcescens* is responsible for urinary tract, respiratory tract and wound infections, septicaemia and endocarditis. Carbapenems are considered as one of the normal treatment options for an infection caused by *S. marcescens* and therefore, descriptions of carbapenemases leading to treatment failure is a serious problem.

### 1.13.9 *Stenotrophomonas maltophilia*

*S. maltophilia* has risen to prominence over the last decade as an important nosocomial pathogen because of the increasing frequency of its isolation and its broad spectrum of antimicrobial resistance, affecting patients with lowered defence mechanisms. *S. maltophilia* is associated with a variety of clinical syndromes including: bacteriaemia, endocarditis and respiratory tract infections (Denton and Kerr, 1998). *S. maltophilia* is, however, often isolated from mixed infection in which its pathogenic role is debatable. The emergence of the organism as a pathogen has been attributed to selective pressure by the use of broad-spectrum antibiotics. *S. maltophilia* is resistant to many currently available broad-spectrum antibiotics and therefore, infections caused by *S. maltophilia* are particularly difficult to treat (Denton and Kerr, 1998). The production of the L1 metallo-β-lactamase is known to confer β-lactam resistance and clinical resistance to imipenem is virtually universal

in all strains (Payne *et al.*, 1994a). It is thought that poor diffusion of β-lactams across the cell membrane may contribute to resistance (Cullman, 1991).

### 1.13.10 Clinical significance of other bacteria that produce IMP-1

*A. xylosoxidans, P. fluorescens, P. putida, and P. rettgeri* are all responsible for hospital acquired infections. *P. aeruginosa* is the *Pseudomonas* spp. most commonly associated with human disease and is a common hospital pathogen. It produces infection of burns and wounds, urinary tract and septicaemia. The lungs of CF patients are particularly susceptible to infection with *P. aeruginosa*. Reports of *K. pneumoniae* strains producing IMP-1 is of particular concern, as this organism is an important hospital pathogen and also a notorious vector of resistance. *K. pneumoniae* causes bacterial pneumonia, urinary tract infections and bacteraemia and has been responsible for large hospital epidemics (Hobson *et al.*, 1996).

## 1.14 Aims of this thesis

1. To carry out a survey on carbapenem resistant clinical isolates from Japan and determine the resistance mechanisms involved, concentrating on the role of β-lactamases.

2. Develop an imipenem substrate based modification to the standard isoelectric focusing technique for the rapid identification of carbapenem-hydrolysing β-lactamases

3. Investigate the resistance mechanism involved in conferring carbapenem resistance in two *A. veronii* bv. *sobria* strains isolated in Vellore, South India.

4. Assess the heterogeneity of metallo-β-lactamases produced by *S. maltophilia* at the molecular level.

# 2: MATERIALS AND METHODS

## 2.1 Bacterial strains and plasmids

The strains and plasmids employed in this study are listed in Tables 2.1 and 2.2. *P. aeruginosa* strains and *Aeromonas* species were both identified using API 20 NE strips (Bio Mérieux, France).

## 2.2 Storage of bacterial cultures

Bacteria were grown overnight at 37°C in cryovials (Alpha Laboratories, Eastleigh, Hampshire) containing 900µl of nutrient broth. After overnight incubation, 100µl of 50% glycerol was added to the vial and mixed, to give a final concentration of 5% glycerol. The cultures were then stored at -70°C.

## 2.3 Reagents

All chemicals were purchased from Sigma Chemicals (Poole, Dorset), unless otherwise stated. All solutions were prepared with either distilled or pyrogen-free water (MilliQPF, Millipore,Watford, Herts).

## Table 2.1 Bacterial strains used in this study

| Bacterial strain | Characteristics | Source or reference |
|---|---|---|
| *E. coli* NCTC 10418 | Laboratory standard | National Collection of Type Cultures, London |
| *S. aureus* NCTC 6571 | Laboratory standard | |
| *P. aeruginosa* NCTC 10662 | Laboratory standard | |
| *S. marcescens* NCTC 1377 | Laboratory standard | |
| *E. coli* 7181 | Produces OXA-7 β-lactamase | Medeiros *et al.*, 1985 |
| *S. marcescens* S6 | Produces Sme-1 β-lactamase | Yang *et al.*, 1990 |
| *E. cloacae* NOR-1 | Produces Nmc-A β-lactamase | Nordmann *et al.*, 1993 |
| *P. aeruginosa* 101/1477 | Produces IMP-1 β-lactamase | Dr. D. J. Payne (SmithKline Beecham) |
| *P. aeruginosa* M18 | Produces IMP-1 β-lactamase | Dr. Y. Arakawa |
| *S. marcescens* TN9106 | Produces IMP-1 β-lactamase | Osano *et al.*, 1994 |
| *S. marcescens* AK9374 | Produces IMP-1 β-lactamase | Ito *et al.*, 1995 |
| *S. marcescens* FHSM4055 | Produces IMP-1 β-lactamase | Marumo *et al.*, 1996 |
| *P. aeruginosa* strains 2, 3, 4, 5, 6, 8, 13, 14, 16, 22, 23, 24, 25, 26, 30, 34, 35, 36, 37, 38, 39, 41, 43, 44, 45, 46, 48, 50, 52, 54, 55, 56, 60, 62, 63, 64, 65, 66, 67, 68, 69, 71, 73, 74, 75, 79, 80, 83, 84, 86, 87, 90, 93, 94, 96, 97, 98, 100, 101, 102, 103, | 61 clinical strains isolated in Japan in 1994 | Dr. K. Sato, Daiichi Pharmaceuticals Co., Ltd., Tokyo, Japan |

## Table 2.1 Bacterial strains used in this study (continued)

| Bacterial strain | Characteristics | Source or reference |
|---|---|---|
| *P. aeruginosa* strains M1405β-con D2⁻, M2297β-con D2⁻ | AmpC derepressed, D2⁻ porin mutants | Livermore, 1992 |
| *P. aeruginosa* strains M1405β-def D2⁻, M2297β-def D2⁻ | AmpC basal, D2⁻ porin mutants | Livermore, 1992 |
| *P. aeruginosa* 2297 | Wild-type strain | Livermore, 1992 |
| *S. maltophilia* 511, | Metallo-β-lactamase producers | Felici *et al*, 1993 |
| *S. maltophilia* strains GEL, 0062, U152, J2323, 37.ED136, A37454 | Metallo-β-lactamase producers | Payne *et al*., 1994a |
| *E. coli* K12 J53-2 (R6K) | Produces TEM-1 β-lactamase | S. G. B. Amyes |
| *A. hydrophila* T429125M | Cefotaxime-resistant mutant derepressed for β-lactamase production | Walsh *et al*., 1997 |
| *A. veronii* bv. *sobria* 163a | Produces AmpS, CepS and ImiS | Walsh *et al*., 1995a |
| *A. veronii* bv. *sobria* strains 13, 27, 36, 47, 51, 52, 99, 110, 115 | Environmental isolates from Vellore, S. India | Isolated by A. K. B. Amyes, July 1996 |
| *E. coli* DH5α | Host for construction of DNA library | Hanahan, 1983 |

## Table 2.2 Plasmids used in this study

| Plasmid | Characteristics | Source or reference |
|---|---|---|
| pK18 | Kanamycin-resistant, lacZ′, p15 origin | Pridmore, 1987 |
| pUB8902 | Recombinant plasmid encoding metallo-β-lactamase $bla_{L1}$ gene of *S. maltophilia* IID 1275 | Walsh *et al.*, 1994 |
| No designated identification | Recombinant plasmid encoding metallo-β-lactamase gene *imiS* from *A. veronii* bv. *sobria* 163a | Walsh *et al.*, 1998 |

## 2.4 Culture media

Media constituents were obtained from either Oxoid (Basingstoke, Hants) or Difco Laboratories (Detroit, MI, USA). All media was made up according to the manufacturer's instructions. Sterilisation of media, to destroy all vegetative cells and bacterial endospores, was achieved by autoclaving at 121°C and 15 pounds of pressure for 15 minutes.

Mac Conkey Agar (Oxoid), Nutrient Agar (Oxoid) and Nutrient Broth No. 2 (Oxoid) were employed for the growth of all bacteria included in this study. Iso-Sensitest Agar (Oxoid), a semi-defined media, was used for antibiotic susceptibility testing.

Luria-Bertani (LB) broth was prepared by adding 10g of Bacto-tryptone (Difco), 5g Bacto-yeast extract and 10g of NaCl to 800mL of distilled water. The pH was then adjusted to 7.0 with 1M NaOH before finally making up the volume to 1litre. LB agar was prepared by adding 1.5% Bacteriological Agar No.1 (Oxoid) to LB broth.

## 2.5 Antibacterial compounds

Table 2.3 lists the antibiotics used, information on their solubility and the suppliers of these compounds. Stock solutions of chloramphenicol, kanamycin, BRL42715 and kanamycin were protected from light and stored at -20°C; all other compounds were prepared on the day of use.

## Table 2.3 Antibacterial compounds

| Antimicrobial | Diluent | Supplier |
|---|---|---|
| Ciprofloxacin | water | Bayer (Newbury, Berkshire) |
| Tazobactam | water | Lederle Laboratories (Cyanamid of Great Britain Ltd., Hants) |
| Ceftazidime | saturated $NaHCO_3$[a] and water | Glaxo-Wellcome (Uxbridge, Middlesex) |
| Cefotaxime | water | Hoechst-Roussel Pharmaceuticals (Uxbridge, Middlesex) |
| Imipenem | water | Merck Sharp & Dohme (Hoddesdon, Herts) |
| Sulbactam | water | Pfizer Central Research (Kent) |
| Ampicillin | water | Sigma Chemicals (Poole, Dorset) |
| Cefoxitin | water | Sigma Chemicals |
| Cephaloridine | water | Sigma Chemicals |
| Chloramphenicol | 70% ethanol | Sigma Chemicals |
| Kanamycin | water | Sigma Chemicals |
| Oxacillin | water | Sigma Chemicals |
| BRL42715 | water | SmithKline Beecham Pharmaceuticals (Surrey) |
| Carbenicillin | water | SmithKline Beecham Pharmaceuticals |
| Nitrocephin | DMSO[a] and 50mM sodium phosphate buffer, pH 7.0 or water | SmithKline Beecham Pharmaceuticals |
| Meropenem | water | Zeneca Pharmaceuticals (Macclesfield, Cheshire) |
| Cefepime | water | Eli Lilly |

[a]Enough to dissolve antimicrobial powder

## 2.6 Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) of antibiotics were determined by the agar dilution method (Working Party of the British Society of Antimicrobial Chemotherapy, 1991). Bacterial strains were grown overnight at 37°C in Nutrient Broth No.2, with shaking. Following incubation cell cultures were diluted to $10^7$ colony forming units (cfu)/ml and 1µl of the diluted cell suspension was inoculated using a multipoint inoculator (Denley, Surrey) onto agar plates containing two-fold serially diluted antibiotics. *E. coli* NCTC 10418, *P. aeruginosa* NCTC 10662 and *S. aureus* NCTC 6571 were used as control strains, with known MIC values for the antibiotics tested.

## 2.7 Preparation of β-lactamases

### 2.7.1 Small-scale preparation of β-lactamases

Bacterial strains were grown overnight on 10ml nutrient agar slopes at 37°C. Following overnight incubation, the bacterial cells were washed off the surface of the slope with 1ml of 50mM sodium phosphate buffer, pH 7.0. The sample was then transferred to a bijoux bottle and placed in an ice/water bath. The cells were burst by ultrasonication for 2 x 15 seconds at an amplitude of 8µm, with a 30 second cooling period (MSE Soniprep 150, MSE Instruments, Crawley). Cell debris was removed from the lysate by centrifugation (MSE Microcentaur Centrifuge) at high speed for 10 minutes at 4°C. β-Lactamase extracts were stored at -20°C until required.

### 2.7.2 Large-scale preparation of β-lactamases

Large-scale β-lactamase extracts were prepared from 100ml cultures according to the method described by Payne and Farmer (1998). A flask containing 100ml of nutrient broth was inoculated with 1ml of an overnight broth culture. The cultures were then grown at 37°C, with shaking, to an optical density of approximately 0.7 at 500nm and at this stage, if appropriate, imipenem was added as a β-lactamase inducer, at a final concentration of ¼ the strains imipenem MIC. After a further 2 hours of continuous shaking at 37°C the cells were harvested by centrifugation (GS3 rotor, Sorvall® RC-5B, Du Pont) at 6000 rpm for 15 minutes and at 4°C. The pellet was resuspended and washed in 10ml of 50mM sodium phosphate buffer, pH 7.0, followed by centrifugation as previously described. The washed cells were resuspended in 1ml of 50mM sodium phosphate buffer, pH 7.0 and placed in an ice/water bath. The cells were burst by ultrasonication for a minimum of 2 x 30 seconds at an amplitude of 8μm with a 1 minute cooling period between each burst. The lysate was cleared of all cell debris by centrifugation (MSE Microcentaur Centrifuge) at high speed for 30 minutes and 4°C. β-Lactamase extracts could then be stored at -20°C.

## 2.8 Analytical isoelectric focusing (IEF)

β-lactamase preparations were examined by IEF based on the method first described by Matthews *et al.* (1975).

### 2.8.1 Casting an IEF gel

β-Lactamases were focused on a horizontal thin layer polyacrylamide gel containing carrier ampholines (pH 3.5-10). The composition of the IEF gel is given in Table 2.4, tetramethylethylenediamine (TEMED) and riboflavin were the last components to be added. The polyacrylamide gel was poured between two clean glass plates, separated by rubber tubing, and approximately 2mm apart. One glass plate was coated with a solution containing 0.5% (w/v) gelatine (ca.225 bloom from calf skin) and 0.05% (w/v) chromium potassium sulphate dodecahydrate, this enabled the gel to stick to one of the glass plates. The other glass plate was siliconised to allow easy separation of the two plates once the gel had polymerised. The gel was left to polymerise for 4-5 hours.

## Table 2.4 Composition of a polyacrylamide IEF gel

| Material | Volume employed (ml) | Final concentration |
|---|---|---|
| 40% (w/v) ampholines | 2.0 | 2% (w/v) |
| 100g acrylamide and 2.7g bisacrylamide in 300ml of water | 9.0 | Acrylamide 75g/l. bisacrylamide 2g/l |
| Distilled water | 25.0 | - |
| 5% (v/v) TEMED | 0.2 | 0.005% (v/v) |
| Riboflavin (20mg/l) | 4.0 | 2mg/l |

### 2.8.2 Pre-cast IEF gels

β-Lactamase extracts were also subjected to IEF on pre-cast polyacrylamide gels (Ampholine® PAGplate) containing 2% (v/v) ampholines of pH range 3.5-9.5 (Pharmacia Biotech, St. Albans, Herts). The pre-cast gel was placed on the cooling plate of a Multiphor II Electrophoresis Unit (Pharmacia Biotech). Paper electrode contacts moistened with 1M $H_3PO_4$ (anode) and 1M NaOH (cathode) were placed on the surface of the gel according to the manufacturer's instructions.

### 2.8.3 Agarose IEF gel

β-Lactamases were focused on a horizontal agarose IEF gel containing carrier ampholines (pH 3.5-10). The composition of the agarose IEF gel is listed in Table 2.5. IEF grade agarose was dissolved in distilled water by heating in a microwave oven, followed by cooling to 60°C in a waterbath. The D-sorbitol and ampholines were added to the melted agarose and pipetted, to a depth of approximately 2mm, onto the hydrophobic surface of a Gelbond sheet (250 x 125 x 0.8mm), placed on the

surface of a Multiphor II Electrophoresis Unit (Pharmacia Biotech). The gel was cooled for a minimum of 30 minutes to ensure gel consistency and strength. Excess moisture was removed from the gel using filter paper. Electrode wicks were soaked in 1M $H_3PO_4$ (anode) and 1M NaOH (cathode) and placed on to the corresponding poles.

**Table 2.5 Composition of an agarose IEF**

| Material | Volume or mass employed | Final concentration |
|---|---|---|
| 40% (w/v) ampholines | 1.5ml | 2% (w/v) |
| Agarose IEF VIII (Sigma) | 240mg | 0.8% (w/v) |
| Distilled water | 15.0ml | - |
| D-sorbitol 20% (w/v) | 13.5ml | 9% (w/v) |

### 2.8.4 Nitrocephin spot testing and sample loading

The quantity of each β-lactamase preparation to be loaded on the gel was determined by nitrocephin spot testing. The amount of sample loaded on to the gel (μl) was equal to the time taken (seconds) for a mixture of 33μl of β-lactamase and 100μl of nitrocephin (50μg/ml), in a microtitre plate, to change from yellow to red. Twelve samples could be loaded on to the self-cast IEF gel and 24 samples could be loaded on to the pre-cast gel. Isoelectric focusing was performed overnight at 4°C at 1W (constant), 500V (limiting) and 20mA (limiting).

### 2.8.5 Detection of β-lactamase activities after IEF

After IEF the polyacrylamide gel was stained by overlaying the gel with a sheet of Whatman No.54 paper (Whatman, Maidstone, Kent) soaked in nitrocephin solution (500μg/ml). The focused β-lactamases appeared as red bands on a yellow background. The β-lactamase isoelectric point (pI) values were determined from a graph of pI against distance migrated for coloured pI markers (pI Calibration Kit Electran®, range 4.7-10.6, BDH Laboratory Supplies, Poole, Dorset). The stained gel was photographed with a Polaroid camera (setting B4, F8), fitted with a Tiffen green filter.

## 2.8.6 Inhibitor studies after IEF

For inhibitor studies, Whatman No. 54 filter paper, soaked with the desired inhibitor was placed on the surface of the focused gel. After 10 minutes the inhibitor soaked paper was removed and a nitrocephin overlay was applied as described above.

## 2.9 β-Lactamase assays

### 2.9.1 Substrate profiling of β-lactamases

β-Lactamase activity was determined by monitoring the hydrolysis of a range of β-lactam agents with a λ2 double beam UV/Vis spectrophotometer, fitted with a temperature controlled cuvette holder (Perkin Elmer). Assays were carried out in 1cm path length quartz cuvettes (working volume -3ml) for 2 minutes at wavelength optimal for each β-lactam (Table 2.6). Assays were performed in either 50mM sodium phosphate buffer, pH 7.0 or 25mM piperazine-*N*,*N*'-bis(2-ethanesulphonic acid) (PIPES) buffer, pH 7.0 at 37°C. For substrate profiling each β-lactam was assayed at a final concentration of 100μM, except for ampicillin which was assayed at 500μM.

β-Lactamase activity was calculated as the amount of substrate (μmoles/nmoles) hydrolysed/minute/ml of enzyme sample using the formula described by Payne and Farmer (1998):

$$\frac{\Delta\text{Absorbance/minute}}{\Delta\Sigma \times 10^{-6}} \times V_r/1000 \times 1/V_e$$

**ΔAbsorbance/minute** – rate of absorbance change.

$\Delta\Sigma$ - molar extinction coefficient of hydrolysis ($\Delta\Sigma$ was determined by measuring the absorbance of a β-lactam solution of known concentration at the optimal wavelength for the compound. The $\Delta\Sigma$ was then determined by inserting the absorbance and concentration of the solution into Beer Lambert's equation, A = ecl, A = absorbance, e = molar extinction coefficient, c = concentration of β-lactam solution and l = cuvette path length).

$\mathbf{V_r}$ – volume of reaction (ml).

$\mathbf{1/V_e}$ – volume of enzyme (ml) added and factor needed for the conversion of sample volume to 1ml

### 2.9.2 β-Lactamase specific activity

The protein concentration of β-lactamase preparations were determined by the method of Waddell (1956). β-Lactamase specific activity could then be calculated as μmoles or nanomoles of β-lactam hydrolysed/minute/mg of protein.

**Table 2.6 Optimal wavelengths for the measurement of β-lactamase hydrolysis**

| β-Lactam | Wavelength (nm) |
|---|---|
| Ampicillin | 238 |
| Cephaloridine | 255 |
| Imipenem | 299 |
| Nitrocephin | 384 |

### 2.9.3 Determination of maximal hydrolysis rates (Vmax) and the Michaelis constant (Km)

β-Lactamases were assayed with decreasing substrate concentrations and decreasing rates of hydrolysis. The reciprocal of the substrate concentration was plotted against the reciprocal of the rate of hydrolysis, according to the method of Lineweaver and Burk (1934), to determine the Vmax and Km. The efficiency of hydrolysis was calculated by dividing Vmax by Km values.

### 2.9.4 Inhibition studies

Determination of the inhibitor concentrations required to inhibit 50% of β-lactamase activity ($ID_{50}$) were performed spectrophotometrically (Hayes, 1995). A range of β-lactamase inhibitor concentrations were incubated with β-lactamase preparations for 10 minutes at 37°C before the addition of the reporter substrate (100μM imipenem or

100μM nitrocephin). The $ID_{50}$ value could be determined from plotting a graph of percentage inhibition against inhibitor concentration.

## 2.10 β-Lactamase purification

Whatman DE52 and QA52 pre-swollen anion exchange celluloses were employed for β-lactamase purification. DE52 cellulose is substituted with a diethylaminoethyl (DEAE) group, QA52 has a quaternary amine group, and these groups define the matrices as positively charge anion exchangers. DE52 and QA52 are designated weak and strong anion exchangers, respectively, and this refers to the pH range over which the exchanger is ionised.

### 2.10.1 Extraction of periplasmic proteins

An overnight culture was used to inoculate 1litre of Nutrient Broth. The culture was incubated at 37°C with shaking for 18 hours. The culture was harvested (GS3 rotor, Sorvall® RC-5B, Du Pont) at 6000 rpm for 15 minutes and at 4°C. The cells were resuspended in 25ml of sodium phosphate buffer, pH 7.0. The bacterial suspension was then mixed with 4.5ml of 100mM EDTA, pH 8.0 (this permeabilises the outer membranes) and 0.5ml of lysozyme (10mg/l) (this digests the cell wall). The mixture was then left to stand at room temperature for 5 minutes. The spheroplasts were stabilised by $CaCl_2$ (final concentration 10mM) and removed by centrifugation (GS3 rotor) at 6,000rpm for 30 minutes at 4°C. The supernatant containing periplasmic

proteins was collected ready for dialysis or for ammonium sulphate precipitation (Section 2. 10.4).

### 2.10.2 Matrix preparation and column packing

The pre-swollen anion exchangers were pre-equilibrated with a concentrated solution (0.5M) of buffer (either sodium phosphate buffer or Tris-Cl, adjusted to the required pH), by stirring the matrix with 6ml of buffer/g of wet ion-exchanger. The slurry was allowed to settle and the supernatant containing any broken down matrix ("fines") was decanted. Removal of fines is important as they can lead to uneven column packing. The above process was repeated several times until the buffer/ion-exchanger slurry was at the desired pH.

When preparing the column care was taken to remove all air bubbles to minimise any problems with column flow rates, by allowing a small amount of buffer to flow through the column outlet. At this stage, the flow rate of the column could be calibrated. The matrix was also degassed, by applying a vacuum to the matrix solution for 10 minutes. Twenty ml of thick slurry (the matrix comprised of 75% of the slurry volume) was poured down a glass rod into a column (dimensions: 1.6cm diameter x 15cm) and the matrix was packed to a height of 10cm. The use of a glass rod prevents the trapping of air bubbles in the matrix as it settles. The column was then attached to a buffer reservoir and the packed column was equilibrated with several column volumes of lower ionic strength starter buffer (either10-20mM sodium phosphate buffer or Tris-Cl buffer at the desired pH). The pH and

conductivity of the buffer was checked before application and after column elution to make sure that they remained the same.

### 2.10.3 Sample application

β-lactamase preparations were placed in dialysis tubing and dialysed against 2 x 1litre starter buffer, with a change of buffer after 4 hours before being applied to the equilibrated column. After the sample was loaded onto the column, the ion exchange matrix was washed with starter buffer to remove any unbound material (3-10 column volumes). The optical density of the eluent at $A_{280}$ was monitored until the flow-through contained negligible contaminants. β-Lactamase was eluted by a linear NaCl gradient, with a steady increase in the ionic strength from 0-1M NaCl prepared in starter buffer. An LKB-BROMMA 2070 fraction collector (Pharmacia Biotech, St Albans, Herts) collected the gradient-eluted samples in 3ml aliquots. Samples were assayed by spectrophotometry and IEF. Following sample elution, the column matrix was regenerated to remove any remaining contaminants according to the manufacturer's instructions.

### 2.10.4 Concentration of protein samples by ammonium sulphate precipitation

Ammonium sulphate precipitation was performed at 4°C with all solutions pre-cooled to this temperature. Ammonium sulphate was added in increments with constant stirring to a final concentration of 80%. Each addition of ammonium sulphate was made only after the previously added amount had completely dissolved.

When all the salt had been added the mixture was stirred for a further 30 minutes to allow equilibration of the solvent and protein. The mixture was then centrifuged (GS3 rotor, Sorvall® RC-5B, Du Pont) at 8000 rpm for 30 minutes at 4°C. The supernatant was decanted off and discarded. The wet precipitate was then ready in a minimum volume of appropriate buffer. The β-lactamase activity of the resuspended pellet and discarded supernatant was assayed by nitrocephin spot testing to check that the β-lactamase had been precipitated. Concentrated protein samples were dialysed against the appropriate buffer to remove all ammonium sulphate before further manipulations.

### 2.10.5 Gel filtration employing the Fast Protein Liquid Chromatography System (FPLC)

A Superose 12 HR 10/30 column (Pharmacia Biotech, St Albans, Herts), mounted on a Pharmacia FPLC system, was used for molecular mass estimations and β-lactamase purification. All solutions used for gel filtration were degassed and filtered through a 0.22μM filter (Millipore, Watford, Herts). The Superose 12 column was stored in 20% ethanol and was washed with pyrogen free water (MilliQPF, Millipore) to remove the ethanol. This wash was followed by equilibration of the column with 2 x 25ml column volumes of column buffer (20mM sodium phosphate buffer, pH 7.0, containing 50mM NaCl).

The sample was centrifuged before application to the column. A maximum of 200μl in volume, containing no more than 5-10mg of protein (or 30mg of protein/ml) was

loaded onto the column. The column was run at a flow rate of 0.4ml/minute. After sample loading the proteins were eluted with the column buffer and collected in 0.5ml fractions. Fractions were assayed by spectrophotometry and IEF.

The column was calibrated by running lmg of four different proteins (bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome C), of known molecular mass, through the column. The separation of these proteins, as they passed through the column, could be detected by measuring the $A_{280}$ on a UV wavelength monitor attached to the column outlet. A plot of molecular mass against elution volume could be generated, forming a standard graph from which the molecular mass of a β-lactamase could be determined.

## 2.11 Preparation of outer membrane proteins (OMPs)

OMPs were prepared from a 250ml nutrient broth culture grown with shaking overnight at 37°C. Cells were harvested by centrifugation (GS3 rotor, Sorvall® RC-5B, Du Pont) at 8000 rpm for 15 minutes and at 4°C. The cell pellet was washed twice in phosphate-buffered saline (PBS) and centrifuged as above. The washed cells were resuspended in 2.5ml of pyrogen-free water (MilliQPF, Millipore, Watford, Herts) and placed in an ice/water bath before bursting the cells by ultrasonication (MSE Soniprep 150) at 8μm for 8 x 30 seconds with 1 minute cooling intervals. Sarkosyl was added to a final concentration of 0.7% to solubilise the cytoplasmic membrane. The lysate was then centrifuged (H1000B rotor, Sorvall® RT 600D) at 3000 rpm for 15 minutes at 4°C to remove any unbroken cells and

debris. The sarkosyl insoluble OMPs were harvested by centrifugation (SM-24 rotor, Sorvall® RC-5B) at 20,000 rpm for 1 hour at 4°C. The OMPs were washed with pyrogen-free water and centrifuged as above. The pellet was finally resuspended in 1ml of pyrogen-free water and stored at -20°C.

## 2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gels, with 12% acrylamide in the separating gel, were prepared according a standard protocol (Maniatis *et al.*, 1982) using a minigel apparatus (ProteinII, Bio-Rad). Prior to electrophoresis, samples were mixed with an equal volume of SDS loading buffer (50mM Tris-Cl buffer adjusted to pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate, 0.1% (w/v) bromophenol blue and 100mM dithiothreitol). The samples were boiled for 5 minutes at 100°C and a maximum of 25µl of each sample could be loaded into each gel well. The gel was run at 25mA for approximately 1 hour until the dye front had nearly reached the bottom of the gel.

## 2.13 Preparation of DNA

### 2.13.1 Small-scale preparation of chromosomal DNA

Small-scale quantities of chromosomal DNA were prepared according to the method described in Unit 2.4A of Current Protocols in Molecular Biology (Wilson, 1994), which includes a hexadecyltrimethyl ammonium bromide (CTAB) extraction for the removal of polysaccharides and proteins. The DNA pellet was redissolved overnight at 4°C in 100µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0) containing ribonuclease A (50µg/ml).

For convenience, chromosomal DNA was also prepared with the Genie genomic DNA extraction kit (Helena BioSciences, Sunderland, Tyne and Wear).

### 2.13.2 Large-scale preparation of chromosomal DNA

Large-scale quantities of chromosomal DNA were prepared according to the method described in Unit 2.4A of Current Protocols in Molecular Biology (Wilson, 1994). Additional purification on a caesium chloride gradient was however, omitted from the protocol, which was essentially a scale up of the genomic DNA miniprep. This procedure was employed to obtain large amounts of genomic DNA for the construction of a genomic DNA library from *A. veronii* bv. *sobria* strain 13.

### 2.13.3 Small-scale plasmid preparation

Small-scale quantities of plasmid DNA were prepared by means of a Hybaid Recovery™ plasmid miniprep kit (Teddington, Middlesex).

### 2.13.4 Large scale plasmid preparation

Large-scale quantities of plasmid DNA was prepared by means of either a QIAGEN midi or QIAGEN maxi plasmid purification kit (QIAGEN Ltd., Crawley, W. Sussex). Purification of high copy number plasmids using the QIAGEN midi kit routinely yielded 75-100μg of DNA, whereas, the maxi kit yielded 300-500μg of DNA.

## 2.14 Agarose gel electrophoresis

Standard agarose gel electrophoresis was applied for both analytical and preparative separation of DNA fragments. Agarose gels were prepared with 1 x Tris/acetate (TAE) buffer diluted from a 10 x TAE stock solution (1litre volume containing 400mM Tris-acetic acid, 20mM disodium EDTA, pH 8.0). The appropriate concentration (typically 1-2%) of electrophoresis grade agarose (Gibco BRL, Life Technologies, Paisley, Glasgow) was added to the electrophoresis buffer and melted in a microwave oven, with intermittent swirling to ensure even mixing. The melted agarose was cooled to 55°C before pouring into a sealed gel casting platform, fitted with a gel comb. After approximately 30 minutes, the gel was placed in an

electrophoresis tank and 1 x TAE buffer was placed in the tank to cover the gel to the depth of about 1mm. DNA samples containing 1 x tracking buffer were loaded onto the gel (10 x tracking buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (w/v) sucrose). A standard molecular weight marker was among the samples loaded onto the gel (λ DNA cut with *Hin*dIII or 100bp ladder) and these were obtained from Gibco BRL, Life Technologies. Electrophoresis was carried out between 50-100V and the progress of the separation was monitored by the migration of the dyes in the tracking buffer (bromophenol blue migrates with DNA molecules around 0.5 kb and xylene cyanol with DNA fragments of around 5kb).

Ethidium bromide (0.5 μg/ml) was incorporated into the gel and electrophoresis tank buffer for direct visualisation of DNA with a UV transilluminator (UV Products, Cambridge), following electrophoresis. Alternatively, gels that were run in the absence of ethidium bromide were stained by covering the gel in a solution of ethidium bromide (0.5 μg/ml in water) for 30 minutes. The gels were photographed with a Polaroid camera fitted with a Tiffen orange filter (setting B, F8).

## 2.15 Enzymic manipulation of DNA

### 2.15.1 Digestion of DNA with restriction endonucleases

Complete restriction endonuclease cleavage of DNA was commonly carried out in 20μl reaction volumes containing 0.1-4μg of DNA, 1 x appropriate restriction buffer (Helena BioSciences, Sunderland, Tyne and Wear) and 1-5 units of restriction

enzyme/μg of DNA. The amount of DNA to be cleaved and/or the reaction volume was increased or decreased if necessary, keeping the proportions of the reaction components constant. The reaction mixture was incubated at the recommended temperature (in general 37°C) for 2 hours. If the products were to be analysed by agarose gel electrophoresis the reaction was stopped by adding 5μl (20% of reaction volume) of 10 x tracking buffer. Otherwise the reaction was terminated by either extracting the DNA with phenol and precipitation in ethanol according to the method described in Current Protocols in Molecular Biology Unit 2.1 (Moore, 1994), or, if appropriate, the restriction enzyme was heat inactivated.

### 2.15.2 Preventing self-ligation of vector termini

Self-annealing of linearised vector during ligation reactions was prevented by pre-treatment with calf intestine alkaline phosphatase (CIP) (Boehringer Mannheim, Lewes, E. Sussex). CIP catalyses the hydrolysis of 5'-phosphate residues from DNA, leaving dephosphorylated products that possess 5'hydroxyl termini. One μg of vector DNA was first restricted with the appropriate restriction enzyme in a total reaction volume of 50μl. One μl of CIP was added to the reaction and incubation was continued for 1 hour at 37°C. The reaction was terminated by extracting the DNA with phenol and precipitation in ethanol according to the method described in Current Protocols in Molecular Biology Unit 2.1 (Moore, 1994).

### 2.15.3 DNA Ligation

Ligation of DNA was performed using T4 DNA ligase (Boehringer Mannheim, Lewes, E. Sussex) with 1x ligation buffer. The total reaction volume was 10µl containing a 3:1 molar ratio of insert DNA to vector. Ligation reactions were incubated overnight at 16°C.

## 2.16 Electrotransformation

### 2.16.1 Preparation of competent cells

1ml of an overnight culture was added to a 200ml prewarmed broth and the culture was incubated with shaking 4 hours until the mid-logarithmic phase had been reached. Cells were harvested by centrifugation (GS3 rotor, Sorvall® RC-5B) at 6000 rpm for 15 minutes and at 4°C and resuspended in 10ml of ice cold sterile distilled water. The suspension was then diluted to 200ml with ice cold water and the cells pelleted as described previously. Cells were washed twice more as described above. The thoroughly washed pellet was resuspended in an ice-cold solution of 30% (v/v) glycerol, where it was further washed before finally being suspended in 500µl of ice-cold 10% (v/v) glycerol. Cells were distributed into 50µl aliquots and stored at -70°C.

### 2.16.2 Transformation

Before transformation, ligated DNA samples were dialysed with a "drop dialysis technique" (Marusyk and Sergeant, 1980). A 0.025μm pore size diameter, Millipore "V" series membrane was floated (shiny side uppermost) in a petri-dish that was filled with 15ml of distilled water. The DNA sample was carefully placed onto the membrane and dialysed for at least 30 minutes at room temperature. The desalted sample was then recovered with a micropipet.

Competent cells and 1-2μl of dialysed DNA were added to a chilled, disposable electroporation cuvette and mixed thoroughly. The outside of the cuvette was wiped dry and the cuvette lightly tapped to remove all air bubbles and ensure that the sample was distributed evenly in the bottom of the cuvette. One pulse was applied (Bio-Rad, 2.5kV, 25μFD, 200Ohm) and immediately 1 ml of pre-warmed nutrient broth medium was added. Electroporated cells were then transferred to a 1.5ml microcentrifuge tube and incubated for 2 hours at 37°C. After incubation 50-200μl aliquots were spread on to selective agar.

## 2.17 Polymerase Chain Reaction (PCR)

### 2.17.1 Design of oligonucleotide primers

Primers were designed with Primer 3 Software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and by reference to previously published data. The primers

were synthesised by Oswell (Southampton University, Southampton), the details of primers are referred to in the relevant chapters

### 2.17.2 PCR procedure

PCR amplification was performed in a 100µl volume with the Techne PHC-2 Dri-Block Cycler (Cambridge Bioscience, Cambridge). Reaction mixtures contained 1 x $MgCl_2$-free PCR buffer (Promega, Southampton), 0.5µM of each primer, approximately 25ng of template DNA, 2.5 units of Taq DNA polymerase (Promega), 200µM (each) deoxynucleoside triphosphate (Boehringer Mannheim, Lewes, E. Sussex), 1-3mM $MgCl_2$ (Promega). A negative control consisting of the reaction mixture minus template DNA was included for each experiment an assay for overt contamination. The contents of each PCR tube were mixed and overlaid with 50µl of mineral oil to prevent evaporation of the PCR reaction mixture. PCR cycling parameters are listed with primers in the relevant chapters. PCR products were run on a 1% agarose gel (Gibco BRL, Life Technologies, Paisley), stained with ethidium bromide and visualised under UV light.

## 2.18 DNA hybridisation techniques

### 2.18.1 Labelling of DNA probes

DNA fragments to be used as probes were labelled with fluorescein-11-dUTP (F1-dUTP) by means of the ECL™ random prime labelling and detection system (Amersham, Life Science, Buckinghamshire) according to the manufacturer's instructions.

### 2.18.2 Dot blotting

A positively charged nylon membrane (Hybond™-N+, Amersham Life Science, Buckinghamshire) was lightly marked with a grid to guide the application of target DNA samples, allowing 1 $cm^2$/2µl sample. DNA samples were diluted with an equal volume of 2x SSC containing carrier DNA (GeneBloc, Helena BioSciences, Sunderland, Tyne and Wear) at a final concentration of 2ng/µl. DNA samples were then denatured by boiling for 5 minutes followed by immediate chilling on ice. Two µl samples of denatured DNA were applied to the membrane, the filter was left to dry and baked for two hours at 80°C in a Bio-Rad slab drier (Hemel Hempstead, Herts).

### 2.18.3 Southern blotting

DNA fragments were separated by agarose gel electrophoresis and Southern blotted on to a positively-charged nylon membrane (Hybond™-N+, Amersham Life Science, Buckinghamshire), according to the method described in Unit 2.9A of Current Protocols in Molecular Biology (Brown, 1993)

### 2.18.4 Hybridisation

Hybridisations with heat-denatured fluorescein-labelled DNA probes were carried out in either heat sealable bags or plastic boxes. Positive hybridisation results were detected by the ECL™ Random prime system (Amersham Life Science, Buckinghamshire). The blots were hybridised overnight at 60°C according to the manufacturer's instructions and sheared denatured heterologous DNA (GeneBloc, Helena BioSciences, Sunderland, Tyne and Wear) was added to the hybridisation buffer to decrease non-specific hybridisation. The following stringency washes were carried out: 2 x 15 minute washes with 1 x SSC and 0.1% (w/v) SDS, followed by 2 x 15 minute washes with 0.05 x SSC and 0.1% (w/v) SDS at 60°C. The hybridisation signals were recorded by placing a blot with a sheet of blue light sensitive autoradiography film (Hyperfilm™-ECL, Amersham Life Science) in a film cassette and exposing the film for 30 minutes.

## 2.19 Elimination of plasmids by ethidium bromide curing

Ethidium bromide has been shown to be efficient in eliminating plasmids that carry drug resistance determinants (Bouanchaud *et al.*, 1969) and was employed to test whether an imipenem-hydrolysing β-lactamase was encoded by an extrachromosomal element.

Curing was performed with a series of tubes containing Nutrient Broth No.2 and ethidium bromide at concentrations ranging from 0.06-64mg/l. The tubes were inoculated with approximately $10^5$ organisms/ml and incubated overnight at 37°C, with shaking. The culture tube that contained the highest concentration of ethidium bromide and still allowed visible growth was plated on to Nutrient Agar and incubated overnight at 37°C. Between 40 and 50 individual colonies were inoculated from the Nutrient Agar plate into individual tubes containing 4.5ml of sterile saline (0.85% (w/v) NaCl). Cured strains were then identified by inoculating 10μl of each inoculated saline onto plates containing sub-inhibitory concentrations of imipenem (1/2 imipenem MIC). Ten μl of each inoculated saline was also inoculated on to plates with no imipenem. Following overnight incubation at 37°C plates were examined for colonies that did not grow on the antibiotic plate. β-Lactamase extracts were prepared from subcultures of the colonies that failed to grow at sub-inhibitory concentrations of imipenem. β-Lactamases were detected as described in Section 2.7 to see if there was a loss of a particular β-lactamase band.

# 3: RESULTS

## A study of the mechanisms involved in carbapenem resistance in *Pseudomonas aeruginosa* isolates from Japan

### 3.1 Introduction

Recently, there have been increasing reports from Japan of carbapenem-resistant clinical isolates, belonging to both the *Enterobacteriaceae* and *Pseudomonaceae* families, some of which produce a metallo-β-lactamase designated IMP-1 (Section 1.11.1.5). Therefore, the following study was undertaken to evaluate the role of β-lactamases in conferring carbapenem resistance in clinical isolates from Japan. The contribution of the IMP-1 β-lactamase, chromosomal cephalosporinases and novel carbapenemases were all examined.

### 3.2 Background to bacterial strains

Sixty-one *P. aeruginosa* clinical isolates were received from Japan. These strains were isolated from patients with respiratory and urinary tract infections from 21 different hospital centres (designated A-U in Table 3.1) in Japan, in 1994.

## 3.3 Susceptibility testing

Fifty-two of the 61 Japanese *P. aeruginosa* strains were originally reported as being imipenem-resistant, having an MIC of >4mg/l (Dr K. Sato - personal communication). On receipt of the strains, their susceptibility to imipenem was re-determined and in addition, the MICs of three other β-lactams, meropenem, carbenicillin and ceftazidime, and the quinolone ciprofloxacin, were also tested.

The susceptibility testing results are shown in Table 3.1; 47 of the isolates were resistant to imipenem (MIC >4mg/l), 25 of these strains were also resistant to meropenem (MIC >4mg/l). Four strains were resistant to meropenem (MIC >4mg/l), but not imipenem. Twenty-two strains were resistant to carbenicillin (MIC >128mg/l), 25 were resistant to ceftazidime (MIC >2mg/l) and 23 isolates were resistant to ciprofloxacin (MIC >4mg/l).

## 3.4 Isoelectric focusing of β-lactamases from *P. aeruginosa* isolates

Isoelectric focusing was performed on small-scale crude cell extracts prepared from the 61 *P. aeruginosa* strains. After staining the IEF gel with nitrocephin, 74% of the *P. aeruginosa* strains were found to possess one or more β-lactamase (Table 3.1). The most prevalent β-lactamase had a pI value of 9.2 was found in 41% of isolates (Table 3.1). Metallo-β-lactamases in *P. aeruginosa* have been reported to have pIs of 9.0 and 9.5 (Watanabe *et al.*, 1991; Minami *et al.*, 1996). The chromosomal cephalosporinases of *P. aeruginosa* also have high pI values (Bush *et al.*, 1995).

## Table 3.1 Antibiotic susceptibility of 61 clinical *P. aeruginosa* isolates and their β-lactamase profiles

| Strain | Hospital Unit (A-U) | MIC (mg/L)[a] IMP | MPM | CARB | CTAZ | CIPRO | pI(s) |
|---|---|---|---|---|---|---|---|
| 10662* | - | 1 | 0.25 | 32 | 1 | 0.12 | 8.7 |
| 2 | A | 8 | 8 | 256 | 16 | 4 | 9.2 |
| 3 | A | 16 | 8 | 64 | 2 | 0.12 | 9.3 |
| 8 | A | 8 | 8 | 128 | 2 | 2 | ND |
| 4 | B | 8 | 32 | >256 | 4 | >8 | 5.4, 8.7 |
| 5 | B | 2 | 8 | 256 | 2 | 0.25 | 8.2 |
| 6 | B | 8 | 2 | 32 | 1 | 0.5 | ND |
| 13 | B | 8 | 32 | >256 | 4 | >8 | 5.3 |
| 14 | B | 8 | 32 | >256 | 16 | >8 | 5.4 |
| 16 | B | 2 | 2 | 128 | 2 | 0.25 | ND |
| 24 | B | 8 | 1 | 32 | 1 | 0.5 | ND |
| 22 | C | 8 | 2 | 64 | 2 | 0.12 | ND |
| 41 | C | 8 | 1 | 16 | 0.25 | 0.064 | 9.2 |
| 23 | D | 8 | 4 | 128 | 32 | 0.064 | 9.2 |
| 25 | E | 16 | 8 | 256 | 32 | >8 | 9.2 |
| 26 | E | 8 | 4 | 32 | 1 | 0.25 | 9.2 |
| 79 | E | 16 | 8 | 128 | 2 | 0.25 | 5.3, 9.2 |
| 101 | E | 4 | 1 | >256 | 32 | >8 | 9.2 |
| 30 | F | 16 | 8 | 128 | 32 | >8 | 9.2 |
| 38 | F | 8 | 2 | 32 | 1 | >8 | 9.2 |
| 39 | F | 8 | 16 | 256 | 4 | 8 | 9.2 |
| 34 | G | 4 | 8 | 256 | 32 | >8 | 8.95 |
| 35 | G | 8 | 8 | 256 | 4 | 0.25 | 9.2 |
| 36 | G | 8 | 0.25 | 64 | 2 | 8 | ND |
| 37 | G | 16 | 32 | 256 | 16 | >8 | 9.2 |

[a] – Blue numbers represent a resistant MIC, black numbers equal sensitive MIC.

**10662*** - *P. aeruginosa* NCTC 10662 = sensitive laboratory control strain.

**IMP**, imipenem; **MPM**, meropenem; **CARB**, carbenicillin; **CTAZ**, ceftazidime; **CIPRO**, ciprofloxacin; **ND**, not detected.

**Table 3.1 Antibiotic susceptibility of 61 clinical *P. aeruginosa* isolates and their β-lactamase profiles (continued)**

| Strain | Hospital Unit (A-U) | MIC (mg/l)[a] IMP | MPM | CARB | CTAZ | CIPRO | pI(s) |
|---|---|---|---|---|---|---|---|
| 43 | H | 8 | 8 | 256 | 4 | >8 | 9.2 |
| 44 | H | 16 | 8 | 64 | 4 | 0.25 | 9.2 |
| 50 | H | 16 | 8 | 128 | 2 | >8 | 9.3 |
| 52 | H | 16 | 4 | 128 | 2 | 0.064 | 9.2 |
| 45 | I | 8 | 8 | 256 | 4 | >8 | 8.7 |
| 46 | I | 8 | 2 | 32 | 1 | 1 | 9.2 |
| 93 | I | 4 | 8 | 128 | 4 | >8 | 8.5, 9.2 |
| 48 | J | 8 | 8 | 64 | 4 | 0.12 | 8.95 |
| 60 | J | 64 | 32 | >256 | 32 | >8 | 8.95 |
| 54 | K | 8 | 4 | 128 | 2 | 0.12 | ND |
| 55 | K | 16 | 16 | 256 | 16 | 8 | 9.2 |
| 64 | K | 2 | 4 | 128 | 4 | >8 | ND |
| 86 | K | 1 | 0.25 | 128 | 2 | 0.25 | 8.3, 8.6 |
| 65 | K | 1 | 0.032 | 32 | 1 | 0.25 | ND |
| 56 | L | 8 | 2 | 64 | 2 | 0.25 | 8.4, 8.95 |
| 83 | L | 8 | 2 | 64 | 1 | 0.064 | 8.9 |
| 67 | M | 8 | 8 | 64 | 2 | 0.12 | 9.2 |
| 68 | M | 16 | 4 | 32 | 2 | 0.25 | 9.2 |
| 69 | M | 16 | 8 | 64 | 2 | 0.25 | ND |
| 84 | M | 2 | 0.25 | 32 | 1 | 0.5 | ND |
| 71 | N | 8 | 2 | 32 | 1 | 0.12 | 8.5, 9.2 |
| 73 | N | 4 | 8 | 128 | 2 | 0.25 | ND |
| 74 | N | 8 | 1 | 16 | 0.5 | 0.064 | ND |
| 80 | O | 8 | 16 | >256 | 2 | >8 | 9.3 |
| 98 | O | 2 | 1 | >256 | 2 | >8 | 8.4, 9.2 |
| 100 | O | 8 | 2 | 32 | 1 | 0.25 | 8.95 |

[a] – Blue numbers represent a resistant MIC, black numbers equal sensitive MIC.

**IMP**, imipenem; **MPM**, meropenem; **CARB**, carbenicillin;

**CTAZ**, ceftazidime; **CIPRO**, ciprofloxacin; **ND**, not detected.

**Table 3.1 Antibiotic susceptibility of 61 clinical *P. aeruginosa* isolates and their β-lactamase profiles (continued)**

| Strain | Hospital Unit (A-U) | MIC (mg/l)[a] IPM | MPM | CARB | CTAZ | CIPRO | pI(s) |
|---|---|---|---|---|---|---|---|
| 87 | P | 2 | 2 | 256 | 4 | 0.5 | 9.2 |
| 94 | P | 8 | 8 | >256 | 2 | >8 | 5.4 |
| 102 | P | 4 | 2 | 32 | 1 | 0.12 | 8.8 |
| 90 | Q | 16 | 4 | 64 | 2 | 0.12 | ND |
| 96 | R | 8 | 2 | 64 | 2 | 0.12 | ND |
| 97 | R | 8 | 4 | 64 | 1 | 0.12 | ND |
| 103 | R | 16 | 2 | 32 | 1 | 0.12 | 8.8 |
| 62 | S | 16 | 64 | >256 | 16 | >8 | 8.3, 9.2 |
| 63 | T | 4 | 2 | 256 | 8 | >8 | 9.3 |
| 66 | U | 16 | 8 | 256 | 4 | 0.5 | ND |
| 75 | U | 8 | 4 | 32 | 4 | 0.25 | 5.3, 9.2 |

[a] – Blue numbers represent a resistant MIC, black numbers equal sensitive MIC.
**IMP**, imipenem; **MPM**, meropenem; **CARB**, carbenicillin;
**CTAZ**, ceftazidime; **CIPRO**, ciprofloxacin; **ND**, not detected.

## 3.5 PCR detection of the metallo-β-lactamase gene $bla_{IMP}$

It has been shown by Senda *et al.*, (1996b) that PCR can be successfully applied to rapidly detect an intragenic 587 bp fragment of the $bla_{IMP}$ gene in clinically isolated Gram-negative rods. Therefore, PCR with $bla_{IMP}$ specific primers was used to determine whether any of the 61 Japanese *P. aeruginosa* isolates possessed this gene.

Five IMP-1 producing strains (*S. marcescens* TN9106, *S. marcescens* AK9374, *S. marcescens* FHSM4055, *P. aeruginosa* M18 and *P. aeruginosa* 101/1477) were employed as positive PCR controls and three standard laboratory strains (*E. coli*

NCTC 10418, *P. aeruginosa* NCTC 10662 and *S. marcescens* NCTC 1377) were employed as negative controls.

The PCR primer sequences are shown in Figure 3.1 (Senda *et al.*, 1996b). The PCR procedure was performed, as described in Section 2.17.2, with 1.5mM $MgCl_2$. The PCR cycling parameters are shown in Table 3.2, however, the annealing temperature was increased from the published 55°C (Senda *et al.*, 1996b) to 60°C because non-specific DNA fragments were amplified for *P. aeruginosa* NCTC 10662 and *S. marcescens* NCTC 1377 when annealing was performed at 55°C.

**Figure 3.1 PCR primers for *bla*$_{IMP-1}$**

| Sequence | Position[a] |
|---|---|
| 5′ CTA CCG CAG CAG AGT CTT TG 3′ | 1241 |
| 5′ AAC CAG TTT TGC CTT ACC AT 3′ | 1808 |

[a] Numbering is according to Arakawa *et al.*, (1995).

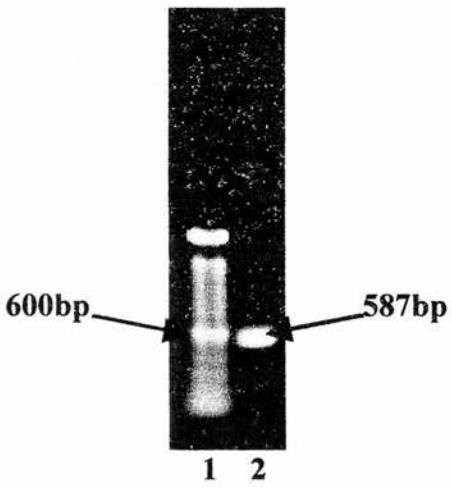
**Table 3.2 PCR cycling parameters for the amplification of *bla*$_{IMP}$**

| Segment | Temperature (°C) | Time (minutes) | Function | No. of cycles |
|---|---|---|---|---|
| 1 | 94 | 2 | Denaturation | 1 |
| 2 | 94 | 1 | Denaturation | x30 |
| | 60 | 1 | Annealing | |
| | 72 | 1.5 | Extension | |
| 3 | 72 | 10 | Final extension | 1 |

## 3.6 PCR detection of *bla*$_{IMP}$ in the 61 *P. aeruginosa* strains from Japan

All of the 61 *P. aeruginosa* strains were subjected to PCR analyses to detect the *bla*$_{IMP}$ gene, however, all these strains were found to be *bla*$_{IMP}$ negative. A 587 bp DNA fragment could be amplified from the five IMP-1 positive control strains (Figure 3.2). Template DNA was prepared from the bacteria strains by using the Genie genomic DNA extraction kit, although boiling a single colony suspended in 25μl of pyrogen-free water for 10 minutes was an equably suitable method and furthermore, it was more convenient.

**Figure 3.2 Amplification of a 587bp intragenic *bla*$_{IMP}$ PCR product from an IMP-1 producing strain**

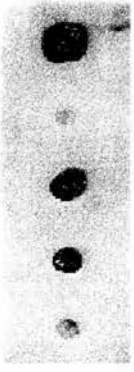



**Lanes: 1**, 100bp DNA ladder; **2**, 587bp PCR product from *bla*$_{IMP}$ positive control strain.

## 3.7 DNA hybridisation with an intragenic *bla*$_{IMP}$ gene probe

Total genomic DNA samples from the 61 *P. aeruginosa* strains were dot blotted and hybridised with an intragenic *bla*$_{IMP}$ gene probe. The probe consisted of the 587bp DNA fragment generated by PCR from *S. marcescens* TN9106. Gene probing was performed because it is less specific than PCR; however, this method also confirmed these 61 Japanese *P. aeruginosa* strains to be negative for an IMP-1-like gene. Figure 3.3 shows a dot blot of the five IMP-1 positive strains hybridised with the *bla*$_{IMP}$ probe.

**Figure 3.3 Dot blot of DNA from five IMP-1 positive strains hybridised with *bla*$_{IMP}$**



*S. marcescens* TN9106

*S. marcescens* AK9374

*P. aeruginosa* M18

*P. aeruginosa* 101/1477

*S. marcescens* FHSM4055

## 3.8 Imipenem hydrolysis assays using small-scale β-lactamase extracts

Hydrolysis of imipenem was assayed for spectrophotometrically, using small-scale β-lactamase preparations. Efficient hydrolysis of imipenem could be easily detected using small scale β-lactamase extracts (prepared in 50mM sodium phosphate buffer, pH 7.0) from IMP-1 positive control strains. However, no imipenem hydrolysis was detected in any of the 61 Japanese *P. aeruginosa* strains by this method.

## 3.9 The role of chromosomal cephalosporinases in imipenem resistance

It is well documented that imipenem resistance in *P. aeru*ginosa is often caused by decreased expression of the outer membrane protein D2 (Section 1.10.1.1). In addition to the loss of D2, imipenem resistance in *P. aeruginosa* is also known to require the activity of the derepressed AmpC chromosomal molecular class C cephalosporinase (Section 1.10.1.1). A simple method described by Zhou *et al.*, (1993) was applied to determine whether the chromosomal cephalosporinase was involved in the imipenem/meropenem resistance mechanism in any of the 61 Japanese *P. aeruginosa* isolates and also IMP-1 positive controls. MICs of imipenem and meropenem were determined either alone or in the presence of a fixed concentration (4mg/l) of the serine β-lactamase inhibitor BRL42715. The MIC of BRL42715 alone was also determined to demonstrate that it is the β-lactamase

inhibitor role of BRL42715 that results in any decrease in the carbapenem MICs and not its antibiotic effect.

Two *P. aeruginosa* mutant strains, M1405β-con D2$^-$ and M2297β-con D2$^-$, that have derepressed AmpC β-lactamase expression and lack D2 were employed as positive controls. Negative controls included M1405β-def D2$^-$, M2297β-def D2$^-$, that are non-inducible β-lactamase basal mutants that lack the D2 porin and, therefore, should show no decrease in imipenem MIC in the presence of BRL42715. The effects of BRL42715 on the imipenem and meropenem MICs of the control strains are shown in Table 3.3.

The MIC for BRL42715 alone was >128mg/L for both control strains and test strains and, therefore, any decrease in MIC was the result of β-lactamase inhibition by BRL42715 and not its antibiotic effect.

In the presence of BRL42715 a four- to 16-fold decrease in the imipenem MIC is indicative of the involvement of a cephalosporinase in the mechanism of imipenem resistance (Zhou *et al.*, 1993) and can be seen in Table 3.3 for the two positive control strains (M1405β-con D2$^-$ and M2297β-con D2$^-$). The meropenem MICs of these control strains are little affected by BRL42715, regardless of the levels of β-lactamase expressed. When this method was carried out on the 61 *P. aeruginosa* strains from Japan, between a four- to 16-fold decrease in imipenem MIC was shown for 57 of the 61 strains examined and only two strains showed a four-fold decrease in their meropenem MIC, the rest being unaffected. By adding BRL42715 in

combination with imipenem the imipenem MICs for the Japanese *P. aeruginosa* strains were all less than the breakpoint for resistance (4mg/L).

The imipenem and meropenem MICs of five IMP-1 positive strains were also tested in the presence and absence of BRL42715 but neither the imipenem nor, meropenem MICs were affected by BRL42715.

**Table 3.3 The effect of the serine β-lactamase inhibitor BRL42715 on imipenem and meropenem MICs of four *P. aeruginosa* mutant control strains**

| Strain | MIC (mg/L) | | | |
|---|---|---|---|---|
| | IMP | IMP + BRL42715 | MPM | MPM + BRL42715 |
| M1405β-con D2⁻ | 8 | 1 | 4 | 2 |
| M2297β-con D2⁻ | 8 | 1 | 2 | 1 |
| M1405β-def D2⁻ | 0.5 | 0.5 | 2 | 2 |
| M2297β-def D2⁻ | 0.5 | 0.5 | 1 | 1 |

**IMP**, imipenem; **MPM**, meropenem.

## 3.10 Determining whether imipenem-resistant *P. aeruginosa* strains are β-lactamase derepressed

Eight of the imipenem-resistant Japanese *P. aeruginosa* strains were chosen for nitrocephin hydrolysis assays. These assays were performed to determine whether β-lactamase derepression is a requirement for imipenem resistance. The eight selected strains included: four strains that showed a four- to 16-fold decrease in their imipenem MICs in the presence of BRL42715 (strains 3, 30, 60, 66), and four strains (2, 4, 39, 43) that showed no decrease in their imipenem MIC in the presence of BRL42715. Nitrocephin hydrolysis assays were performed with imipenem induced and non-induced large-scale β-lactamase extracts prepared in 25mM PIPES buffer, pH 7.0.

Strains 3, 30, 60 and 66 decreased their imipenem MICs in the presence of BRL42715 (Table 3.4); however this was not necessarily associated with derepressed β-lactamase activity, as three out of the four strains (3, 60, 66) showed a >25-fold increase in β-lactamase activity following induction with imipenem. The four strains (2, 4, 39, 43) that are resistant to imipenem but unaffected by BRL42715 also had variable levels of β-lactamase activity.

**Table 3.4 The specific activities of crude β-lactamase extracts from four *P. aeruginosa* strains that show between a four- to 16-fold decrease in their imipenem MIC in the presence of BRL42715 and from four strains that show no decrease in their imipenem MIC in the presence of BRL42715 (measured against nitrocephin)**

| Strain | Specific activity (sp act)* | | Induction ratio (induced/non-induced sp act) |
|---|---|---|---|
| | Noninduced | Induced | |
| 10662[a] | 1 | 479 | 479 |
| M18[b] | 460 | 2433 | 5 |
| 3[c] | 2 | 72 | 36 |
| 30 | 3649 | 3706 | 1 |
| 60 | 32 | 844 | 26 |
| 66 | 3 | 844 | 281 |
| 2[d] | 17 | 2021 | 118 |
| 4 | 462 | 594 | 1 |
| 39 | 115 | 5160 | 45 |
| 43 | 63 | 2220 | 35 |

* Nanomoles of nitrocephin hydrolysed/minute/mg of protein.

[a] *P. aeruginosa* NCTC 10662 sensitive laboratory control.

[b] *P. aeruginosa* M18 = IMP-1 positive strain.

[c] Four *P. aeruginosa* strains that show a four- to 16-fold decrease in their imipenem MIC in the presence of BRL42715.

[d] Four *P. aeruginosa* strains that show no decrease in their imipenem MIC in the presence of BRL42715.

## 3.11 Imipenem hydrolysis assays using large-scale β-lactamase extracts

*P. aeruginosa* strains 3, 30, 60, 66, 2, 4, 39 and 43 were chosen for further biochemical study. β-Lactamase extracts were prepared on a large scale in the presence and absence of imipenem as an inducer. 50mM sodium phosphate buffer, pH7.0 was replaced with 25mM PIPES buffer, pH 7.0 because some metallo-β-lactamases have been reported to be unstable in 50mM sodium phosphate buffer, pH 7.0 (Watanabe *et al.*, 1991) and 25mM PIPES, pH 7.0 has been recommended for metallo-β-lactamase assays (Payne and Farmer, 1998). Both induced and non-induced crude β-lactamase extracts were also prepared in the presence and absence of both 1μM and 100μM $ZnSO_4$ to ensure that any potential metallo-β-lactamases were clearly identified.

Hydrolysis of imipenem could not, however, be detected with any of the β-lactamase extracts prepared from these eight *P. aeruginosa* strains, under each condition used.

## 3.12 IEF and β-lactamase typing

Isoelectric focusing was repeated using the large-scale induced β-lactamase preparations from the eight *P. aeruginosa* representative strains (3, 30, 60, 66, 2, 4, 39, 43). Focusing of the β-lactamases was followed by overlaying the gels with BRL42715 (100μM), cloxacillin (100μM) and clavulanic acid (1mM) before staining with nitrocephin. The purpose of these inhibitor overlays was to provide further evidence that it is a molecular class C, Bush group 1 cephalosporinase that is involved in the imipenem resistance mechanism in the Japanese *P. aeruginosa* strains. BRL42715 inhibits serine β-lactamases, although it cannot be used to distinguish between the different classes of serine β-lactamases. Cloxacillin, however, is recognised as a good inhibitor of the molecular class C, Bush group 1 cephalosporinases, and clavulanic acid is a good inhibitor of molecular class A β-lactamases, but not class C cephalosporinases (Bush *et al.*, 1995). The TEM-1 β-lactamase was employed as a positive molecular class A β-lactamase control. The β-lactamase extract from *P. aeruginosa* NCTC 10662 was employed as a positive class C cephalosporinase control. The effect of these inhibitors on the β-lactamases from the eight *P. aeruginosa* strains are summarised in Table 3.5, where it can be seen that the high pI β-lactamases from the *P. aeruginosa* strains are inhibited by BRL42715 and cloxacillin, but not clavulanic acid. This suggests that all the β-lactamases are of molecular class C.

**Table 3.5 Effects of BRL42715, cloxacillin and clavulanic acid on β-lactamases from eight *P. aeruginosa* strains**

| Strain | β-lactamase pI(s) | Bands seen after treatment with: BRL42715 (100μm) | Cloxacillin (100μm) | Clavulanic acid (1mM) |
|---|---|---|---|---|
| TEM-1 | 5.4 | ND | 5.4 | ND |
| 10662 | 8.7 | ND | ND | 8.7 |
| 3 | 9.3 | ND | ND | 9.3 |
| 30 | 9.2 | ND | ND | 9.2 |
| 60 | 8.95 | ND | ND | 8.95 |
| 66 | 8.95 | ND | ND | 8.95 |
| 2 | 5.4, 8.7 | ND | 5.4 | 8.7 |
| 4 | 9.2 | ND | ND | 9.2 |
| 39 | 9.2 | ND | ND | 9.2 |
| 43 | 9.2 | ND | ND | 9.2 |

**ND**. not detected.

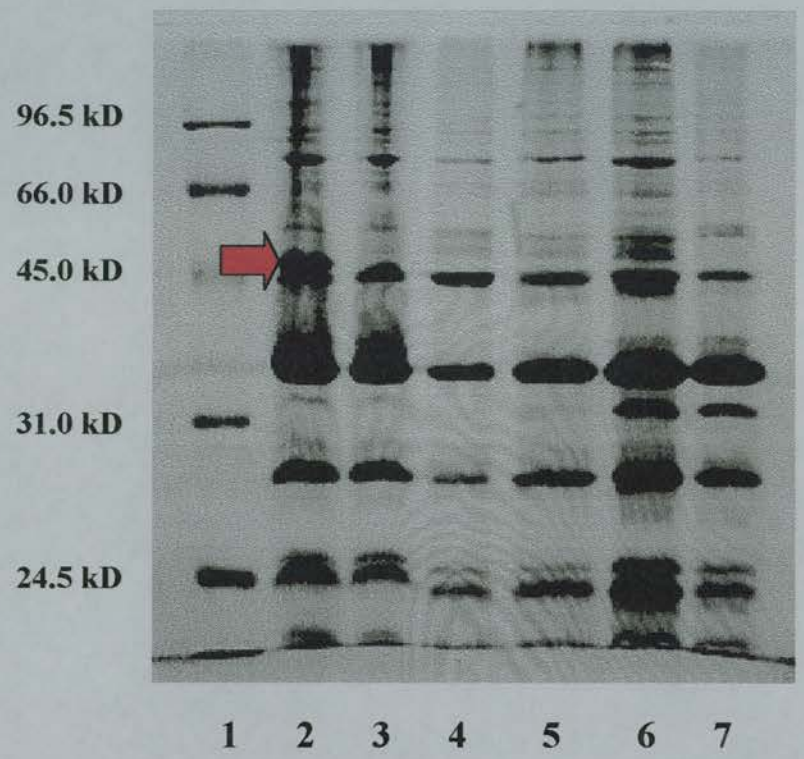
## 3.13 Analysis of outer membrane proteins

Outer membrane proteins were prepared from the eight selected *P. aeruginosa* strains (3, 30, 60, 66, 2, 4, 39, 43). The outer membrane proteins from these strains were examined to determine whether these isolates lacked the D2 outer membrane protein. Unfortunately, a comparison between the outer membrane proteins from the imipenem-resistant Japanese *P. aeruginosa* strains and their isogenic impenem-susceptible parents could not be made because the parent strains were not available. Instead the outer membrane profiles from these strains were compared with *P.*

*aeruginosa* M2297β-con and *P. aeruginosa* M2297β-con D2$^-$, these are control strains that produce and are deficient in the D2 porin respectively.

Figure 3.4 shows the outer membrane proteins profiles from four randomly selected *P. aeruginosa* strains (3, 30, 60, 66) that show a four- to 16-fold decrease in their imipenem MICs in the presence of BRL42715. These strains appear to have diminished production of the D2 porin when the SDS polyacrylamide gel was stained with Coomassie blue. Figure 3.5 shows that the four *P. aeruginosa* strains (2, 4, 39, 43), showing no decrease in imipenem MIC in the presence of BRL42715 are also deficient in the D2 porin.

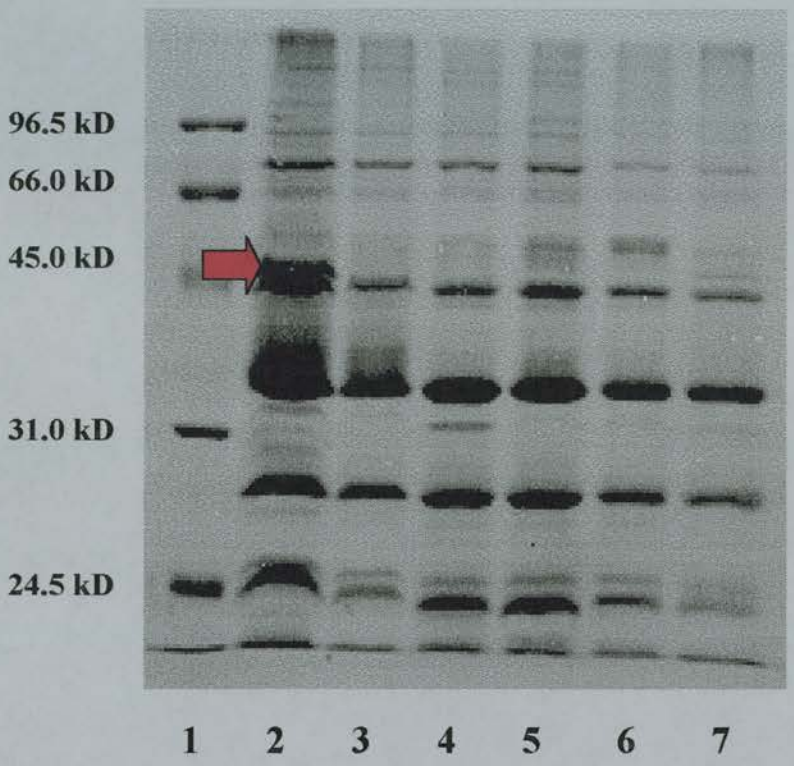
**Figure 3.4 Outer membrane protein profiles of four *P. aeruginosa* strains that show a four- to –16-fold decrease in their imipenem MIC in the presence of BRL42715**




Red arrowhead indicates D2 porin

**Lanes**: **1**, molecular weight markers (molecular weights listed to the left); **2**, M2297β-con; **3**, M2297β-con D2$^-$; **4**, strain 3; **5**, strain 30; **6**, strain 60; **7**, strain 66.

**Figure 3.5 Outer membrane protein profiles of four *P. aeruginosa* strains that show no decrease in their imipenem MIC in the presence of BRL42715**




Red arrowhead indicates D2 porin

**Lanes**: **1**, molecular weight markers (molecular weights listed to the left); **2**, M2297β-con; **3**, M2297β-con D2$^-$; **4**, strain 2; **5**, strain 4; **6**, strain 39; **7**, strain 43.

## 3.14 Discussion

When the 61 clinical *P. aeruginosa* isolates, included in this study were subjected to PCR with $bla_{IMP}$ primers they were all found to be negative for this apparently highly conserved gene. Furthermore, DNA hybridisation with an intragenic $bla_{IMP}$ gene probe also confirmed these strains to be negative for an IMP-1-like enzyme. These results are in agreement with previous studies (Senda *et al.*, 1996b) where all strains that have so far been found to harbour the $bla_{IMP}$ gene are highly resistant to almost every cephem, in particular ceftazidime (MIC >128mg/l). From Table 3.1 it can be seen that for the *P. aeruginosa* strains in this study, the highest recorded ceftazidime resistance was 64mg/l and therefore, none of these isolates fit the profile of previously reported IMP-1 producing strains.

Chromosomal cephalosporinases are ubiquitous amongst *P. aeruginosa.* By determining the MIC of imipenem alone and in the presence of a fixed concentration of the serine β-lactamase BRL42715, it was possible to show that the chromosomal cephalosporinase contributed significantly to imipenem resistance in the majority of the 61 strains included in this survey. A greater than two-fold decrease in the imipenem MIC was demonstrated in all but four of the 61 strains, which showed only a two-fold decrease. In contrast, inhibition of the chromosomal cephalosporinase by BRL42715 has little effect on the meropenem MICs and, therefore, the *P. aeruginosa* cephalosporinase is not a major contributor to meropenem resistance. This phenomenon is thought to be related to the greater stability of meropenem to the *P.*

*aeruginosa* chromosomal cephalosporinase when compared with imipenem (Livermore, 1992a).

The imipenem MIC of the five IMP-1 producing strains were not affected by BRL42715 and therefore the chromosomal cephalosporinase does not appear to be a contributing factor to imipenem resistance in these isolates.

Isoelectric focusing of β-lactamases from selected strains, followed by overlays with the inhibitors BRL42715, cloxacillin and clavulanic acid provided further evidence that the Japanese *P. aeruginosa* strains possess a class C cephalosporinase that is involved in the imipenem resistance mechanism. The results of Table 3.4 additionally show that derepression and thus high level production of the cephalosporinase is not necessarily a requirement for imipenem resistance because the strains in this study were either constitutive or inducible producers of β-lactamase. These results are in agreement with the findings of Zhou *et al.*, (1993). Analysis of the outer membranes of selected *P. aeruginosa* strains, however, did confirm that these strains are deficient in the carbapenem specific porin channel D2 (Figures 3.4 and 3.5).

Efficient hydrolysis of imipenem could not be detected from small-scale β-lactamase extracts prepared from the *P. aeruginosa* strains. Additionally, no efficient hydrolysis of imipenem could be detected from large-scale extracts, prepared under different conditions, from four strains that were unaffected by BRL42715, or from four randomly selected strains that displayed a significant decrease in their imipenem

MIC in the presence of BRL42715. Therefore, these results ruled out the involvement of a novel carbapenemase in the imipenem resistance mechanism. Active efflux systems (Nikaido, 1994) or other porin species (Livingstone *et al.*, 1995) may be contributing factors in imipenem resistance in the four strains that are unaffected by BRL42715.

Determining the mechanism of bacterial resistance to carbapenems in *P. aeruginosa* is a complex process. Synergism between a cephalosporinase and lowered outer membrane permeability is by far the most prevalent mechanism of imipenem resistance in the strains studied in this survey, which also highlights that the IMP-1-mediated mechanism of resistance is currently a much rarer event in *P. aeruginosa*. It remains to be seen whether the IMP-1 resistance mechanism becomes as widespread in *P. aeruginosa* as the one that involves the combination of a class C cephalosporinase and impermeability.

# 4: RESULTS

## A rapid substrate-based technique for the detection of carbapenemases after isoelectric focusing

### 4.1 Introduction

Matthews first described the application of analytical IEF for the characterisation of β-lactamases in 1975. IEF is a very useful technique to employ initially when comparing the β-lactamases produced by different bacteria. In the past IEF has been described as 'one of the most critical assays used to verify the identity of β-lactamases' (Bush *et al.*, 1988). The vast array of β-lactamases identified over the last 20 years (see Section 1.4.4) has meant that pI values are no longer sufficient to classify a β-lactamase and that additional biochemical and molecular data are now an essential requirement. Furthermore, it is now known that the carbapenem-hydrolysing metallo-β-lactamases of the genus *Aeromonas* poorly hydrolyse nitrocephin, the substrate conventionally used for the detection of β-lactamases after IEF and, therefore, go undetected in IEF gels (Hayes *et al.*, 1994; Walsh *et al.*, 1995a).

For the above reasons several modifications to IEF have been described to enhance the utility of this procedure including the identification of *Aeromonas* metallo-β-lactamases (Massida *et al.*, 1991; Payne *et al.*, 1994*a*; Walsh *et al.*, 1995a).

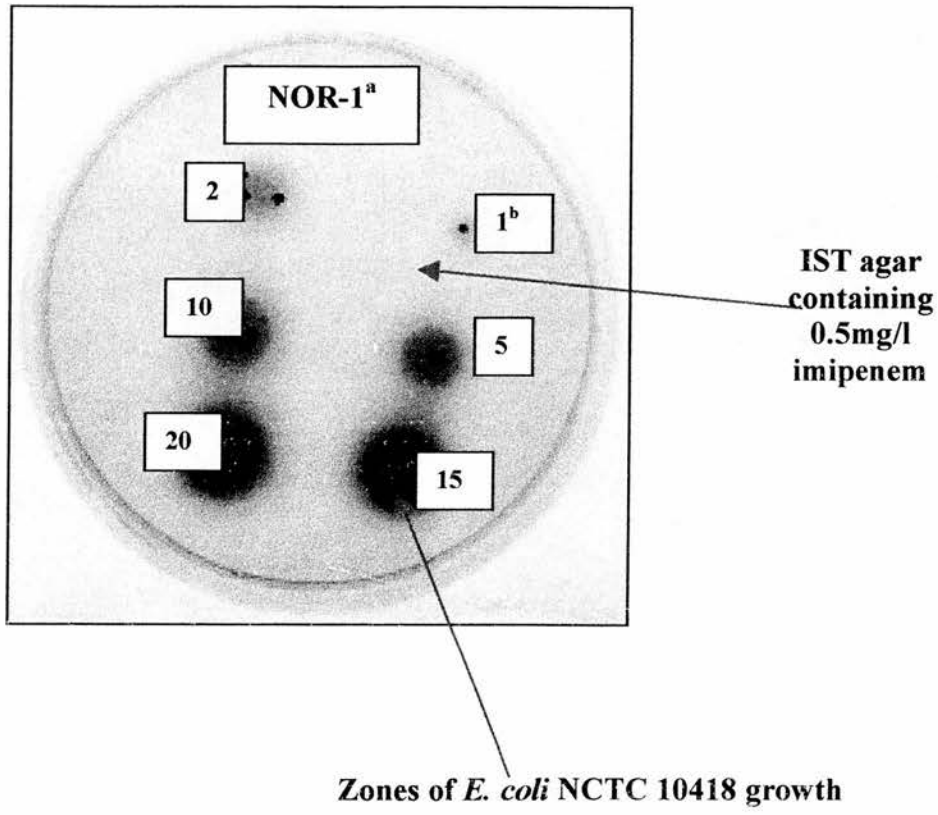
This section now describes the development of a modified substrate-based technique for the identification of carbapenemases after IEF. The principle behind the technique is that β-lactamases are allowed to focus on an IEF gel and this is then overlaid with agar containing imipenem (0.5mg/l) and an indicator organism (*E. coli* NCTC 10418) that is susceptible to imipenem (imipenem MIC - 0.12mg/l). After overnight incubation at 37°C, no growth of the indicator organism should be seen, except above the position where β-lactamases have focused and hydrolysed the imipenem.

## 4.2 Assessing the ability of a carbapenemase to hydrolyse imipenem in an agar overlay

A microbiological screening method was designed to assess whether the carbapenem-hydrolysing β-lactamase (NMC-A) from *Enterobacter cloacae* strain NOR-1 (Nordmann *et al.*, 1993) was able to hydrolyse imipenem in an agar overlay, allowing the *E. coli* NCTC 10418 indicator organism to grow. This involved pouring a layer of electrophoresis grade agarose into a petri dish and spotting different volumes (μl) of a large scale imipenem-induced (1/4 MIC) β-lactamase extract prepared from *E. cloacae* NOR-1 on top of the set agarose. The layer of agarose was covered with Iso-Sensitest (IST) agar containing 0.5mg/l imipenem and 100μl of a 1/100 dilution of an overnight *E. coli* NCTC 10418 broth culture. The volume of IST agar poured over the agarose surface was varied to determine the depth of agar that would give optimum results.

Figure 4.1 shows the results of this microbiological screening assay. The zone of *E. coli* NCTC 10418 growth was dependent on the amount of β-lactamase extract spotted onto the agarose and on the depth of agar poured over the surface of the agarose. This experiment demonstrated that it was important to apply only a thin layer of agar and a depth of approximately 2.5mm was considered to give optimum results. This method was also successfully repeated with imipenem-induced (1/4 MIC) β-lactamase extracts from carbapenemase-producing *Aeromonas hydrophila* strain T429125 (Walsh *et al.*, 1997) and *Serratia marcescens* S6 that produces the carbapenemase Sme-1 (Yang *et al.*, 1990).

**Figure 4.1 Microbiological screening assay**




[a] β-Lactamase extract from *E. cloacae* strain NOR-1

[b] Volume (μl) of β-lactamase extract spotted onto the layer of agarose

## 4.3 Imipenem/agar overlay modification with a polyacrylamide IEF gel

Once it had been established that carbapenemases could hydrolyse imipenem in an agar overlay, allowing *E. coli* NCTC 10418 to grow, this method could be applied after IEF. β-Lactamase extracts prepared from *E. cloacae* NOR-1, *S. marcescens* S6 and *A. hydrophila* T429125 were focused on a self-cast polyacrylamide gel. Repeated attempts were made to detect the carbapenemases by the imipenem/agar

overlay and an ampicillin/agar overlay. None of the carbapenemases could be detected with either of these β-lactams.

## 4.4 Imipenem/agar overlay modification with an agarose IEF gel

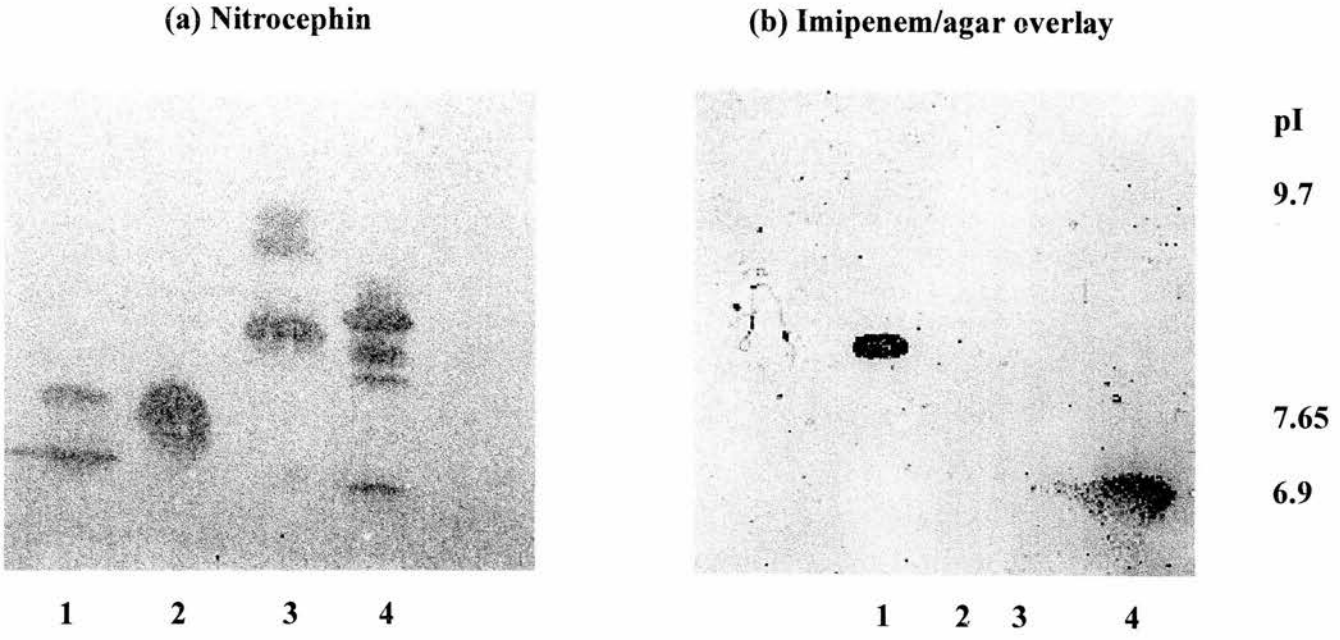
Two possible reasons why the carbapenemases could not be detected with the imipenem/agar overlay modification to a self-cast polyacrylamide IEF gel were (i) the pH gradient of the IEF gel was interfering with the growth of the *E.coli* NCTC 10418; or (ii) the polyacrylamide in the IEF gel, particularly unpolymerised acrylamide monomers, may have an adverse effect on the growth of *E.coli* NCTC 10418.

*E. coli* has a growth optimum between pH 6.0 and 7.0, although it will grow in pH conditions as low as 4.4 and as high as 9.0. Therefore, although the pH gradient may affect the growth of *E.coli* NCTC 10418 it would be expected that a β-lactamase with a pI value between 6.0 and 7.0 such as NMC-A (pI 6.9) would be detectable.

The effect of polyacrylamide was investigated by substituting a self-cast polyacrylamide IEF gel with an agarose IEF gel (Section 2.8.3). Replacing polyacrylamide gels with agarose gels confirmed that polyacrylamide gels were not suitable for the modified IEF technique, because carbapenemases that had previously gone undetected could now be identified. Figure 4.2 shows an agarose IEF gel that has been stained with (a) nitrocephin, compared with an agarose IEF gel that has been (b) overlaid with IST agar containing imipenem and *E. coli* NCTC 10418. In

Figure 4.2 two areas of growth can be seen in lanes 1 and 4. Lane 1 corresponds to *A. hydrophila* T429125 –the area of growth does not line up with either of the two β-lactamase bands that are visualised by nitrocephin overlay. This observation is in agreement with what is known about the metallo-β-lactamases of *Aeromonas*, that is they are not detectable with nitrocephin (Hayes *et al.*, 1994; Walsh *et al.*, 1995a). Lane 4 corresponds to *E. cloacae* NOR-1 and growth can be seen where NMC-A has focused (pI 6.9). Lane 3 contains the β-lactamase extract from *S. marcescens* S6, however the carbapenemase Sme –1 (pI 9.7) could not be detected. The β-lactamase OXA-7 (Medeiros *et al.*, 1985) was included on the gel as negative control (Lane 2).

**Figure 4.2 An agarose IEF gel overlaid with (a) nitrocephin and (b) IST agar containing imipenem (0.5mg/l) and *E. coli* NCTC 10418**

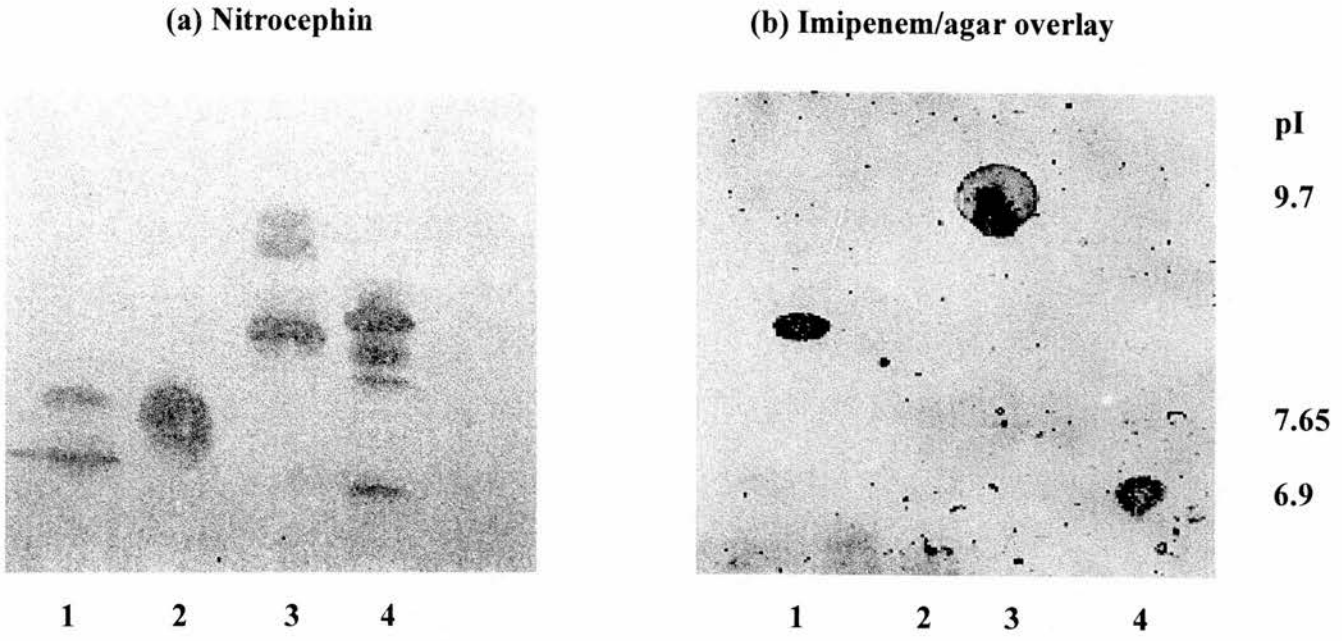



**Lanes:** **1**, *A. hydrophila* T419125 (pIs between 6.9-8.0); **2**, OXA-7 (pI 7.65); **3**, *S. marcescens* S6 (pIs 8.2, 9.7); **4**, *E. cloacae* NOR-1 (pIs 6.9, 9.3).

## 4.5 Employing a pH tolerant indicator for the detection of high pI carbapenemases

A pH tolerant *E. coli* NCTC 10418M mutant was selected by growing the organism on nutrient agar plates made up with Tris-Cl buffer, pH 10.0. *E. coli* NCTC 10418M was then employed as the indicator organism in the imipenem/agar overlay to determine whether the pH gradient of the IEF gel was affecting the growth of the *E. coli* NCTC 10418 parent strain above the Sme-1 carbapenemase, which has a much more basic pI value (pI 9.7) compared with the carbapenemases from *A. hydrophila* T429125 and *E. cloacae* NOR-1. Figure 4.3 shows that when the IEF gel was overlaid with agar containing imipenem and the pH tolerant mutant the Sme-1 carbapenemase from *S. marcescens* could be detected and, therefore, the pH of the IEF gel does affect the growth of the indicator organism.

**Figure 4.3 An agarose IEF gel overlaid with (a) nitrocephin and (b) IST agar containing imipenem (0.5mg/l) and *E. coli* NCTC 10418M**




**Lanes: 1**, *A. hydrophila* T419125 (pIs between 6.9-8.0)); **2**, OXA-7 (pI 7.65); **3**, *S. marcescens* S6 (pIs 8.2, 9.7); **4**, *E. cloacae* NOR-1 (pIs 6.9, 9.3).

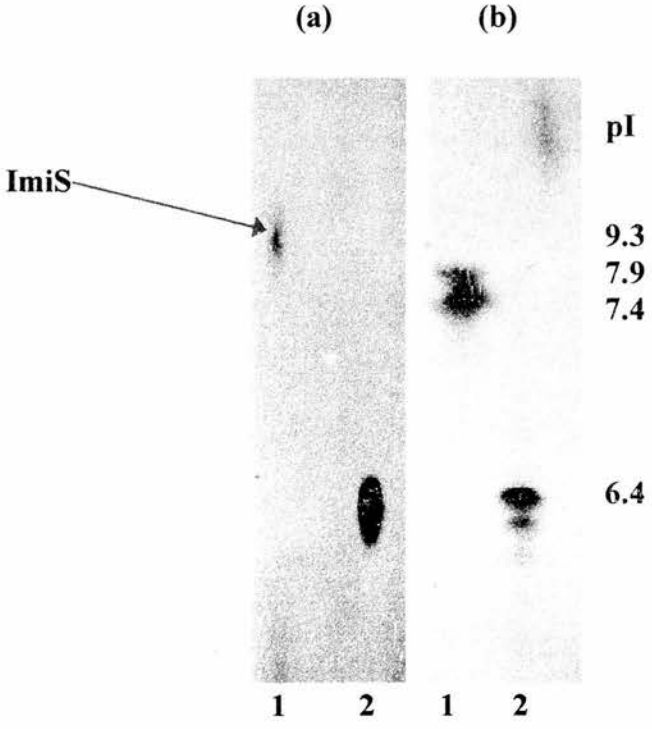
## 4.6 Imipenem agar modification with a pre-cast IEF gel

During the development of this substrate-based technique for the detection of carbapenemases after IEF a paper was published by Yang and Bush (1996) on the biochemical characterisation of a carbapenem-hydrolysing AsbM1 β-lactamase from *A. jandaei* AER14M (formerly identified as *A. sobria* AER14M). The study by Yang and Bush (1996) included the application of an imipenem/agar overlay following IEF to show the presence of AsbM1 (pI 9.1). The method described by Yang and Bush (1996) involved using commercially prepared pre-cast polyacrylamide gels and the spreading of the indicator organism (*E.coli* ACTC 25922) over the surface of the agar overlay, as opposed to mixing the diluted indicator organism with molten agar and pouring this over the surface of the IEF gel.

Only polyacrylamide gels prepared in the laboratory had been tested in this current study, therefore the IEF/overlay technique was repeated this time employing pre-cast IEF gels and swabbing the diluted *E. coli* NCTC 0418 over the solidified agar surface and it became apparent that this technique could be successfully performed with commercially prepared polyacrylamide gels. Figure 4.4 shows the detection of a carbapenemase (pI 6.4) produced by *Stenotrophomonas maltophilia* strain 511 (Felici *et al.*, 1993; Payne *et al.*, 1994a) and the ImiS metallo-β-lactamase (pI 9.3) produced by *A. veronii* bv. *sobria* 163a (Walsh *et al.*, 1995a), following the procedure of Yang and Bush (1996).

Carbapenemases could additionally be detected when the procedure described by Yang and Bush (1996) was repeated using a self-cast polyacrylamide, the only difference between this method and the one employed in Section 4.3 being the streaking of the indicator organism over the agar surface.

**Figure 4.4 A polyacrylamide IEF gel overlaid with (a) IST agar containing imipenem (0.5mg/l), with *E. coli* NCTC 10418 streaked over the agar surface and (b) nitrocephin**




**Lanes: 1,** *A. veronii bv.* sobria 163a (pIs 7.4, 7.9); **2,** *S. maltophilia* 511 (pI 6.4, 9.7).

## 4.7 Discussion

Substrate-based overlay studies after IEF provide a simple approach for the characterisation of β-lactamases. This methodology offers the advantage that enzyme activities can be detected without firstly having to perform β-lactamase purification and is particularly beneficial when bacteria produce more than one β-lactamase.

This section has described the development of a technique that specifically enables carbapenemases to be detected, including the metallo-β-lactamases of two *Aeromonas* species (Figures 4.2 - 4.4). Both an iodometric method (Massida *et al.*, 1991) and a bromothymol blue/imipenem IEF overlay method (Walsh *et al.*, 1995a) have previously been employed to detect the *Aeromonas* carbapenemases. However, Hayes (1995) could not detect the *Aeromonas* carbapenemases by either of these methods which implies that they are both problematic. In contrast the imipenem/agar overlay has been successful in detecting the metallo-β-lactamases from two *Aeromonas* species in this study, and Yang and Bush (1996) have also reported the identification of carbapenemases from another *Aeromonas* species by an imipenem/agar overlay method published recently.

From this study, it has been revealed, however, that an important factor in determining the success of this technique is whether the indicator organism is inoculated over the surface of the imipenem/agar layer. When a broth culture of *E. coli* NCTC 10418 was mixed with molten agar carbapenemases could only be

detected when focused on agarose IEF gels and Sme-1 with a pI of 9.7 could only be identified with a pH tolerant mutant. However, when the *E. coli* NCTC 10418 was streaked over the surface of the agar carbapenemases could be detected when focused on self-cast and pre-cast polyacrylamide gels in addition to agarose IEF gels. *Aeromonas* carbapenemases with reported pIs of > 9.0 could also be revealed (Figure 4.4). The reason for the importance of this step may possibly be that by swabbing *E. coli* NCTC 10418 on the agar surface the organism is not in direct contact with the polyacrylamide or subject to the influence of the IEF gel pH gradient.

# 5: RESULTS

## The isolation of imipenem-resistant *Aeromonas veronii* biovar (bv.) *sobria*, that possess a novel carbapenemase from a water source in India

### 5.1 Introduction

*Aeromonas veronii* bv. *sobria* (formerly *A. sobria*) is a major pathogenic species of the genus *Aeromonas*. In common with most *Aeromonas* species, it has the ability to express up to three different chromosomally encoded, inducible β-lactamases a molecular class C cephalosporinase, a molecular class A or class D penicillinase and a molecular class B metallo-carbapenemase (Section 1.11.2.1).

This section reports the identification of two imipenem-resistant *A. veronii* bv. *sobria* strains isolated from a water source in Vellore, South India; and the preliminary evidence for the production of a novel *Aeromonas* carbapenemase in these two isolates.

### 5.2 Background to bacterial strains

During July 1996, a study was undertaken by Amyes to examine antibiotic resistance in environmental bacteria from water sources in the town of Vellore, South India.

*Aeromonas* species were found to be particularly abundant in the water sources sampled and more significantly seven *Aeromonas* strains were recorded as being resistant to imipenem (MIC >4mg/l), with six of these strains additionally resistant to meropenem (MIC >4mg/l) (Amyes, 1996). Therefore, ten *Aeromonas* isolates from the survey by Amyes (1996), including the strains identified as being carbapenem-resistant, were obtained for further investigations outlined in this chapter.

## 5.3 Species identification and β-lactam sensitivities of ten *Aeromonas* isolates

The speciation of the ten *Aeromonas* isolates was determined with API 20NE strips (Table 5.1). The sensitivities of these ten strains to various β-lactams (including the carbapenems) were determined by the agar dilution method (Table 5.1). In Table 5.1 it can be seen that only two *A. veronii* bv. *sobria* isolates, strains 13 and 99, were found to be resistant to imipenem (MIC >4mg/l) by the agar dilution method, the meropenem MIC of these two strains was at the breakpoint for resistance (4 mg/l). Previously it had been shown by the Etest method that seven of the ten strains were resistant to imipenem and five of these strains also resistant to meropenem (Amyes, 1996). These conflicting results can however be explained by an inoculum effect (see Section 5.4). When preparing a bacterial culture for the Etest method it is recommended that the density of culture should be adjusted to equal that of a 0.5 McFarland turbidity standard, this contains approximately $10^8$ cfu/ml, whereas an inoculum of $10^4$ cfu is recommended for the agar dilution method (see below – Section 5.4).

All of the *A. veronii* bv. *sobria* strains were highly resistant to both ampicillin and oxacillin (MIC ≥ 512 mg/l), whereas *A. hydrophila* strain 98 was more susceptible to ampicillin (Table 5.1). *A. veronii* bv. *sobria* strains 13 and 99 were additionally highly resistant to cephaloridine and showed reduced susceptibility to cefoxitin, although these two strains remain sensitive to both the third and fourth generation cephalosporins (Table 5.1).

## Table 5.1 Species identification and β-lactam sensitivities of ten *Aeromonas* isolates

| Strain | API 20NE identification | MIC (mg/l) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **IMP** | **MERO** | **AMP** | **OXA** | **CEPH** | **CFOX** | **CTAZ** | **CTAX** | **CPIME** |
| 13 | *A. veronii* bv. *sobria* | 8 | 4 | >1024 | >512 | >256 | 4 | 0.25 | 0.12 | 0.12 |
| 27 | *A. veronii* bv. *sobria* | 2 | 0.12 | >1024 | >512 | 8 | 1 | 0.25 | 0.032 | 0.064 |
| 36 | *A. veronii* bv. *sobria* | 1 | 0.12 | >1024 | 512 | 8 | 1 | 0.25 | 0.032 | 0.064 |
| 47 | *A. veronii* bv. *sobria* | 1 | 0.064 | >1024 | 512 | 4 | 2 | 0.5 | 1 | 1 |
| 51 | *A. veronii* bv. *sobria* | 0.25 | 0.064 | 1024 | 512 | 8 | 1 | 0.12 | 0.016 | 0.064 |
| 52 | *A. veronii* bv. *sobria* | 1 | 0.064 | 1024 | 512 | 4 | 1 | 0.12 | 0.032 | 0.064 |
| 98 | *A. hydrophila* | 0.25 | 0.016 | 128 | 256 | 2 | 0.25 | 0.12 | 0.016 | 0.064 |
| 99 | *A. veronii* bv. *sobria* | 8 | 4 | >1024 | >512 | >256 | 4 | 0.25 | 0.25 | 0.12 |
| 110 | *A. veronii* bv. *sobria* | 1 | 0.12 | >1024 | >512 | 8 | 1 | 0.25 | 0.032 | 0.064 |
| 115 | *A. veronii* bv. *sobria* | 0.5 | 0.12 | >1024 | >512 | 8 | 1 | 0.12 | 0.016 | 0.064 |

**IMP**, imipenem; **MERO**, meropenem; **AMP**, ampicillin, **OXA**, oxacillin; **CEPH**, cephaloridine; **CFOX**, cefoxitin; **CTAZ**, ceftazidime, **CTAX**, cefotaxime; **CPIME**, cefepime.

## 5.4 Inoculum size

An experiment was carried out to analyse the inoculum effect on the imipenem MIC of the ten *Aeromonas* strains. The imipenem MIC was determined by the agar dilution method and inoculum sizes of $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ cfu were tested. *A. veronii* bv. *sobria* strains 13 and 99 were the only isolates resistant to imipenem when inocula of $10^4$ or $10^5$ cfu were tested (Table 5.2). With an inoculum of $10^6$ cfu, six of the nine *A. veronii* bv. *sobria* strains were resistant to imipenem (MIC ≥8mg/l). All of the *A. veronii* bv. *sobria* strains were resistant to imipenem with an inoculum of $10^7$ cfu. *A. hydrophila* strain 98 remains sensitive to imipenem even with an inoculum of $10^8$ cfu.

**Table 5.2 The effect of bacterial inoculum size on imipenem sensitivity**

| | Imipenem MIC (mg/l) with $10^x$ cfu | | | | |
|---|---|---|---|---|---|
| **Strain** | **$10^4$** | **$10^5$** | **$10^6$** | **$10^7$** | **$10^8$** |
| 13 | 8 | 8 | 64 | >256 | >256 |
| 27 | 2 | 4 | 32 | >256 | >256 |
| 36 | 1 | 2 | 16 | >256 | >256 |
| 47 | 0.5 | 2 | 8 | 128 | 128 |
| 51 | 0.25 | 1 | 2 | 32 | 32 |
| 52 | 1 | 2 | 4 | 256 | >256 |
| 98 | 0.12 | 0.25 | 0.25 | 0.25 | 0.25 |
| 99 | 8 | 16 | 16 | 32 | >256 |
| 110 | 2 | 2 | 16 | 32 | >256 |
| 115 | 0.5 | 0.5 | 0.5 | 256 | >256 |

## 5.5 β-Lactamase assays

Small-scale β-lactamase extracts were prepared in 50mM sodium phosphate buffer, pH 7.0 from the ten *Aeromonas* strains and assayed spectrophotometrically against 100μM imipenem. Efficient hydrolysis of imipenem could only be detected with β-lactamase extracts from imipenem-resistant strains 13 and 99.

Hydrolysis assays were also performed with induced (imipenem ¼ MIC) and non-induced large-scale β-lactamase extracts prepared from *A. veronii* bv. *sobria* strains 13, 27, 99, and 110 to analyse further the β-lactamase activities in carbapenem-sensitive and –resistant strains. Enzyme activity was assayed against ampicillin (500μM), cephaloridine (100μM) and imipenem (100μM). Imipenem was additionally recorded after pre-incubation at 37°C for 10 minutes with 10mM EDTA (Table 5.3).

Table 5.3 shows that β-lactamase extracts from the two carbapenem-resistant strains 13 and 99 can hydrolyse all three β-lactams tested in the absence of a β-lactamase inducer, and in the presence of the imipenem inducer there is a less two-fold increase in enzyme activity. In contrast, with the two sensitive *A. veronii* bv. *sobria* strains, 27 and 110, β-lactam-hydrolysis is only evident in extracts prepared from induced cultures, where a dramatic increase in β-lactamase activity against imipenem and ampicillin can be noted. No hydrolysis of cephaloridine was detectable in induced β-lactamase preparations from strain 27 and 110. A marked difference is also seen in

the effects of imipenem hydrolysis in the presence of the metallo-β-lactamase inhibitor EDTA. Pre-incubation of the metallo-β-lactamase extracts from strains 27 and 110 results in >95% inhibition of imipenem hydrolysis, whereas, only partial (≤ 33%) inhibition of imipenem hydrolysis is achieved when β-lactamase extracts from imipenem-resistant strains 13 and 99 were pre-incubated with EDTA.

## 5.6 IEF of induced and non-induced β-lactamase preparations from ten *Aeromonas* isolates

IEF was performed on induced (imipenem ¼ MIC) and non-induced large-scale β-lactamase extracts prepared from the ten *Aeromonas* strains. Induced and non-induced β-lactamase preparations from *A. veronii* bv. *sobria* 163a, a strain which expresses three different β-lactamases characterised in detail by Walsh *et al.*, (1995a) was also included on the IEF gel. Figure 5.1 shows that the β-lactamase banding patterns were essentially the same for both non-induced and imipenem induced cells, although the β-lactamase bands were more intense when the strains are induced. Exceptions to this were the extracts from induced strain 163a and induced strain 36 where additional bands can be seen, compared with the non-induced extracts. In the induced extract from 163a two β-lactamases bands are detectable with nitrocephin as reported previously (Walsh *et al.*, 1995a), these correspond to the penicillinase AmpS (pI 7.9) and the cephalosporinase CepS (pI 7.4); however, only one band is detectable in the non-induced 163a strain. The most notable feature of Figure 5.1 is, however, the distinctive β-lactamase banding patterns of the two imipenem-resistant

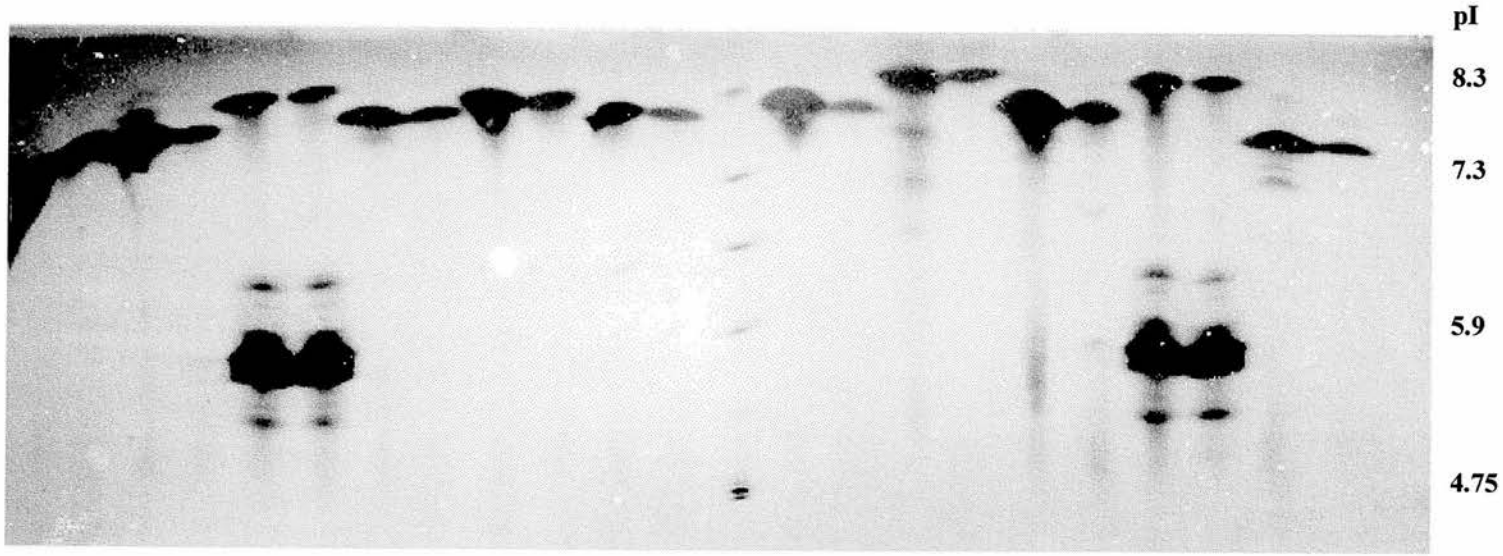
strains 13 and 99, which in addition to a basic β-lactamase of pI 8.3 also includes an intense band with an acidic pI value (5.84). Less intense bands are also seen above and below the pI 5.84 band of strain 13 and 99 and are possibly 'satellite bands', that is different forms of the main pI 5.84 β-lactamase band (Payne and Farmer, 1998). The pI values written in parenthesis in Figure 5.1 were all determined with the aid of commercial pI markers as described in Section 2.8.4.

**Table 5.3 An assessment of the hydrolytic activities of β-lactamase extracts from four *A. veronii* bv. *sobria* strains**

| Strain | IMP (specific activity)* | Hydrolysis of AMP relative to IMP (%) | Hydrolysis of CEPH relative to IMP (%) | % inhibition of IMP hydrolysis by EDTA |
|---|---|---|---|---|
| 13 non-induced | 487 | 44 | 17 | 33 |
| 13 induced | 903 | 23 | 17 | 33 |
| 27 non-induced | ND | ND | ND | NT |
| 27 induced | 316 | 12 | ND | 96 |
| 99 non-induced | 385 | 63 | 65 | 9 |
| 99 induced | 511 | 54 | 61 | 10 |
| 110 non-induced | ND | ND | ND | NT |
| 110 induced | 798 | 7 | ND | 99 |

**IMP**, imipenem; **AMP**, ampicillin; **CEPH**, cephaloridine.
* Units of activity: nanomoles of β-lactam hydrolysed per minute per milligram of protein.
ND – not detected.
NT – not tested.

**Figure 5.1 Isoelectric focusing of induced (I) and non-induced β-lactamase preparations from *Aeromonas* isolates**



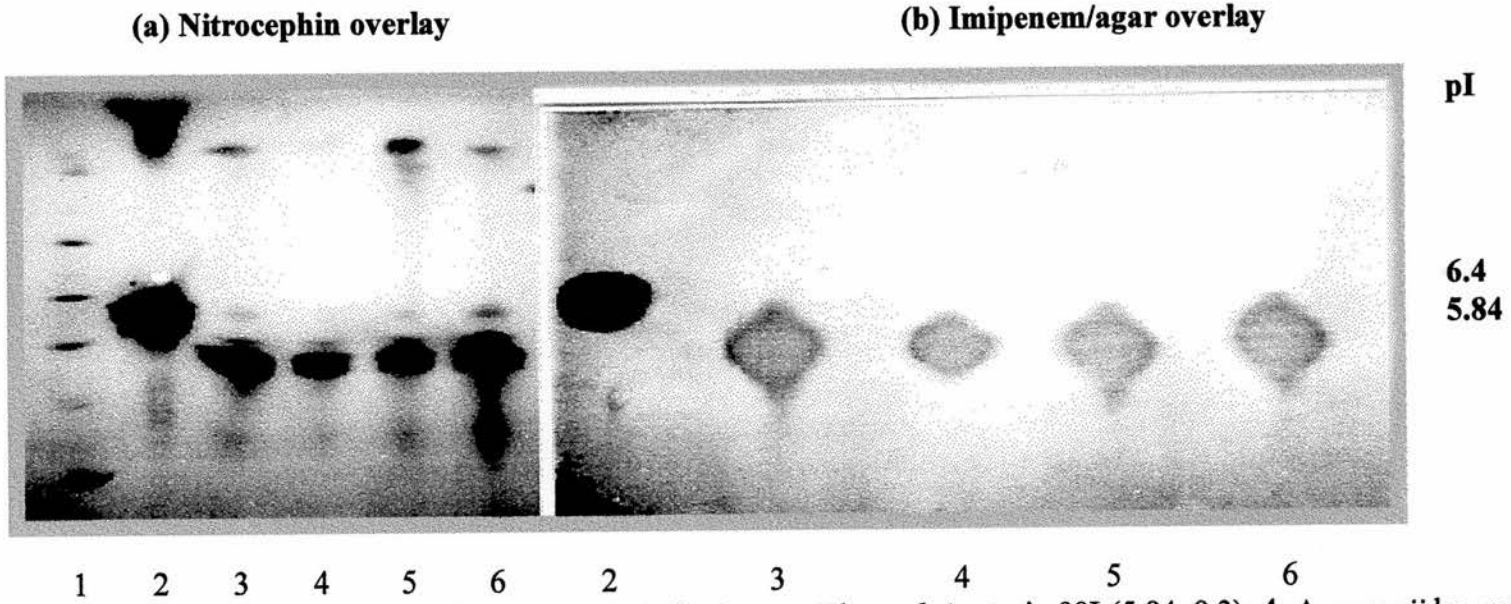

**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23**

**Lanes:** **1**, strain 115I (pI 7.9); **2**, strain 115 (pI 7.9); **3**, strain 110I (pI 7.9); **4**, strain 110 (pI 7.9); **5**, strain 99I (pIs 5.84, 8.3); **6**, strain 99 (pIs 5.84, 8.3); **7**, strain 98I (pI 8.1); **8**, strain 98 (pI 8.1); **9**, strain 52I (pI 8.2); **10**, 52 (pI 8.2); **11**, strain 51I (pI 8.1); **12**, strain 51 (pI 8.1); **13**, pI markers; **14**, strain 47I (pI 8.1); **15**, strain 47 (pI 8.1); **16**, strain 36I (pIs 8.3, 7.9, 7.4); **17**, strain 36 (pI 8.3); **18**, strain 27I (pI 8.2); **19**, strain 27 (pI 8.2); **20**, strain 13I (pIs 5.84, 8.3); **21**, strain 13 (pIs 5.84, 8.3); **22**, strain 163aI (pIs 7.9, 7.4); **22**, strain 163a (pI 7.9).

## 5.7 Identification of imipenem-hydrolysing β-lactamases after IEF

The imipenem/agar overlay of Yang and Bush (1996), and modified as previously described in Section 4.6 was applied to determine the pI of the imipenem-hydrolysing enzyme produced by the two imipenem-resistant *A. veronii* bv. *sobria* strains 13 and 99. This method was performed on non-induced and imipenem-induced β-lactamase preparations from strains 13 and 99. A cell extract from *S. maltophilia* that produces two β-lactamases (L1-type carbapenem-hydrolysing metallo-β-lactamase – pI 6.4 and an L2 cephalosporinase – pI 9.7) was employed as both a positive and negative control. Figure 5.2 shows the IEF gel overlaid with (a) nitrocephin and (b) agar containing 0.5mg/l imipenem with a diluted overnight *E.coli* NCTC 10418 broth culture streaked over the surface of the agar. A single zone of *E. coli* NCTC 10418 growth can be seen in the lanes (3-6) corresponding to induced and non-induced β-lactamase extracts from *A. veronii* bv. *sobria* strains 13 and 99 (Figure 5.2b). This zone of *E. coli* NCTC 10418 growth indicates the presence of a carbapenemase that focuses at pI 5.84, which is also detectable with nitrocephin. A zone of growth is also seen in lane 2 of Figure 5.2b and this corresponds to the *S. maltophilia* L1 carbapenem-hydrolysing metallo-β-lactamase (pI 6.4).

**Figure 5.2 β-Lactamase from two imipenem-resistant *A. veronii* bv. *sobria* strains (13 and 99), (a) detected with nitrocephin and (b) detected with an imipenem/agar overlay**




**Lanes: 1**, pI markers; **2**, *S. maltophilia* strain 511 (pIs 6.4, 9.7); **3**, *A. veronii* bv. *sobria* strain 99I (5.84, 9.3); **4**, A. *veronii* bv. *sobria* strain 99 (pIs 5.84, 8.3); **5**, *A. veronii* bv. *sobria* strain 13I (pIs 5.84, 8.3); 6, *A. veronii* bv. *sobria* strain 13 (pIs 5.84, 8.3).

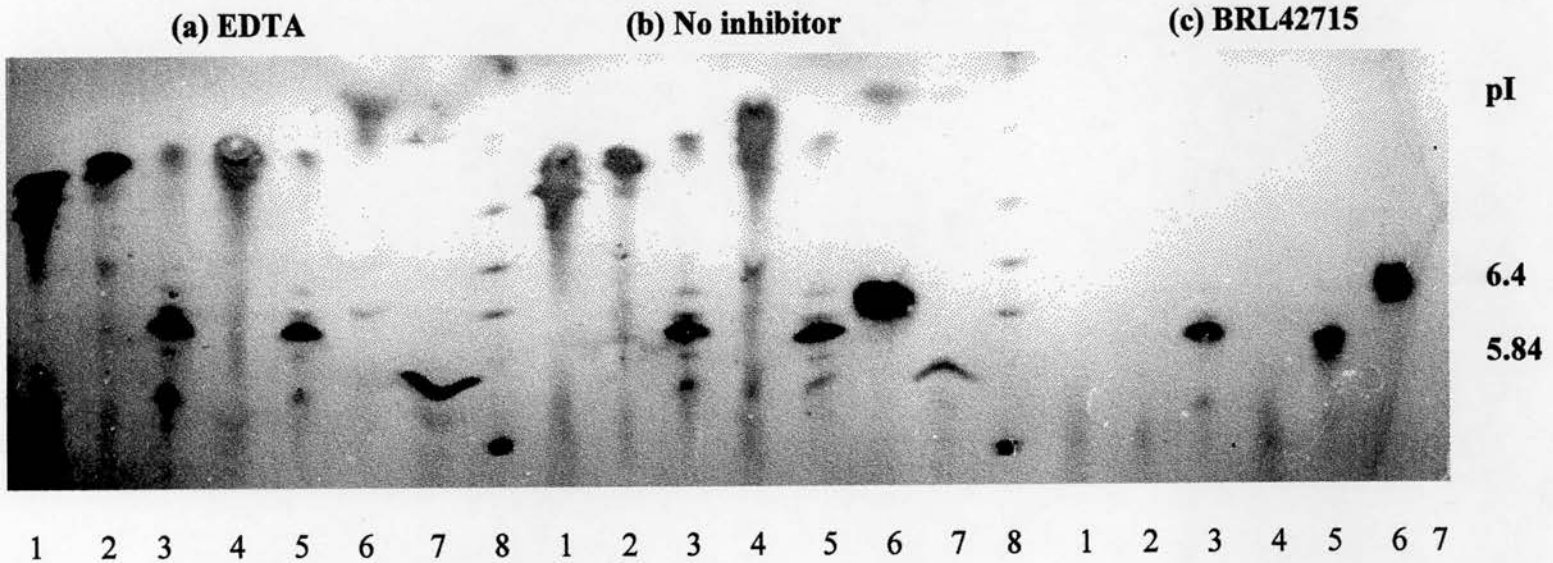
## 5.8 IEF with EDTA and BRL42715 inhibitor overlays

IEF followed by overlays with metallo-β-lactamase inhibitor EDTA (100mM) and the serine β-lactamase inhibitor BRL42715 (100μM) prior to nitrocephin staining has previously been shown to rapidly distinguish metallo-β-lactamases from serine β-lactamases (Payne *et al.*, 1994a). Therefore, this method was applied to further characterise the pI 5.84 and pI 8.3 β-lactamase bands of *A. veronii* bv. *sobria* strains 13 and 99. β-Lactamases from three additional *A. veronii* bv. *sobria* strains (27, 110 and 163a) were also focused on the gel. A TEM-1 (serine-based) β-lactamase and a cell extract from *S. maltophilia* 511 (described in Section 5.7) were included as controls.

It can be seen from Figure 5.3a that the *S. maltophilia* L1 metallo-β-lactamase (lane 6, pI 6.4) was the only β-lactamase band to be affected by the metallo-β-lactamase inhibitor EDTA. When the gel was overlaid with the serine β-lactamase inhibitor BRL42715 (Figure 5.3c) the only bands that are not inhibited were the two pI 5.84 β-lactamase bands belonging to *A. veronii* bv. *sobria* strains 13 and 99 and the L1 metallo-β-lactamase of *S. maltophilia* 511. The results from Figure 5.3, therefore, show that the pI 8.3 β-lactamase of strains 13 and 99 were serine-based β-lactamases, as were the nitrocephin detectable β-lactamases of the three other *A. veronii* bv. *sobria* strains (27, 110 and 163a) included on the gel. However, the pI 5.84 β-lactamase band of strains 13 and 99 was not inhibited by either EDTA or

BRL42715. It should be noted, however, that the satellite bands above and below the pI 5.84 band do appear to be inhibited by BRL42715.

**Figure 5.3 Effect of EDTA (100mM) and BRL42715 (100μM) on the β-lactamase bands from *A. veronii* bv. *sobria* isolates**



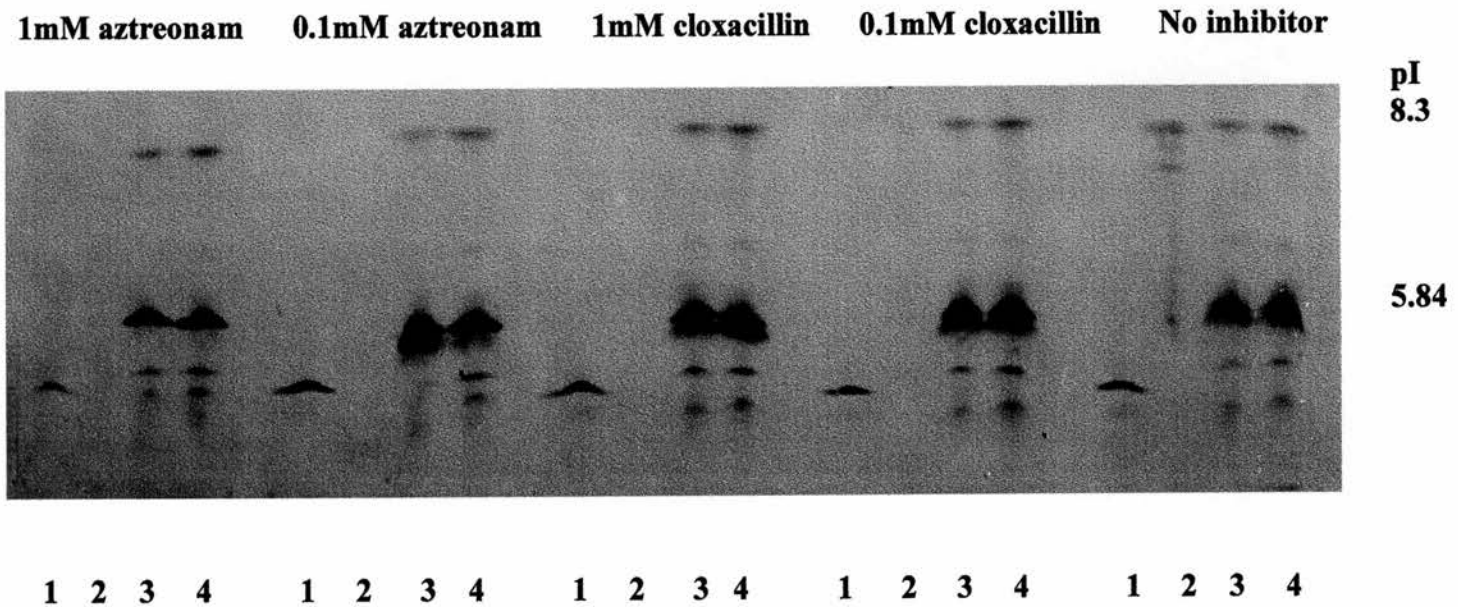

**Lanes: 1**, strain 163a (pIs 7.4, 7.9); **2**, strain 110 (pI 7.9); **3**, strain 99 (pIs 5.84, 8.3); **4**, strain 27 (pI 8.2); **5**, strain 13 (pIs 5.84, 8.3); **6**, *S. maltophilia* 511 (pIs 6.4, 9.7); **7**, TEM-1 (pI 5.4); **8**, pI markers

## 5.9 IEF with aztreonam, cloxacillin and clavulanic acid inhibitor overlays

Additional aztreonam, cloxacillin and clavulanic acid inhibitor overlays were performed on the focused β-lactamases of *A. veronii* bv. *sobria* strains 13 and 99. Figure 5.4 shows that two different concentrations (0.1 and 1mM) of the molecular class C cephalosporinase inhibitors aztreonam and cloxacillin have no significant effect on either the pI 5.84 or the pI 8.3 β-lactamase bands of strains 13 and 99, or on the molecular class A β-lactamase TEM-1. In contrast the AmpC-type class C cephalosporinase of *P. aeruginosa* strain 2297 (included on the gel as a positive control) is completely inhibited by 0.1 and 1mM concentrations of both aztreonam and cloxacillin.

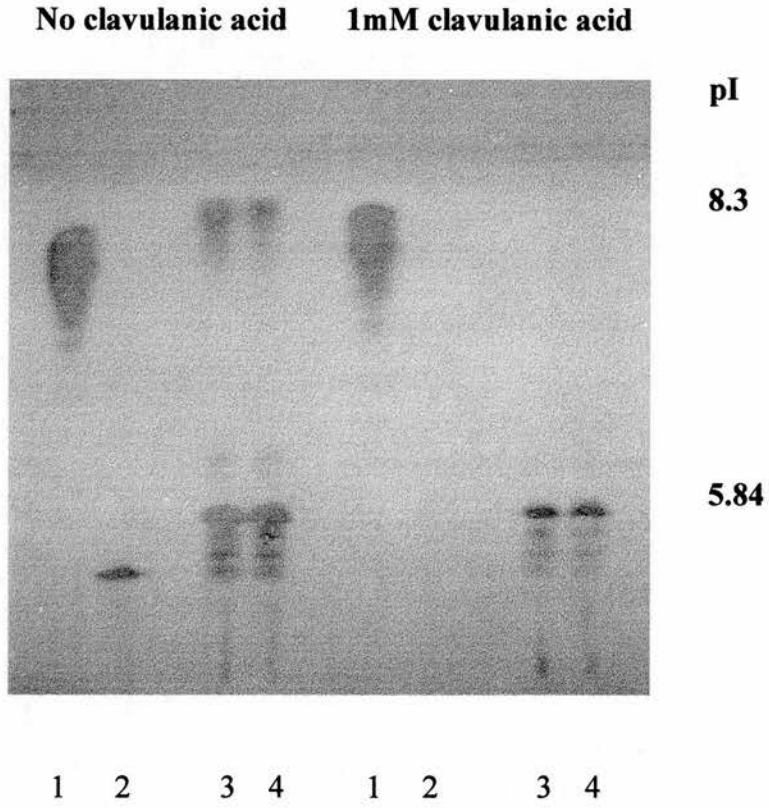
Figure 5.5 shows that the molecular class A β-lactamase inhibitor clavulanic acid (1mM) completely inhibited the pI 8.3 β-lactamase band of strains 13 and 99 and the TEM-1 β-lactamase. There also appeared to be some partial inhibition by clavulanic acid of the pI 5.84 β-lactamase band from strains 13 and 99. The class C cephalosporinase of *P. aeruginosa* strain 2297 was not affected by clavulanic acid.

**Figure 5.4 Effect of aztreonam (0.1 and 1mM) and cloxacillin (0.1 and 1mM) on the β-lactamase bands from *A. veronii* bv. *sobria* strains 13 and 99**




**Lanes: 1**, TEM-1 (pI 5.4); **2**, *P. aeruginosa* 2297 (pI 8.3); **3**, strain 13 (pI 5.84, 8.3); **4**, strain 99 (pI 5.84, 8.3).

**Figure 5.5 Effect of clavulanic acid (1mM) on the β-lactamase bands from *A. veronii* bv. *sobria* strains 13 and 99**

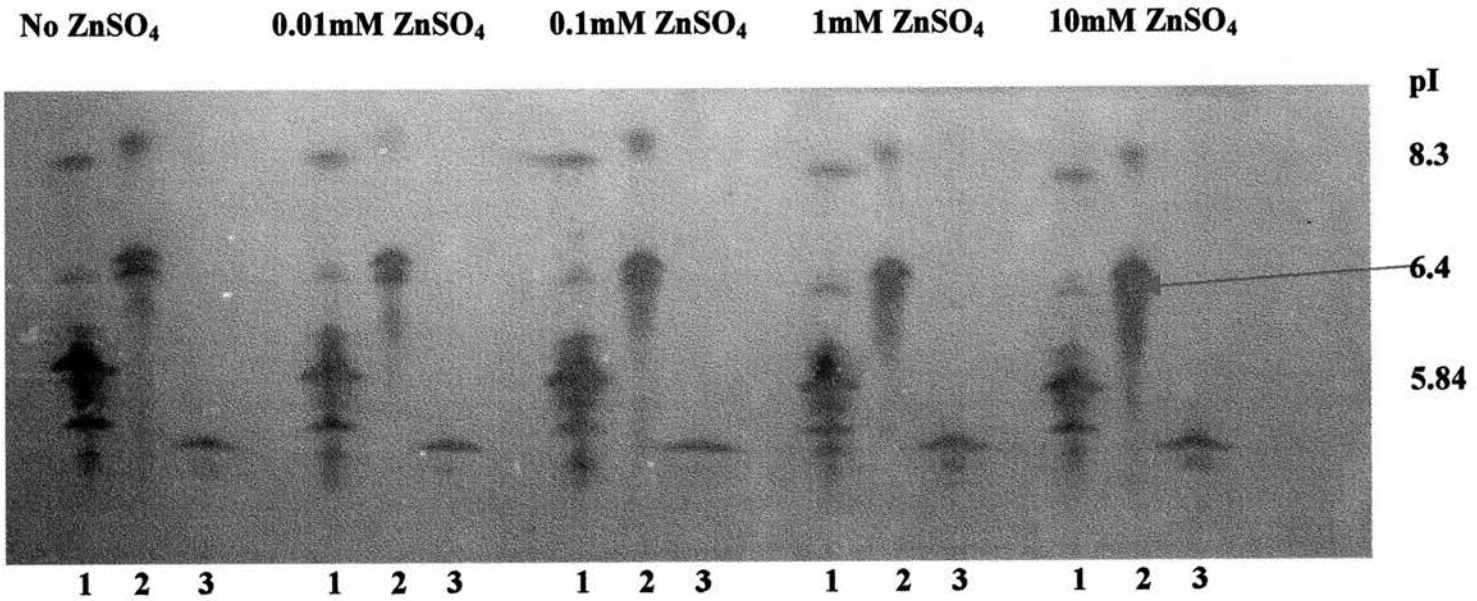



**Lanes: 1**, *P. aeruginosa* 2297 (pI 8.3); **2**, TEM-1 (pI 5.4); **3**, strain 13 (pIs 5.84, 8.3); **4**, strain 99 (pIs 5.84, 8.3).

## 5.10 IEF followed by a zinc sulphate gel overlay

It has previously been demonstrated that the application of a zinc sulphate overlay to an IEF gel before nitrocephin staining can enhance the identification of zinc-dependent metallo-β-lactamases (Payne *et al.*, 1994*a*). Therefore, an experiment was performed to establish whether the pI 5.84 β-lactamase band common to both *A. veronii* bv. *sobria* strains 13 and 99 was affected by increasing concentrations of $ZnSO_4$. The β-lactamases from *A. veronii* bv. *sobria* strain 13, *S. maltophilia* 511 and the TEM-1 β-lactamase were focused on an IEF gel and overlaid with 0.01, 0.1, 1.0 and 10mM zinc sulphate concentrations. In Figure 5.6 the L1 metallo-β-lactamase band (pI 6.4) was visibly enhanced by overlaying with increasing concentrations of $ZnSO_4$. None of the other β-lactamase bands were affected by $ZnSO_4$, including the pI 5.84 band of *A. veronii* bv. *sobria* strain 13.

**Figure 5.6 Effect of zinc sulphate (0.01 - 10mM) on the β-lactamase bands from *A. veronii* bv. *sobria* strain 13**




**Lanes: 1**, strain 13 (pI 5.84); **2**, *S. maltophilia* 511 (pIs 6.4, 9.7); **3**, TEM-1 (pI 5.4)

## 5.11 Discussion

This section has reported the identification of two imipenem-resistant *A. veronii* bv. *sobria* (strains 13 and 99), isolated from a water source in South India. Strains 13 and 99 were found to have an imipenem MIC of 8mg/l by the agar dilution method, employing a conventional inoculum of $10^4$ cfu (Table 5.1). To date, only a handful of naturally occurring carbapenem-resistant *Aeromonas* isolates have been recorded when testing with a conventional inoculum size, these being usually derepressed mutants for carbapenemase production (Rossolini *et al.*, 1996).

It is well known that the carbapenem MICs of *Aeromonas* strains are subject to an inoculum effect (Rossolini *et al.*, 1996). Carbapenem-resistance is normally only detectable when a large inoculum of a carbapenemase-producing strain ($10^8$ cfu) is used in susceptibility testing (Rossolini *et al.*, 1996). Carbapenemase-negative *Aeromonas* strains remain always below the breakpoint for susceptibility irrespective of the inoculum size (Rossolini *et al.*, 1996). The results of Table 5.2 illustrate the effect of the inoculum size on imipenem MICs. Nine of the ten *Aeromonas* strains tested were found to be imipenem-resistant with an inoculum of $10^7$ cfu. It is interesting that *A. hydrophila* strain 98 remains sensitive to imipenem even with an inoculum of $10^8$ cfu and, therefore, this strain may not possess carbapenemase activity.

β-Lactamase extracts from *A. veronii* bv. *sobria* strains 13 and 99 were found to possess penicillinase, cephalosporinase and carbapenemase activity (Table 5.3).

Following β-lactam induction, there was a less than two-fold increase in β-lactamase activity was detected (Table 5.3) and, therefore, these two strains appear to be constitutive β-lactamase producers.

The carbapenemase activity of strains 13 and 99 could only be partially inhibited (≤33%) by 10mM EDTA (Table 5.3). The carbapenemase activity of *Aeromonas* strains of several different species is usually found to be completely inhibited by 10mM EDTA and thus demonstrates their metallo-enzyme nature (Rossolini *et al.*, 1995, Walsh *et al.*, 1997). Partial inhibition with 10mM EDTA has only previously been reported for a single isolate, *A. hydrophila* T429125, where activity against imipenem decreased by only 55% in the presence of the EDTA chelating agent (Walsh *et al.*, 1997). More recent data, however, suggest that *A. hydrophila* T429125 produces a metallo-β-lactamase that requires a greater concentration of EDTA (55mM) to achieve complete inhibition of the carbapenemase activity (T. Walsh – personal communication). Therefore, the carbapenemase produced by *A. veronii* bv. *sobria* strain 13 and 99 may also be less sensitive to EDTA compared with the metallo-β-lactamases produced by other *Aeromonas* isolates.

IEF has shown that both strains 13 and 99 produce two β-lactamases with pI values of 5.84 and 8.3 (Figure 5.1). The application of an imipenem overlay modification to IEF demonstrated the presence of a single imipenem-hydrolysing enzyme of pI 5.84 in both strains 13 and 99 (Figure 5.2). These results suggest, therefore, that either the carbapenemase in these two strains is unlike all other previously described *Aeromonas* carbapenemases and can be identified with nitrocephin, or alternatively,

the carbapenemase is co-focusing with a separate nitrocephin hydrolysing enzyme (see below). Nevertheless, the pI value (5.84) is considerably different from the *Aeromonas* carbapenemases that have been reported previously, which are typically pI 8.0 or greater (Rossolini *et al.*, 1996).

IEF/inhibitor overlays facilitated further characterisation of the pI 5.84 and 8.3 β-lactamases of strains 13 and 99 (Figure 5.3). The pI 8.3 β-lactamase of both strains 13 and 99 were found to be serine-based because they were inhibited by BRL42715, but not EDTA. The pI 8.3 β-lactamase band was also sensitive to 1mM clavulanic acid and this suggests that it is either a molecular class A or D β-lactamase (Figure 5.5). Insensitivity of the pI 8.3 β-lactamase band to cloxacillin (Figure 5.4) would imply that, in addition to being not of class C origin, this β-lactamase is also not a class D enzyme; however, the cloned class D AmpS penicillinase of *A. veronii* bv. *sobria* 163a also does not hydrolyse cloxacillin, although it can readily hydrolyse oxacillin (Walsh *et al.*, 1995b).

IEF followed by inhibitor overlays further highlighted the unusual nature of the pI 5.84 carbapenem-hydrolysing β-lactamase from strains 13 and 99 (Figure 5.3). The pI 5.84 β-lactamase band could not be inhibited by either EDTA (100mM) or BRL42715 (100μM) and therefore cannot be classified as either a metallo- or serine β-lactamase. *Aeromonas* strains are known to produce a penicillinase and cephalosporinase readily identifiable by nitrocephin staining after IEF, in addition to a carbapenemase that is not detectable with nitrocephin. Both the penicillinase and cephalosporinase β-lactamase bands have previously been shown to be inhibited by

100μM BRL42715 (Walsh *et al.*, 1995a). It was suggested above that strains 13 and 99 could produce two β-lactamases that co-focus at pI 5.84 – one β-lactamase a carbapenemase, not detectable with nitrocephin and the other a β-lactamase detectable with nitrocephin. In the knowledge that the pI 8.3 β-lactamase from these two strains is most likely the clavulanic acid sensitive penicillinase it could be speculated that the nitrocephin detectable enzyme of pI 5.84 is a cephalosporinase. However, if this were true BRL42715 would be expected to inhibit the cephalosporinase. Furthermore, the class C inhibitors aztreonam and cloxacillin also failed to inhibit the pI 5.84 β-lactamase band, providing further evidence that cephalosporinase is not co-focusing at pI 5.84.

The observation of inhibition with BRL42715 of the 'satellite bands' above and below the pI 5.84 β-lactamase band (Figure 5.3c) could imply that either these are additional β-lactamases or that they are different forms of the pI 5.84 enzyme display variation in their inhibitor binding affinities. Cloxacillin and aztreonam have no affect on these 'satellite bands', although there is some partial inhibition with clavulanic acid (Figures 5.4, 5.5).

Zinc has been shown to serve as a metallo-β-lactamase activator (Sabath and Abraham, 1966; Bicknell *et al.*, 1985; Payne *et al.*, 1994a) and metallo-β-lactamase inhibitor in the case of the AsbM1 carbapenemase from *A. veronii* bv. *sobria* AER14M (Yang and Bush, 1996). In this study, zinc sulphate overlays prior to nitrocephin staining of an IEF gel failed to have any effect on the pI 5.84 carbapenem-hydrolysing β-lactamase band (Figure 5.6) from strains 13 and 99 and,

therefore, this enzyme appears to be insensitive to zinc, in addition to a lack of sensitivity to EDTA.

The above results all indicate the presence of a completely novel carbapenemase in *A. veronii* bv. *sobria* strains 13 and 99. The hyper-production of this carbapenemase can also be clearly correlated with an increase in carbapenem resistance. The pI 5.84 β-lactamase is designated AVS-1 and the pI 8.3 β-lactamase AVS-2.

# 6: RESULTS

## Purification of a novel carbapenemase (AVS-1) from *A. veronii* bv. *sobria* strain 13

### 6.1 Introduction

Section 5 has described the identification of two *A. veronii* bv. *sobria* strains 13 and 99 that possess both a novel carbapenemase named AVS-1 with a pI value of 5.84 and a β-lactamase with a pI of 8.3. This section describes the partial purification of AVS-1 from strain 13, followed by kinetic analysis of this enzyme.

### 6.2 Separation of AVS-1 by DE52 anion exchange chromatography

Periplasmic proteins were extracted from *A. veronii* bv. *sobria* strain 13 (Section 2.10.1), dialysed against 20mM sodium phosphate buffer, pH 7.0 and loaded onto a DE52 anion exchange column also equilibrated with 20mM sodium phosphate buffer, pH 7.0. At pH 7.0 the AVS-1 β-lactamase should be negatively charged and, therefore, have affinity for the DE52 anion exchanger; however, under the conditions described above the enzyme failed to bind to the column.

It has been recommended that the pH of the anion exchange column should be at least one pH unit above the pI of the protein of interest to allow it to reversibly bind

to the anion exchange column. Therefore, the DE52 column was re-equilibrated with 20mM sodium phosphate buffer at pH 7.5. This pH was chosen to ensure that it was far enough away from the AVS-1 pI to achieve binding. Sample dialysed in 20mM sodium phosphate buffer pH 7.5 was applied to the column, but again the pI 5.84 β-lactamase failed to bind.

Another reason as to why the protein elutes in the wash step, before the application of a salt gradient, can be that the ionic strength of the buffer is too high. The sample application step was thus repeated with 10mM sodium phosphate buffer, pH 7.0 instead of 20mM; however, once again the pI 5.84 protein failed to bind.

Finally, the sample was applied to a column equilibrated with 10mM Tris-Cl buffer pH 8.5 and under these conditions the pI 5.84 β-lactamase did bind to the column and could be subsequently eluted with 30mM NaCl. Despite this, the DE52 may not be the most suitable anion exchange matrix for purifying this protein, because it shows weak affinity for the column under conditions that should be extremely favourable for its binding, that is very low ionic strength and a pH >2.5 units away from its pI.

## 6.3 Separation of AVS-1 by a QA52 anion exchange matrix

An experiment was carried out to determine whether AVS-1 demonstrated greater binding affinity for QA52 anion exchange matrix compared with DE52. A series of five test tubes each containing 1.5ml of QA52 matrix were set up, and each tube was then equilibrated to a different pH (pH 6.5, 7.0, 7.5, 8.0, 8.5) with either 20mM sodium phosphate or 20mM Tris-Cl buffer. A known, constant amount of sample was added to each tube and mixed with the gel matrix. After the matrix had settled, the supernatant was assayed for carbapenemase activity; if no activity was found in the supernatant this indicated binding of the AVS-1 β-lactamase to the matrix. No carbapenemase activity was detected in the supernatant from the tube equilibrated with Tris-Cl buffer, pH 7.5 and, therefore, AVS-1 appears to show greater affinity for the QA52 matrix compared with DE52.

A QA52 chromatography column was then prepared and equilibrated with 20mM Tris-Cl, pH 8.0, to ensure adequate binding of the AVS-1 enzyme to the column. The AVS-1 β-lactamase (pI 5.84) was subsequently eluted with 90mM NaCl. Figure 6.1 shows the β-lactamase activity of fractions eluted after the application of the salt gradient. Figure 6.2 shows that a single β-lactamase band (pI 5.84) was present in the fractions that demonstrated carbapenemase activity. The pI 8.3 β-lactamase from strain 13 could bind to the column under the conditions employed; therefore, this step separated these two β-lactamases from each another.

**Figure 6.1 Fractions collected from a QA52 anion exchange column after the application of a salt gradient**

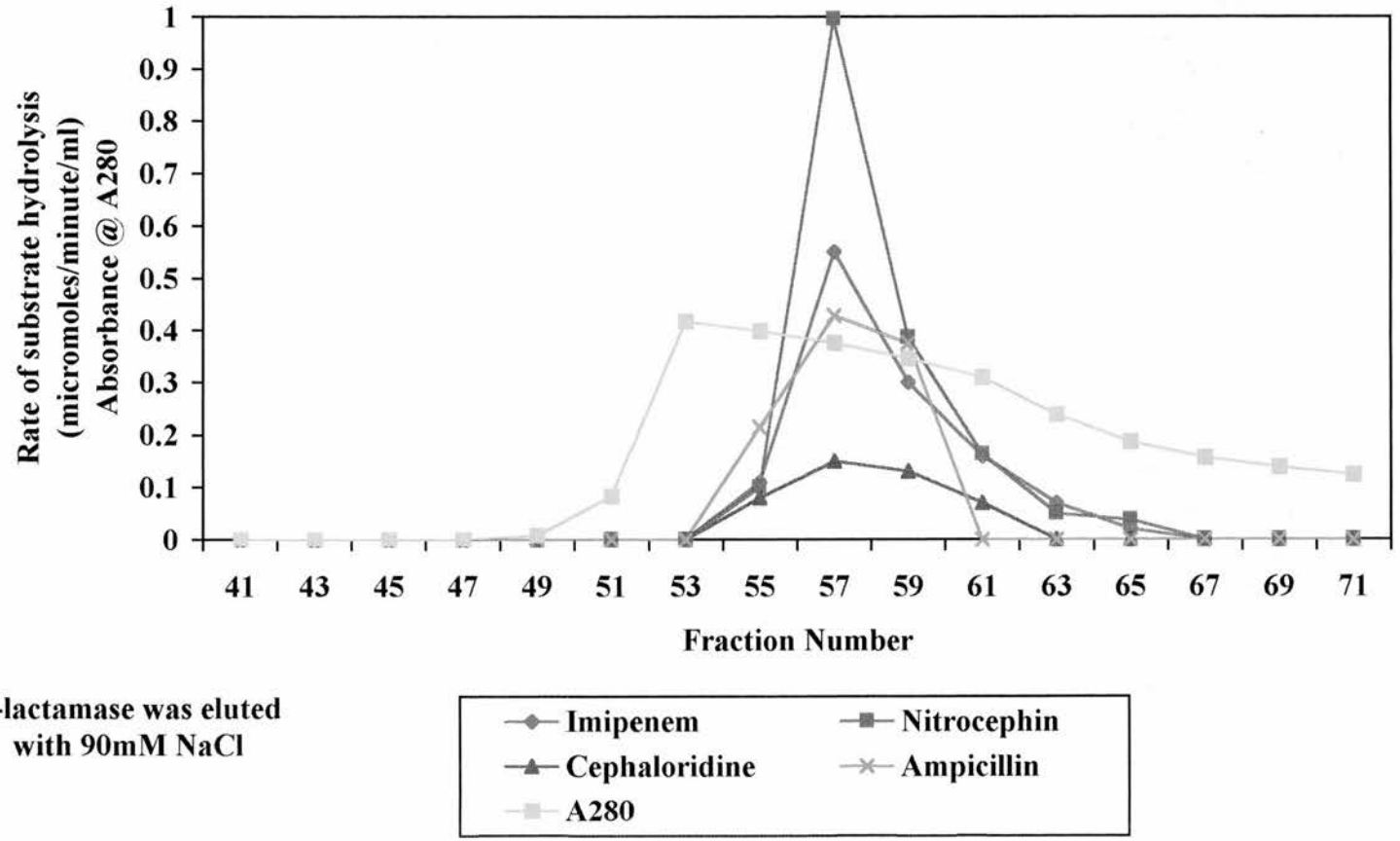
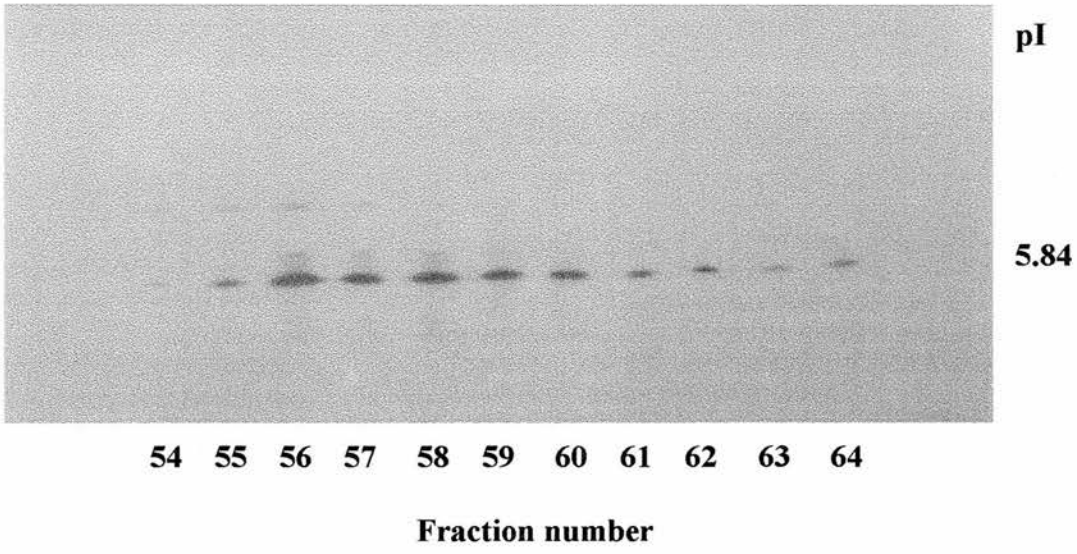


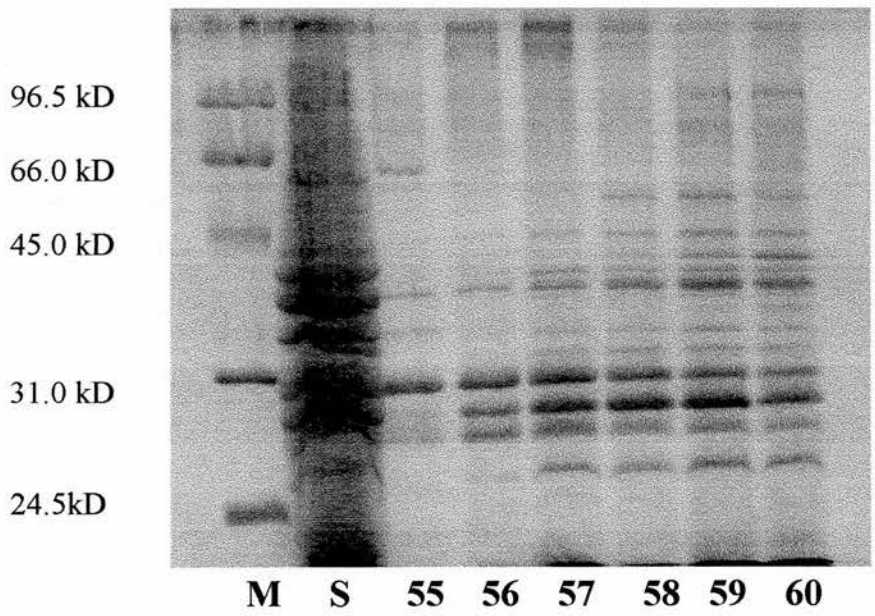
**Figure 6.2 IEF gel of fractions eluted from the QA52 anion exchange column**




## 6.4 SDS-PAGE analysis of fractions eluted from the QA52 column

The purity of the pI 5.84 AVS-1 β-lactamase after the anion exchange step was assessed by SDS-PAGE analysis. Figure 6.3 shows that fractions 55-60 which demonstrate good β-lactamase activity contain several proteins other than just β-lactamase. Peak β-lactamase activity was recorded with fractions 56-58, although it was not possible to determine the molecular weight of the β-lactamase from the SDS-PAGE gel because no one protein band was significantly prominent in these fractions.

**Figure 6.3 SDS-PAGE of carbapenem-hydrolysing fractions eluted from a QA52 column**




**Lanes: M**, molecular weight markers; **S,** crude sample applied to the QA52 column; **55-56**, column fractions.

## 6.5 Gel filtration chromatography

Gel filtration chromatography was employed as a further purification step after ion exchange chromatography, this time separating proteins by size exclusion rather than charge. Carbapenemase active QA52 fractions were pooled, concentrated by 80% ammonium sulphate precipitation and dialysed in sodium phosphate buffer before loading on to a Superose 12 HR 10/30 column. The proteins emerging from the column were collected in fractions and tested for β-lactamase activity as before. The β-lactamase active fractions were analysed by SDS-PAGE; however 5 or 6 other proteins of similar size and charge were always found in those fractions that displayed carbapenemase activity. Therefore, purification of the AVS-1 protein to homogeneity was a difficult process and furthermore, it was still not possible to determine the size of the β-lactamase by SDS-PAGE with coomassie blue staining.

## 6.6 Determining the size of AVS-1 β-lactamase by gel filtration

Although the molecular mass of the AVS-1 could not be established by SDS-PAGE, the molecular mass could be estimated from a standard graph obtained after calibration of the gel filtration column with standard proteins of known size. The size of AVS-1 was estimated to be 26kDa.

## 6.7 Enzyme kinetics of AVS-1 β-lactamase

Fractions demonstrating carbapenemase activity and a single β-lactamase band (pI 5.84) were pooled after QA52 anion exchange chromatography for further biochemical examination. The Michaelis constant ($K_m$) and the maximum rate of hydrolysis ($V_{max}$) of AVS-1 are shown in Table 6.1. Partially purified AVS-1 demonstrated hydrolysis of imipenem, nitrocephin, cephaloridine and ampicillin, although nitrocephin and imipenem were hydrolysed with the greatest efficiency.

**Table 6.1 Hydrolysis of β-lactam antibiotics by AVS-1**

| Substrate | $V_{max}$ (μM/min/ml) | $K_m$ (μM) | $V_{max}/K_m$ |
|---|---|---|---|
| Imipenem | 0.869 | 172 | 0.005 |
| Nitrocephin | 0.625 | 69 | 0.009 |
| Cephaloridine | 1.0 | 800 | 0.00125 |
| Ampicillin | 1.1 | 1100 | 0.001 |

## 6.8 Inhibitor studies

Purified AVS-1 was pre-incubated for 10 minutes at 37°C with the β-lactamase inhibitors listed in Table 6.2. Enzyme inhibition ($ID_{50}$) was recorded with both imipenem and nitrocephin as the reporter substrates. EDTA (100mM) had no effect on AVS-1 activity against imipenem or nitrocephin; instead, the hydrolytic activity of AVS-1 was sensitive to the presence of the serine β-lactamase inhibitors clavulanic acid, tazobactam, sulbactam and BRL42715. These results are in marked

contrast to those obtained by Yang and Bush (1996) for the *A. jandaei* AER 14M (formerly *A. sobria*) metallo-carbapenemase AsbM1 (Figure 6.2).

**Table 6.2 A comparison of the inhibitor profile of AVS-1 with that of another *Aeromonas* carbapenemase AsbM1[b]**

| Inhibitor | ID50 (μM) AVS-1[a] | ID50 (μM) AsbM1[b] |
|---|---|---|
| EDTA | >100000 | 18 |
| *p*-Chloro-<br>-mercuribenzoate | 120 | 2 |
| Clavulanic acid | 9 | >1000 |
| Tazobactam | 2 | 250 |
| Sulbactam | 20 | >1000 |
| BRL42715 | 4 | NT |

[a] nitrocephin and imipenem were both used as the reporter substrate and the ID50 values listed here were the same for both of these substrates.
[b] AsbM1 metallo-β-lactamase purified from an *A. jandaei* isolate, with imipenem as the reporter substrate only – data taken from Yang and Bush, 1996.

## 6.9 Discussion

Ion exchange chromatography has been applied to purify the unusual *A. veronii* bv. *sobria* carbapenemase (AVS-1) that has been identified in two strains, 13 and 99. The carbapenemase was partially purified from strain 13 on a QA52 cellulose matrix. Attempts were made to purify AVS-1 to homogeneity by employing gel filtration chromotography; however, this proved to be difficult because the periplasmic protein solution extracted from strain 13 appears to contain several proteins of similar pI and size.

Enzyme kinetics were determined with partially purified AVS-1 β-lactamase. AVS-1 was found to efficiently hydrolyse nitrocephin in addition to imipenem, a property not normally associated with the *Aeromonas* carbapenemases. Ampicillin and cephaloridine hydrolysis was also detected, although the $K_m$s for these substrates are much higher compared with imipenem and nitrocephin and, therefore, AVS-1 has a lower affinity for these β-lactams. The $K_m$ of AVS-1 for imipenem is similar to that of the metallo-β-lactamase ImiS from *A. veronii* bv. *sobria* (formerly *A. sobria*) 163a (Walsh *et al.*, 1996).

Inhibition assays were determined with both imipenem and nitrocephin as the reporter substrates and $ID_{50}$ values were found to be the same, regardless of the reporter substrate used, these results are consistent with the pooled fractions containing a single β-lactamase. The AVS-1 inhibitor profile was found to differ radically from previously purified *Aeromonas* metallo-carbapenemases. The AVS-1 EDTA $ID_{50}$ value measured >100mM, whereas other *Aeromonas* carbapenemases are generally highly sensitive to EDTA. One exception to this statement is an EDTA-resistant strain of *A. hydrophila*, alluded to in section 5.11.

In addition to EDTA resistance, AVS-1 was found to be sensitive to the serine β-lactamase inhibitors clavulanic acid, tazobactam, sulbactam and BRL42715, with tazobactam showing the greatest inhibitory effect. These results suggest that either AVS-1 is a serine-based carbapenemase or alternatively these serine β-lactamase inhibitors are actually behaving as competitive substrates; that is they are competing with either imipenem or nitrocephin for the AVS-1 active site and this manifests

itself as inhibition spectrophotometrically. It is already known that molecular class B metallo-β-lactamases (including Aeromonas metallocarbapenemases) can hydrolyse BRL42715 (Matagne *et al.*, 1995) and tazobactam is a substrate for the *Stenotrophomonas maltophilia* and *B. cereus* metallo-β-lactamases (Felici and Amicosante, 1995). It is however puzzling as to why these inhibitors should appear so effective when assayed spectrophotometrically, yet when BRL42715 and clavulanic acid are used as IEF inhibitor overlays they have little effect on the AVS-1 β-lactamase band (Section 5.8 and 5.9). Again, the explanation for these inconsistencies may arise from the fact that these inhibitors could be substrates for this enzyme. Once the AVS-1 β-lactamase has hydrolysed the serine β-lactamase inhibitor in the IEF overlay, it is then able to hydrolyse nitrocephin, as normal and therefore, the inhibitors show no effect on an IEF gel.

Finally, the size of AVS-1 could only be determined by gel filtration chromatography and was found to be 26kDa, however, it should be noted that gel filtration can underestimate the molecular weights of β-lactamases (Bush, 1989). The cloned and sequenced *Aeromonas* metallo-carbapenemases genes encoding CphA and ImiS have been reported to have a molecular mass of 28kDa (Massida *et al.*, 1991; Walsh *et al.*, 1996).

# 7: RESULTS

## PCR amplification of a metallo-β-lactamase gene from *A. veronii* bv. *sobria* strains 13 and 99

### 7.1 Introduction

The sequences of three *Aeromonas* metallo-carbapenemases (*cphA*, *cphA2* and *imiS*) have been previously determined and share greater than 95% sequence identity to one another (Section 1.11.2.1). The following section describes the identification of a metallo-β-lactamase gene sequence highly homologous to *cphA*, *cphA2* and *imiS* in *A. veronii* bv. *sobria* strains 13 and 99.

### 7.2 Initial primer design

An experiment was devised to determine whether *A. veronii* bv. *sobria* 13 and 99 harboured an *imiS*-like metallo-β-lactamase gene, previously identified in *A. veronii bv. sobria* 163a (Walsh *et al.*, 1998). The strategy was to generate an intragenic *imiS* gene probe by PCR, using a recombinant plasmid that carries *imiS*, as the template DNA. Intragenic primers (1 and 2) were designed from the nucleotide sequence of *imiS* (Walsh *et al.*, 1998), with the aid of Primer 3 software (Section 2.17.1). The *imiS* primers shown in Figure 7.1 are also completely conserved within the *cphA* metallo-β-lactamase sequence from *A. hydrophila* AE036 (Massidda *et al.*, 1991). A single nucleotide in each primer (highlighted in red in Figure 7.1) was not

conserved in the metallo-β-lactamase gene *cphA2* from *A. hydrophila* AER19 (Genbank, accession number U60294). The PCR reaction was performed as described in Section 2.17.2, with 3mM $MgCl_2$, using the amplification parameters listed in Table 7.1. Under these conditions a 516bp DNA fragment was amplified from the recombinant plasmid harbouring *imiS*. The identity of this fragment DNA fragment could be confirmed by digesting the 516bp PCR product with the restriction enzyme *Sal*I, which cuts once within the PCR product to yield two fragments that are 384 and 132bp in length.

Before labelling the intragenic *imiS* PCR product ready for DNA hybridisation, the PCR reaction was repeated but this time with the extracted DNA from *A. veronii* bv. *sobria* strains 13 and 99. A PCR product of the correct size (516bp) was also amplified from strains 13 and 99 and this PCR product could also be cut with *Sal*I. These results strongly suggested that *A. veronii* bv. *sobria* 13 and 99 possess an *imiS*-like metallo-β-lactamase. Therefore, rather than performing DNA hybridisation it was decided that the PCR reaction should be repeated with new primers that would amplify up the entire metallo-β-lactamase gene from strains 13 and 99, which could then be sequenced.

**Figure 7.1 Intragenic PCR primers homologous to the *imiS* metallo-β-lactamase gene that yield a 516bp product**

| | Sequence | Position[a] |
|---|---|---|
| (1) | 5′ ACA AGC TGA TCA AAC GGG TC 3′ | 358 |
| (2) | 5′ TGA TCA GCG CTT CGT AGT GA 3′ | 873 |

[a] Numbering is according to Walsh *et al.*, 1998. Guanine (G) is replaced by adenine (A) and thymine (T) is replaced by cytosine (C) in the *cphA2* metallo-β-lactamase gene (Genbank, accession number U60294).

**Table 7.1 PCR cycling parameters for the amplification of *imiS***

| Segment | Temperature (°C) | Time (minutes) | Function | No. of cycles |
|---|---|---|---|---|
| 1 | 94 | 5 | Denaturation | 1 |
| 2 | 94 | 1 | Denaturation | x30 |
| | 50 | 1 | Annealing | |
| | 72 | 1.5 | Extension | |
| 3 | 72 | 10 | Final extension | 1 |

## 7.3 The selection of new primers

A second set of primers was designed by looking for regions of homology that exist between the DNA that is upstream and downstream of the start and stop codons of *imiS* and *cphA*. Only nucleotide sequence data on the open reading frame (ORF) of *cphA2* were available (Genbank, accession number U60294), therefore, nucleotides

upstream and downstream of *cphA2* could not be compared with those from *imiS* and *cphA*. The nucleotides upstream of the *imiS* ORF show a high sequence identity with the nucleotides that precede the start codon of *cphA*. Unfortunately the same cannot be said of the sequences immediately downstream of *imiS* and *cphA* where there is no sequence homology (Massidda *et al.*, 1991; Walsh *et al.*, 1998). Figure 7.2 shows the primers (3 and 4) chosen to amplify the entire *imiS* open reading frame as featured in the *imiS* sequence (Walsh *et al.*, 1998). A single nucleotide in the left hand primer (3) is not conserved in the sequence of *cphA*. In the sequence of the right hand primer (4), there are several mismatches between the sequence of *imiS* and *cphA*, which are unavoidable because of the lack of homology that occurs between these genes downstream. A section of the right hand primer sequence also falls within the *imiS* ORF (highlighted in bold type). The primers illustrated in Figure 7.2 produced a DNA fragment, that was 870bp in length, with the positive control recombinant plasmid encoding *imiS*, using 1.5mM MgCl2 and the cycling parameters in Table 7.1. No PCR product was obtained with DNA from *A. veronii* bv. *sobria* strains 13 and 99, even when the cycling conditions were varied.

## Figure 7.2 PCR primers that yield an 870bp DNA fragment

| | Sequence | Position[a] |
|---|---|---|
| (3) | 5′ TTC CCC TCA CAA ATC CC ATT 3′ | 28 |
| (4) | 5′ CGG CAG **CTT ATG ATT GTG AA** 3′ | 900 |

[a] Numbering is according to Walsh *et al.*, 1998. In the left hand primer (3) cytosine (C) is replaced by thymine (T) in the *cphA* metallo-β-lactamase gene (Massidda *et al.*, 1991). In the right hand primer (4) cytosine (C) is replaced by guanine (G), guanine (G) is replaced by adenine (A), adenine (A) is replaced by guanine (G), thymine (T) is replaced by cytosine (C), thymine (T) replaced by guanine (G), adenine (A) is replaced by guanine (G) and adenine (A) is replaced by thymine (T) in the *cphA* metallo-β-lactamase gene sequence (Massidda *et al.*, 1991).
Bold type in right hand primer (4) indicates sequence that falls within the *imiS* ORF.

## 7.4 PCR reaction with primers 2 and 3

It was decided that the PCR reaction should be repeated this time employing primers 2 and 3, which have a similar melting temperature and therefore, form a suitable primer pair. A combination of primers 2 and 3 amplifies an 843bp DNA fragment from the positive control recombinant plasmid DNA harbouring *imiS*. This 843bp fragment includes 745 nucleotides of the *imiS* ORF, which is only 20 bases short of the entire *imiS* gene (from start to stop codon). The PCR protocol was followed according to Section 2.17.2 using 3mM $MgCl_2$. The PCR cycles described in Table 7.1 were employed except that an annealing temperature of 52°C was required to prevent amplification of non-specific DNA fragments. A positive PCR reaction was then achieved with extracted DNA from strains 13 and 99.

## 7.5 Sequence analysis of the 843bp PCR products amplified from strains 13 and 99

The nucleotide sequences of the PCR products amplified from *A. veronii* bv. *sobria* strains 13 and 99 were sequenced automatically on an ABI PRISM™ 377 DNA Sequencer (SmithKline Beecham Pharmaceuticals). The 786bp determined from the PCR products were identical for both strains 13 and 99 and are shown in Figure 7.3 along with the deduced amino acid sequences of the truncated metallo-β-lactamase gene.

The metallo-β-lactamase amino acid sequence encoded by this 843bp PCR product from strain 13 and 99 was compared with the amino acid sequences of CphA, CphA2 and ImiS. The differences in the amino acid sequences of these enzymes are shown in Figure 7.4. Figure 7.4 and Table 7.2 show that the amino acid sequence encoded by metallo-β-lactamase gene detected by PCR in strains 13 and 99 is very closely related to the protein sequences of the metallo-β-lactamases CphA, CphA2 and ImiS (≥95% homology).

Five residues were identified that were conserved in the amino acid sequences of CphA, CphA2 and ImiS, but not in the amino acid sequence deduced from the PCR products of strains 13 and 99 (highlighted in bold green type in Figure 7.3). The significance of these five amino acid changes were assessed by aligning the metallo-β-lactamase amino acid sequence from strains 13 and 99 with the sequences of the metallo-β-lactamases CcrA3 (from *Bacteroides fragilis*) and β-lactamase II (from

*Bacillus cereus*). The position of the five amino acids that are different with respect to the other three *Aeromonas* metallo-β-lactamases were then identified on the crystal structures of CcrA3 and β-lactamase II. The position of these five amino acids could not be identified on a crystal structure of an *Aeromonas* metallo-β-lactamase because there are no resolved structures available at present. Only two residues were found anywhere near the substrate binding site, these were lysine (K) at position 49 and asparagine (N) at 206. Lysine 49 is in the CcrA3/β-lactamase II flexible loop region and was identified as possibly an important residue, asparagine 206 on the other hand does not appear to affect anything in the CcrA3/β-lactamase II model (Brian Clarke, SmithKline Beecham Pharmaceuticals - personal communication). There are also no significant differences in the locations of lysine 49 and asparagine 206 in the CcrA3 and β-lactamase II crystal structures. Figure 7.5 shows a three dimensional side by side stereo image of the CcrA3 metallo-β-lactamase with the locations of lysine 49 and asparagine 206 highlighted. The location of threonine 175 is also indicated in Figure 7.5, however, this should be ignored as this is not a residue conserved throughout the sequences from CphA, CphA2 and ImiS – Figure 7.4.

**Figure 7.3 DNA and deduced amino acid sequence of the PCR products from *A. veronii* bv. *sobria* strains 13 and 99**

```
  CCGGCTTGCCACAGGGGTGGCGAGGGGGTGGTTGGTTCGAGGGAGCAAG ATG ATG AAA   58
                                                      M   M   K

GGT TGG ATG AAG TGC GGG TTG GCC GGA GCC GTG GTG CTG ATG GCG AGT  106
 G   W   M   K   C   G   L   A   G   A   V   V   L   M   A   S

TTC TGG GGG GGC AGC GTG CGG GCG GCG GGG ATC TCC CTT AAG CAG GTG  154
 F   W   G   G   S   V   R   A   A   G   I   S   L   K   Q   V

AGC GGC CCT GTC TAT GTG GTA GAG GAC AAC TAC TAC GTC AAA GAG AAC  202
 S   G   P   V   Y   V   V   E   D   N   Y   Y   V   K   E   N

TCC ATG GTC TAT TTC GGG GCC AAG GGG GTG ACG GTG GTG GGG GCG ACC  250
 S   M   V   Y   F   G   A   K   G   V   T   V   V   G   A   T

TGG ACG CCG GAT ACC GCC CGC GAG CTG CAC AAG CTC ATT AAA CGG GTC  298
 W   T   P   D   T   A   R   E   L   H   K   L   I   K   R   V

AGC AGC AAG CCG GTG CTG GAG GTG ATC AAC ACC AAC TAC CAC ACC GAT  346
 S   S   K   P   V   L   E   V   I   N   T   N   Y   H   T   D

CGG GCG GGC GGT AAC GCC TAC TGG AAG TCC ATC GGG GCC AAG GTG GTC  394
 R   A   G   G   N   A   Y   W   K   S   I   G   A   K   V   V

TCG ACC CGC CAG ACC CGG GAT CTG ATG AAG AGC GAC TGG GCC GAG ATT  442
 S   T   R   Q   T   R   D   L   M   K   S   D   W   A   E   I

GTC GCC TTT ACC CGC AAG GGG CTG CCA GAG TAT CCG GAT CTG CCG CTG  490
 V   A   F   T   R   K   G   L   P   E   Y   P   D   L   P   L

GTG CTG CCC AAC GTG GTG CAC GAT GGC GAC TTC ACC CTG CAA GAG GGC  538
 V   L   P   N   V   V   H   D   G   D   F   T   L   Q   E   G

AAG GTG CGT GCC TTC TAT GCG GGC CCG GCC CAC ACG CCG GAC GGC ATC  586
 K   V   R   A   F   Y   A   G   P   A   H   T   P   D   G   I

TTT GTC TAC TTC CCC GAT GAG CAG GTG CTC TAT GGC AAC TGC ATC CTC  634
 F   V   Y   F   P   D   E   Q   V   L   Y   G   N   C   I   L

AAG GAG AAG CTG GGC AAC CTG AGC TTT GCC AAT GTG AAG GCC TAT CCG  682
 K   E   K   L   G   N   L   S   F   A   N   V   K   A   Y   P

CAG ACC ATC GAG CGG CTG AAA GCG ATG AAG CTG CCG ATC AAG ACG GGT  730
 Q   T   I   E   R   L   K   A   M   K   L   P   I   K   T   V

ATT GGC GGT CAC GAC TCG CCG CTG CAC GGC CCC GAG CTG ATT GAT CAC  778
 I   G   G   H   D   S   P   L   H   G   P   E   L   I   D   H

TAC GAA GC *                                                    786
 Y   E
```

* 28 nucleotides short of entire gene.

**Figure 7.4 A comparison between the amino acid sequence encoded by the metallo-β-lactamase gene amplified by PCR from *A. veronii* bv. *sobria* strains 13 and 99 with the amino acid sequences of three other *Aeromonas* metallo-β-lactamases (CphA, CphA2 and ImiS).**

```
                                                    49
13/99 MMKGWMKCGLAGAVVLMASFWGGSVRAAGISLKQVSGPVYVVEDNYYVKENSMVYFGA  58
CphA  MMKGWMKCGLAGAVVLMASFWGGSVRAAGMSLTQVSGPVYVVEDNYYVQENSMVYFGA
CphA2 MMKGWMKCGLAGAVVLMASFWGGSVRAAGMSLTQVSGPVYVVEDNYYVQENSMVYFGA
ImiS  MMKGWIKCGLAGAVVLMASFWGGSVRAAGMSLTQVSGPVYVVEDNYYVQENSMVYFGA

13/99 KGVTVVGATWTPDTARELHKLIKRVSSKPVLEVINTNYHTDRAGGNAYWKSIGAKVVS 116
CphA  KGVTVVGATWTPDTARELHKLIKRVSRKPVLEVINTNYHTDRAGGNAYWKSIGAKVVS
CphA2 KGVTVVGATWTPDTARELHKLIKRVSRQPVLEVINTNYHTDRAGGNAYWKSIGAKVVS
ImiS  KGVTVVGATWTPDTARELHKLIKRVSRKPVLEVINTNYHTDRAGGNAYWKSIGAKVIS

13/99 TRQTRDLMKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHDGDFTLQEGKVRAFYAGPAH 174
CphA  TRQTRDLMKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHDGDFTLQEGKVRAFYAGPAH
CphA2 TRQTRDLMKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHEGDFTLQEGKLRAFYAGPAH
ImiS  TRQTRDLMKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHEGDFTLQEGKLRAFYLGPAH

                                   206
13/99 TPDGIFVYFPDEQVLYGNCILKEKLGNLSFANVKAYPQTIERLKAMKLPIKTVIGGHD 232
CphA  TPDGIFVYFPDEQVLYGNCILKEKLGNLSFADVKAYPQTIERLKAMKLPIKTVIGGHD
CphA2 TPDGIFVYFPDQQVLYGNCILKEKLGNLSFADVKAYPRTIERLKAMKLPIKTVVGGHD
ImiS  SPDGIFVYFPDQQVLYGNCILKEKLGNLSFADVKAYPQTIERLKAMKLPIKTVVGGHD

13/99 SPLHGPELIDHYE*                                             254
CphA  SPLHGPELIDHYEALIKAAPQS
CphA2 SPLHGPELIDHYEALIKAAPQS
ImiS  SPLHGPELIDHYEALIKAASQS
```

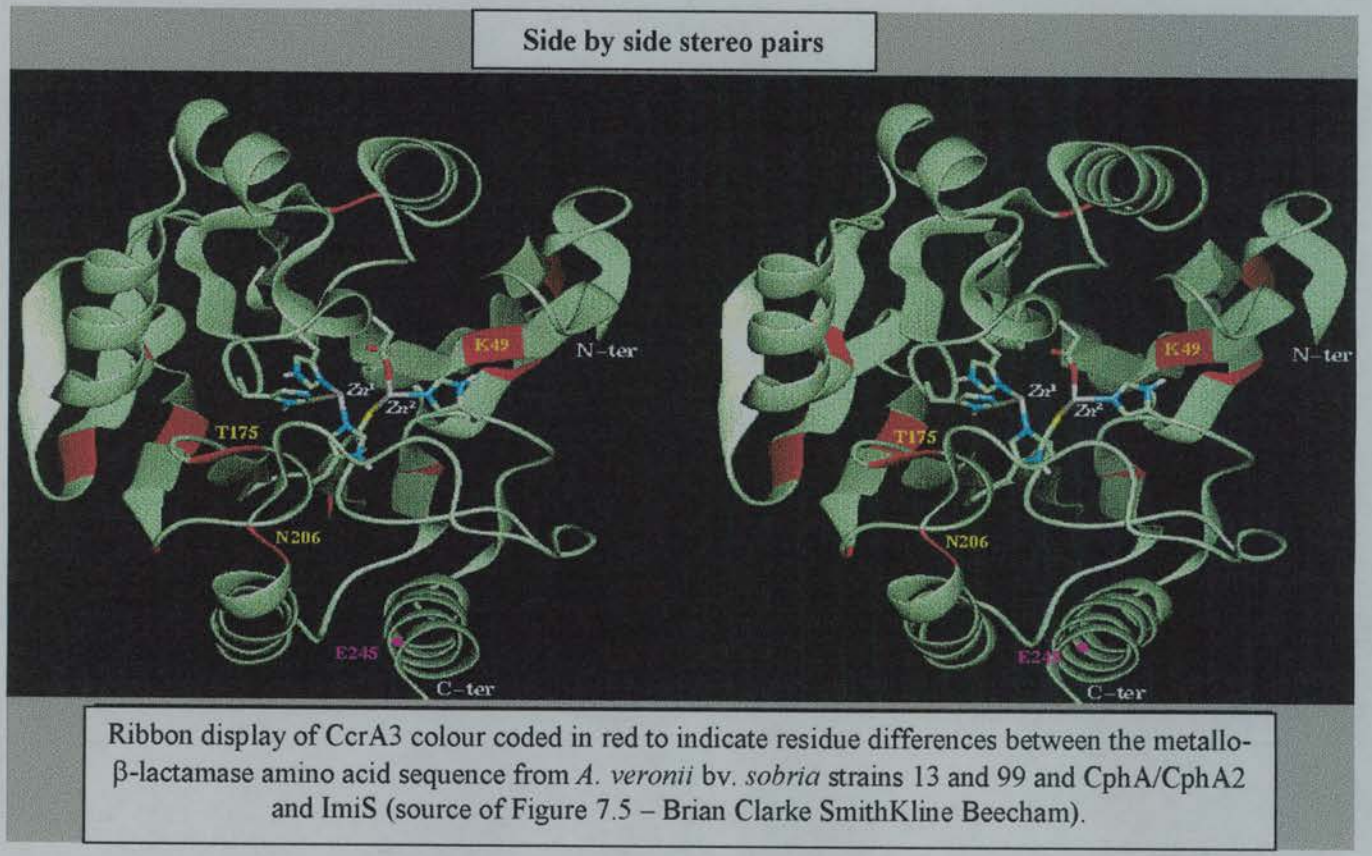
* The sequence is nine amino acids short of the entire unmodified protein.
Shaded amino acids indicate ligands involved in zinc binding in the *B. fragilis* and *B. cereus* metallo-β-lactamase crystal structures (Concha *et al.*, 1997; Carfi *et al.*, 1998*b*).

**Table 7.2 Amino acid identity between the 246 amino acid residues of a metallo-β-lactamase from *A. veronii* bv. *sobria* strains 13 and 99 and the *Aeromonas* metallo-β-lactamases CphA, CphA2 and ImiS**

Identity values are expressed as a percentage calculated from an amino acid sequence that is 246 residues in length.

| | 13/99 | CphA | CphA2 | ImiS |
|---|---|---|---|---|
| **13/99** | - | - | - | - |
| **CphA** | 98 | - | - | - |
| **CphA2** | 95.5 | 98 | - | - |
| **ImiS** | 95 | 97 | 98 | - |

**Figure 7.5 The location of amino acid substitutions in the metallo-β-lactamases from *A. veronii* bv. *sobria* strains 13 and 99 identified on the x-ray crystal structure of the *B. fragilis* CcrA3 metallo-β-lactamase**




Ribbon display of CcrA3 colour coded in red to indicate residue differences between the metallo-β-lactamase amino acid sequence from *A. veronii* bv. *sobria* strains 13 and 99 and CphA/CphA2 and ImiS (source of Figure 7.5 – Brian Clarke SmithKline Beecham).

## 7.6 Discussion

PCR with *imiS*-specific primers has identified a metallo-β-lactamase gene in *A. veronii* bv. *sobria* strains 13 and 99. Although a metallo-β-lactamase gene has been detected in strains 13 and 99, it cannot be conclusively stated that this sequence encodes the pI 5.84 AVS-1 carbapenemase, only cloning or N-terminal sequencing can provide this answer. However, if the metallo-β-lactamase gene possessed by strains 13 and 99 does not code for the AVS-1 carbapenemase, this implies that not only must there be another carbapenemase gene encoded by these two strains but also the metallo-β-lactamase or other genes involved in its regulation must have undergone silencing mutational events.

The amino acid sequence encoded by the metallo-β-lactamase gene in strains 13 and 99 was found to be very similar to ImiS, CphA and CphA2 and possess the putative active site sequence, asparagine-$x_1$-histidine-$x_2$-aspartate (residues 95-99 of Figure 7.4), that is unique to the *Aeromonas* metallo-β-lactamases (Rasmussen and Bush, 1997). Additional zinc binding ligands (histidine -position 174, cysteine -position 193 and histidine –position 243) identified from the crystal structures of the *B. fragilis* and *B. cereus* enzymes were found to be conserved in the metallo-β-lactamase sequence from strains 13 and 99, as they are in the other sequenced *Aeromonas* metallo-β-lactamases.

Only five amino acid residues were found to be among the amino acids conserved in the sequences of CphA, CphA2 and ImiS but not in the 246 amino acids deduced

from the metallo-β-lactamase from strains 13 and 99. There may, however, be further amino acid substitutions amongst the nine amino acid residues at the C-terminus of the metallo-β-lactamase from 13 and 99 not determined in this study. If however, AVS-1 is a metallo-β-lactamase then one or more of these five amino acid differences (or any further substitutions at the metallo-β-lactamase C-terminus) must be responsible for the differences in AVS-1 substrate and inhibitor profiles compared with other *Aeromonas* metallo-β-lactamases. Two possible candidates for the differences in hydrolytic activity are lysine at position 49 and asparagine at 206 because they are located near the β-lactam binding site. The importance of these residues and any additional residues could only be determined by site directed mutagenesis experiments.

# 8: RESULTS

## The cloning of two β-lactamase genes *bla*$_{AVS-2}$ and *bla*$_{AVS-3}$ from *A. veronii* bv. *sobria* 13

### 8.1 Introduction

Chapter 6 has described the purification the AVS-1 carbapenemase, which exhibits an extended substrate profile, including the hydrolysis of ampicillin and cephaloridine. Additionally, a metallocarbapenemase sequence has been identified in both *A. veronii* bv. *sobria* 13 and 99 that is closely related in sequence to previously identified *Aeromonas* carbapenemases, although it could not be concluded whether the metallocarbapenemase sequence encodes the AVS-1 carbapenemase. The following chapter describes the application of different cloning strategies aimed at cloning *bla*$_{AVS-1}$, to determine its sequence. However, the protocols employed result in the isolation of two chromosomal genes encoding the pI 8.3 β-lactamase -AVS-2 and an additional β-lactamase designated AVS-3.

### 8.2 Determining the genetic location of AVS-1

Ethidium bromide has been shown to be efficient in eliminating plasmids that carry drug resistance determinants (Bouanchaud *et al.*, 1969). Ethidium bromide curing was employed to determine whether the AVS-1 carbapenemase was encoded on a

plasmid (Section 2.19). The curing experiment, however, could not demonstrate loss of resistance to imipenem. Furthermore, no plasmid could be isolated from strain 13. Therefore, these results suggest that $bla_{AVS-1}$ is located on the bacterial chromosome.

## 8.3 Creation of a gene bank from *A. veronii* bv. *sobria* 13

*A. veronii* bv. *sobria* 13 chromosomal DNA was isolated (Section 2.13.2) and digested to completion with *Bam*HI. Size fractionated DNA fragments of between 3 and 8kb, obtained following separation in a 0.7% agarose gel and extraction from the agarose with Geneclean II, were ligated into a cloning vector (Section 2.15.3). The chosen cloning vector was pK18 which is a 2.7kb pUC-derived plasmid that has a high copy number, a versatile multiple cloning site and carries a kanamycin resistance marker. The pK18 cloning vector was also digested with *Bam*HI and treated with calf intestine alkaline phosphatase to prevent re-annealing (Section 2.15.2). Recombinant DNA was introduced into competent *E. coli* DH5α cells by electrotransformation (Section 2.16.1 – 2.16.2).

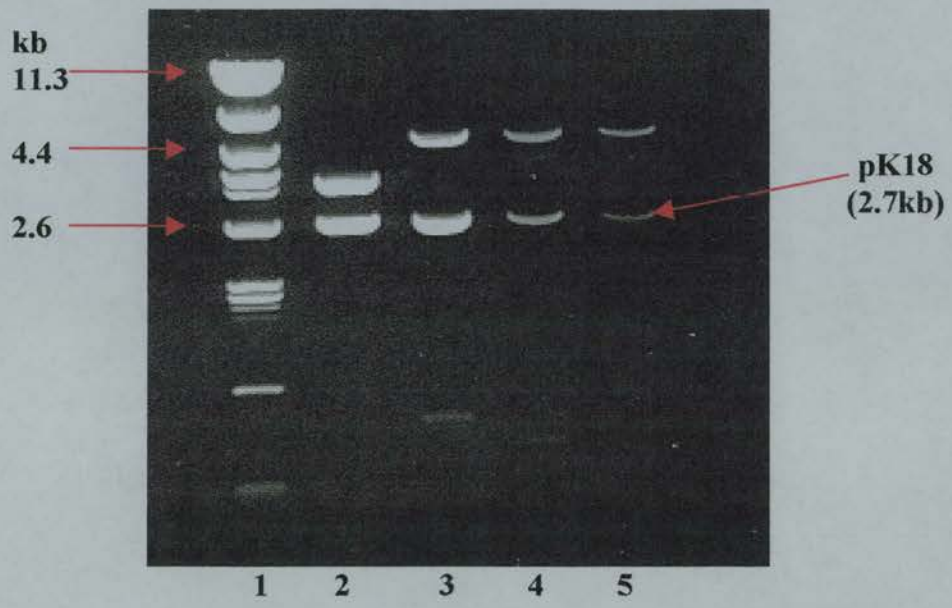
Transformants resistant to β-lactams were selected on two types of media, the first containing kanamycin (30mg/l) and ampicillin (20mg/l) and the other containing kanamycin (30mg/l) and cephaloridine (20mg/l). Ampicillin and cephaloridine were chosen for the selection of the carbapenemase because purified AVS-1 had demonstrated hydrolysis of these substrates. Imipenem was not employed for the selection of the AVS-1 carbapenemase because it is probable that the enzyme will

not confer imipenem resistance in the *E. coli* cloning vector host, as this organism is likely to be more permeable to imipenem than *A. veronii* bv. *sobria*.

Four resistant transformants, numbered clones 1-4, were recovered on agar containing kanamycin and ampicillin but not kanamycin and cephaloridine. Recombinant plasmids were extracted with the Hybaid Recovery™ plasmid miniprep kit, three of the ampicillin resistant transformants possessed a recombinant plasmid with an insert of 5.3kb (clones 2-4) and one recombinant plasmid (clone 1) had an insert of 3.8kb (Figure 8.1).

β-Lactamase extracts were prepared from clones 1-4 by inoculating single colonies into 10ml nutrient broths containing kanamycin (30mg/l) and ampicillin (20mg/l). after six hours of growth at 37°C the cultures were centrifuged, washed in 50mM sodium phosphate buffer and subjected to ultra-sonication. The crude cell extracts were assayed against imipenem; however, no imipenem hydrolysis was detected.

**Figure 8.1 Extracted recombinant plasmids from clones 1-4, digested with *Bam*HI**




**Lanes: 1**, λcut with *Cla*I; **2**, clone 1 (insert – 3.8kb); **3**, clone 2 (insert 5.3kb); **4**, clone 3 (insert 5.3kb); **5**, clone 4 (insert 5.3kb).

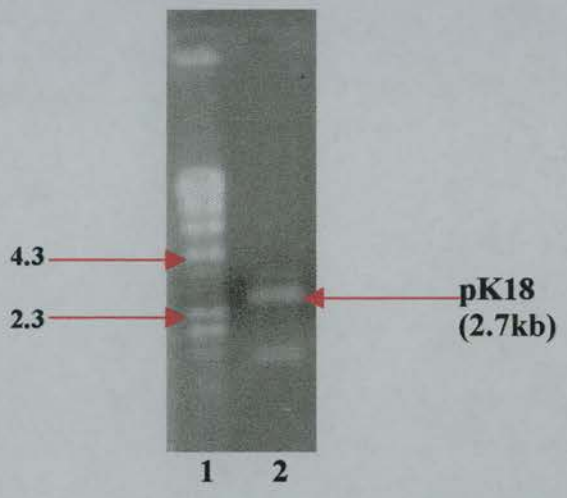
## 8.4 Directional cloning in pK18

Cloning of $bla_{AVS-1}$ was attempted again, this time digesting the *A. veronii* bv. *sobria* 13 chromosomal DNA with two restriction enzymes. This process is known as directional cloning because foreign DNA is inserted in only one orientation within the recombinant plasmid. Three different combinations of restriction enzymes were used to cut the pK18 vector and the chromosomal DNA of strain 13. These combinations were *Eco*RI and *Sal*I, *Eco*RI and *Bam*HI and *Bam*HI plus *Sal*I. Transformants resistant to β-lactams were again selected for on media containing

kanamycin (30mg/l) plus ampicillin (20mg/l) and kanamycin (30mg/l) plus cephaloridine (20mg/l).

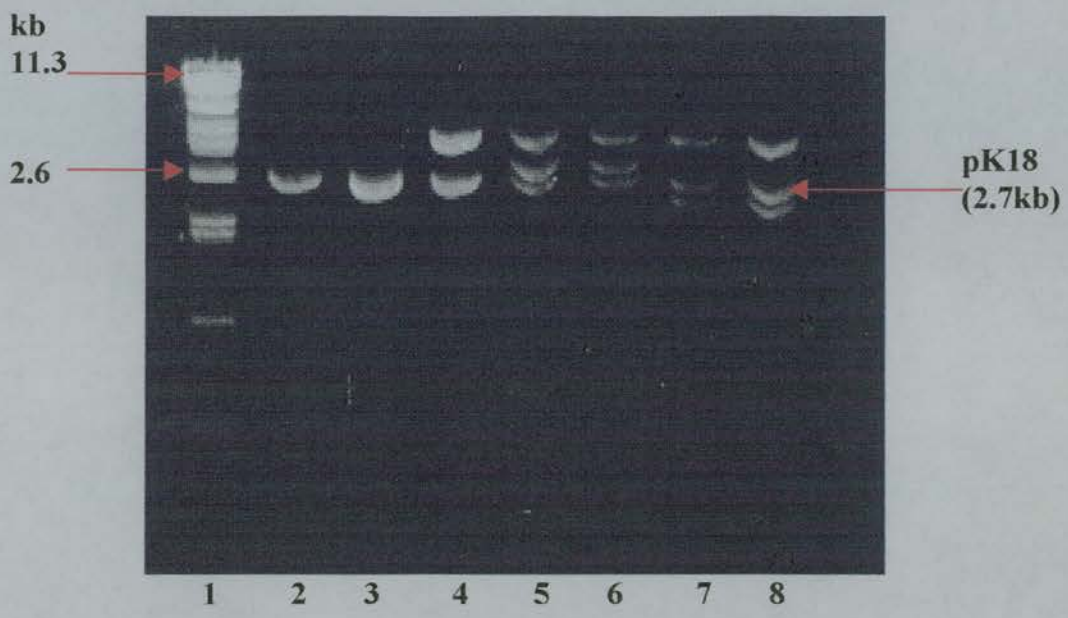
One ampicillin resistant clone (clone 5) was recovered from the gene bank created by digesting the chromosomal and vector DNA with *Eco*RI and *Sal*I. Two cephaloridine resistant clones (clones 6 and7) and five ampicillin resistant clones (clones 8-12) were recovered from the gene bank created by digesting chromosomal and vector DNA with *Eco*RI and *Bam*HI. No β-lactam resistant clones were obtained from the gene bank that involved the *Bam*HI/*Sal*I digest. The insert of the recombinant plasmid extracted from clone 5 measured 1.2kb in length (Figure 8.2). The recombinant plasmids extracted from clones 8-12 had an insert of 4.3kb (Figure 8.3). An additional DNA fragment was also observed next to the pK18 vector of clones 9-12. This might, however, be a DNA artefact (Figure 8.3). The plasmids extracted from the cephaloridine resistant clones 6 and 7 were found to contain no insert (Figure 8.3).

**Figure 8.2 Extracted recombinant plasmid from clone 5 digested with *Eco*RI/*Sal*I**




**Lanes: 1**, λ cut with *Hin*dIII; **2**, clone 5 (insert 1.2kb).

**Figure 8.3 Extracted recombinant plasmids from clones 6-12, digested with *Eco*RI/*Bam*HI**



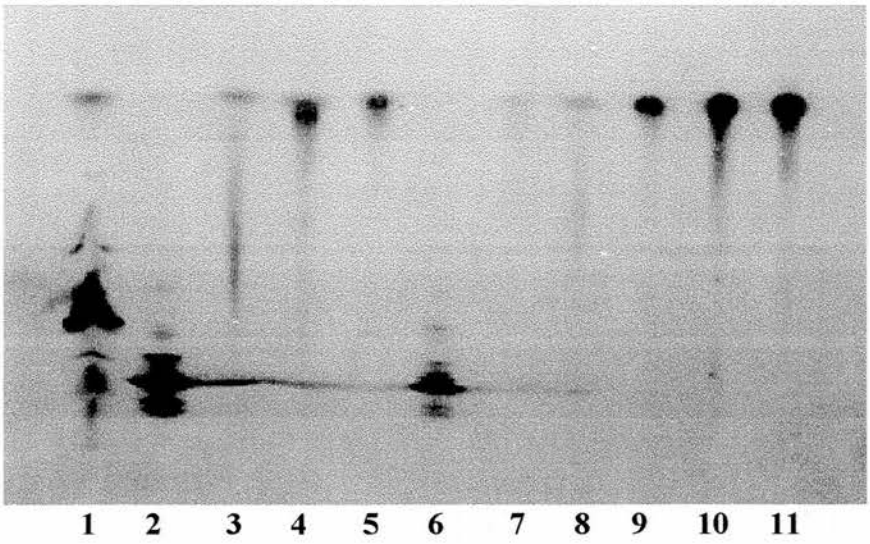

**Lanes: 1,** λcut with *Cla*I, **2**, clone 6 (no insert); **3**, clone 7 (no insert); **4**, clone 8 (4.3kb); **5**, clone 9 (insert 4.3kb); **6**, clone 10 (insert 4.3kb); **7**, clone 11 (insert 4.3kb); **8**, clone 12 (insert 4.3kb).

## 8.5 IEF of β-lactamase active clones

Crude cell extracts prepared from *E. coli* clones 1-12 were tested by nitrocephin spot testing for β-lactamase activity. β-Lactamase activity was detected in clones 1, 2, 3, 4, 5, 8, 9, 10, 11 and 12; however, the cephaloridine-resistant clones 6 and 7 that did not contain a DNA insert failed to produce a colour change with the spot testing reaction. β-Lactamase preparations from clones 1, 2, 3, 4, 5, 8, 9, 10, 11 and 12 were

focused on an IEF gel. IEF assays showed that clones 2, 3, 4, 8, 9, 10, 11 and 12 encoded the AVS-2 enzyme that focused at pI 8.3 (Figure 8.4). Clones 1 and 5 were found to encode a different β-lactamase, named AVS-3, that had a pI value of 4.75 (Figure 8.4), this β-lactamase band was previously thought to be a satellite band of the pI 5.84 AVS-1 β-lactamase.

**Figure 8.4 IEF of β-lactamase extracts from clones 1, 2, 3, 4, 5, 8, 9, 10, 11 and 12**




**Lanes:** **1**, *A. veronii* bv. *sobria* 13; **2**, clone 1 (pI 4.75); **3**, clone 2 (pI 8.3); **4**, clone 3 (pI 8.3); **5**, clone 4 (pI 8.3); **6**, clone 5 (pI 4.75); **7**, clone 8 (pI 8.3); **8**, clone 9 (pI 8.3); **9**, clone 10 (pI 8.3); **10**, clone 11 (pI 8.3); **11**, clone 12 (pI 8.3).

## 8.6 β-Lactam MICs and β-lactam hydrolysis by *E. coli* DH5α clones

β-Lactam susceptibility testing was performed on clones numbered 1-5 and 8-12. All ten clones were found to be resistant to ampicillin, clones 1 and 5 additionally demonstrated a reduced susceptibility to cephaloridine, none of the clones however, showed resistance to imipenem (Table 8.1).

Cell extracts prepared from six representative *E.coli* DH5α clones (1, 2, 5, 8, 9 and 11) were assayed against ampicillin, cephaloridine and imipenem. Extracts from clones 1 and 5 that carry a recombinant plasmid encoding a pI 4.75 β-lactamase from *A. veronii* bv. *sobria* 13 showed strong hydrolytic activity against ampicillin, but much weaker cephaloridine hydrolysis. β-Lactamase preparations from clones 2 ,8, 9 and 11 showed good hydrolytic activity against ampicillin, but only poor cephaloridine activity. None of these clones demonstrated any hydrolysis of imipenem.

## Table 8.1 β-Lactam susceptibility of ten *E. coli* DH5α clones

| Clone | AMP | CEPH | IMP |
|---|---|---|---|
| *E.coli* DH5α* | 4 | <8 | 0.25 |
| 1 | >256 | 64 | 0.25 |
| 2 | >256 | <8 | 0.25 |
| 3 | >256 | <8 | 0.25 |
| 4 | >256 | <8 | 0.25 |
| 5 | >256 | 32 | 0.25 |
| 8 | >256 | <8 | 0.25 |
| 9 | >256 | <8 | 0.25 |
| 10 | >256 | <8 | 0.25 |
| 11 | >256 | <8 | 0.25 |
| 12 | >256 | <8 | 0.25 |

**AMP**, ampicillin; **CEPH**, cephaloridine; **IMP**, imipenem.

* *E. coli* DH5α host containing no recombinant vector.

## Table 8.2 β-Lactam hydrolysis of cell extracts of *E. coli* DH5α carrying *A. veronii* bv. *sobria* 13 β-lactamase genes

| Clone | Hydrolytic activity* | | |
|---|---|---|---|
| | AMP | CEPH | IMP |
| 1 | 149 | 7 | ND |
| 2 | 19.6 | 0.08 | ND |
| 5 | 49.64 | 2.6 | ND |
| 8 | 6.1 | 0.035 | ND |
| 9 | 12.74 | 0.023 | ND |
| 11 | 22.83 | 0.046 | ND |

* μmoles of substrate hydrolysed per minute per ml of enzyme preparation.
**AMP**, ampicillin; **CEPH**, cephaloridine; **IMP**, imipenem.
**ND** – not detected.

## 8.7 Back probing recombinant plasmid inserts to the chromosomal DNA from *A. veronii* bv. *sobria* strain 13

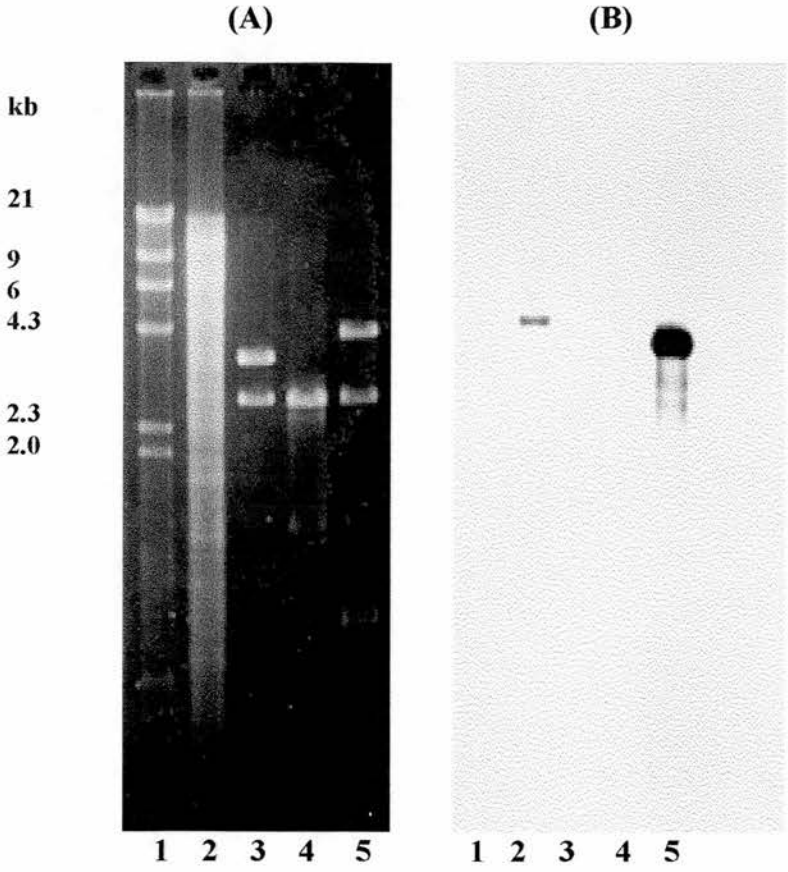
It was decided that the recombinant plasmids from clones 5, and 8 should be extracted and the DNA of the inserts, encoding the AVS-3 and AVS-2 β-lactamase genes, should be automatically sequenced. Before sending the plasmids away for automatic DNA sequencing (SmithKline Beecham Pharmaceuticals, Collegeville, P.A.), back probing of these plasmid inserts to the chromosomal DNA from *A. veronii* bv. *sobria* 13 was performed to confirm their origin.

The purified recombinant plasmids from clones 5, and 8 were digested with the appropriate restriction enzymes (*Eco*RI/*Sal*I and *Eco*RI/*Bam*HI, respectively) to release the inserts. The two different inserts could then be purified from the vector DNA by running the digested plasmids on an agarose gel, slicing the insert from the gel and extracting the DNA fragment from the agarose. The inserts were then labelled with F1-dUTP (Section 2.18.1), ready for hybridisation. Chromosomal DNA from *A. veronii* bv. *sobria* 13 was cut with *Bam*HI and blotted onto a nylon membrane along with the digested recombinant plasmids from clones 1, 5 and 8, after agarose gel electrophoresis (Section 2.18.3).

Figure 8.5 shows the labelled 4.3kb insert of clone 8 back probing to the chromosomal DNA from *A. veronii* bv. *sobria* 13, confirming its origin. The 4.3kb insert from clone 8 does not hybridise with the insert from either clone 1 or clone 5.

Figure 8.6 shows the hybridisation of the labelled 1.2kb insert from clone 5 to the *A. veronii* bv. *sobria* 13 chromosomal DNA and to the insert from clone 1 (3.8kb). Unfortunately the labelled insert from clone 5 was contaminated with vector DNA, even though repeated attempts were made to completely purify the insert away from the pK18 vector DNA. Vector DNA in clones 1, 5 and 8 was also detected. Figure 8.6 does, however, show that the labelled insert from clone 5 does not hybridise to the insert from clone 8.

**Figure 8.5 Hybridisation of the insert from clone 8 to *A. veronii* bv. *sobria* 13 chromosomal DNA**

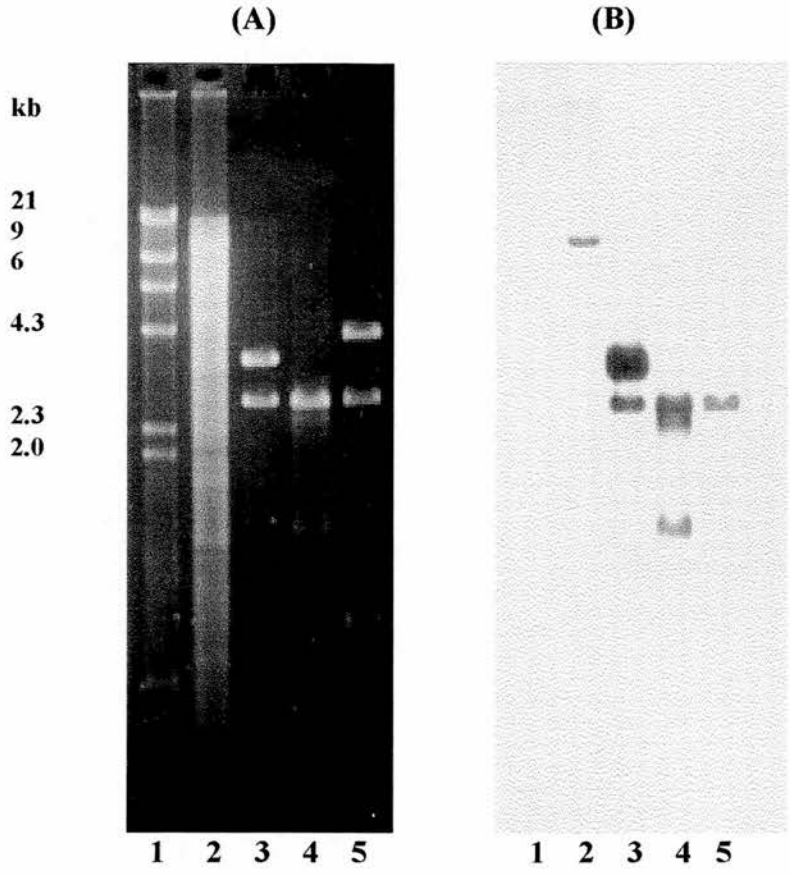



**(A)** Separated fragments in a 0.7% agarose gel.

**(B)** Hybridisation of the 4.3kb insert of clone 8 to the DNA fragments on the blotted gel.

**Lanes: 1**, λ cut with *Hin*dIII; **2**, *A. veronii* bv. *sobria* 13 chromosomal DNA cut with *Bam*HI; **3**, recombinant plasmid from clone 1 cut with *Bam*HI; **4**, recombinant plasmid from clone 5 cut with *Eco*RI/*Sal*I; **5**, recombinant plasmid from clone 8 cut with *Eco*RI/*Bam*HI.

**Figure 8.6 Hybridisation of the insert from clone 5 to *A. veronii* bv. *sobria* 13 chromosomal DNA**




**(A)** Separated fragments in a 0.7% agarose gel.

**(B)** Hybridisation of the 1.2kb insert of clone 5 to the DNA fragments on the blotted gel.

**Lanes: 1**, λ cut with *Hin*dIII; **2**, *A. veronii* bv. *sobria* 13 chromosomal DNA cut with *Bam*HI; **3**, recombinant plasmid from clone 1 cut with *Bam*HI; **4**, recombinant plasmid from clone 5 cut with *Eco*RI/*Sal*I; **5**, recombinant plasmid from clone 8 cut with *Eco*RI/*Bam*HI.

## 8.8 Sequence analysis of clones 1, 5 and 8

DNA sequencing of the inserts from clones 5 and 8 was performed on an ABI PRISM™ 377 DNA Sequencer with the universal primers M13F (forward) and M13R (reverse), using a primer walking strategy. Unfortunately, it was only possible to sequence the pI 8.3 β-lactamase gene $bla_{AVS-2}$ harboured on the clone 8 recombinant plasmid. The pI 4.75 β-lactamase gene $bla_{AVS-3}$, encoded on the recombinant plasmid of clone 5 and clone 1, could not be sequenced. Clone 5 was sequenced using M13F and M13R, however, the sequencing reaction was only successful in one direction (L. Katz, SmithKline Beecham – personal communication). When the sequencing information from clone 5 was entered into NCBI BLAST the sequence was found to be part of the pK18 vector. The primers were checked for correct labelling, however, it appears that clone 5 had lost its insert and this explains why only one of the universal primers was successful data (L. Katz - SmithKline Beecham, personal communication). Clone 1 was reported to give high background peaks, which is indicative of two clones being present and therefore, also could not be sequenced (L. Katz - personal communication).

Sequencing the insert of clone 8 was carried out until an open reading frame of potential interest was located. Figure 8.7 shows the DNA sequence and deduced amino acid sequence of the $bla_{AVS-2}$ gene. The AVS-2 β-lactamase was found to be closely related to the molecular class D penicillinases AsbB1 from *A. jandaei* AER 14M (formerly *A. sobria*) and AmpS from *A. veronii* bv. *sobria* 163a (formerly *A. sobria*) (Rasmussen, *et al.*, 1994a; Walsh *et al.*, 1995b). The $bla_{AVS-2}$ gene has an

open reading frame of 795 nucleotides which encodes a protein of 264 amino acids. A comparison between the amino acid sequences of AVS-2, AsbB1 and AmpS is shown in Figure 8.8. The amino acid identity between these three enzymes was found to be ≥95.5% (Table 8.3).

**Figure 8.7 DNA and deduced amino acid sequence of the *A. veronii* bv. *sobria* 13 $bla_{AVS-2}$ β-lactamase gene**

```
ATG TCC CGC CTG CTT CTC TCC AGC CTG CTG GCT GCC GGT CTG CTC GCA  48
 M   S   R   L   L   L   S   S   L   L   A   A   G   L   L   A

GCC CTG CCT GCC TCC GCC GCC AGC GGC TGC TTT CTC TAT GCT GAC GGC  96
 A   L   P   A   S   A   A   S   G   C   F   L   Y   A   D   G

AAC GGC CAG ACC CTC TCC AGC GAA GGG GAC TGC TCC AGC CAG CTG CCA 144
 N   G   Q   T   L   S   S   E   G   D   C   S   S   Q   L   P

CCC GCG TCC ACC TTC AAG ATC CCG CTC GCG CTG ATG GGT TAC GAC AGC 192
 P   A   S   T   F   K   I   P   L   A   L   M   G   Y   D   S

GGC TAT CTG GTG GAT GAA GAG CAT CCG GCA CTG CCT TAC AAA CCG AGC 240
 G   Y   L   V   D   E   E   H   P   A   L   P   Y   K   P   S

TAT GAC GGC TGG CTG CCC GCC TGG CGT GAA ACC ACC ACC CCG CGC CGC 288
 Y   D   G   W   L   P   A   W   R   E   T   T   T   P   R   R

TGG GAA ACC TAC TCG GTG GTC TGG TTC TCC CAG CAG ATC ACC GAA TGG 336
 W   E   T   Y   S   V   V   W   F   S   Q   Q   I   T   E   W

CTG GGG ATG GAG CGC TTC CAG CAA TAC GTC GAC CGC TTC GAC TAC GGC 384
 L   G   M   E   R   F   Q   Q   Y   V   D   R   F   D   Y   G

AAC CGG GAT CTC TCC GGC AAT CCG GGC AAA CAT GAC GGC CTG ACC CAG 432
 N   R   D   L   S   G   N   P   G   K   H   D   G   L   T   Q

GCC TGG CTC AGC TCC AGC CTC GCC ATC AGT CCG GAG GAG CAG GCT CGC 480
 A   W   L   S   S   S   L   A   I   S   P   E   E   Q   A   R

TTC CTC GGC AAG ATG CTG AGC GGC AAG CTG CCG GTC TCG GCG CAG ACC 528
 F   L   G   K   M   L   S   G   K   L   P   V   S   A   Q   T

CTG CAG TAC ACC GCC AAT ATC CTC AAG GTG AGC GAG ATC GAC GGC TGG 576
 L   Q   Y   T   A   N   I   L   K   V   S   E   I   D   G   W

CAG ATC CAC GGC AAA ACC GGC ATG GGC TAC CCG AAG AAG CTG GAT GGC 624
 Q   I   H   G   K   T   G   M   G   Y   P   K   K   L   D   G

AGC CTC AAC CGC GCT CAG CAG ATT GGC TGG TTC GTC GGC TGG GCC AGC 672
 S   L   N   R   A   Q   Q   I   G   W   F   V   G   W   A   S

AAA CCG GGC AAA CAG CTG ATC TTC GTC CAT ACC GTG GTG CAA AAG CCC 720
 K   P   G   K   Q   L   I   F   V   H   T   V   V   Q   K   P

GGC AAG CAG TTC GCC TCC CTC AAG GCG AAA GAA GAG GTG CTG GCT GCC 768
 G   K   Q   F   A   S   L   K   A   K   E   E   V   L   A   A

CTG CCT GCA CAA CTT AAA AAG CAG TGA                             795
 L   P   A   Q   L   K   K   Q   *
```

**Figure 8.8 Comparison of the amino acid sequences of AVS-2 with the molecular class D enzymes AsbB1 and AmpS from *A. jandaei* AER14M and *A. veronii* bv. *sobria* 163a[a]**

```
AVS-2   MSRLLLSSLLAAGLLAALPASAASGCFLYADGNGQTLSSEGDCSSQLPPA
AsbB1   MSRLLLSGLLATGLLCAVPASAASGCFLYADGNGQTLSSEGDCSSQLPPA
AmpS    MSRLLLSSLLAAGLLAALPASAASGCFLYADGNGQTLSSEGDCSSQLPPA

AVS-2   STFKIPLALMGYDSGYLVDEEHPALPYKPSYDGWLPAWRETTTPRRWETY
AsbB1   STFKIPLALMGYDSGFLVDEEHPALPYKPSYDGWLPAWRETTTPRRWETY
AmpS    STFKIPLALMGYDSGFLVDEEHPALPFKPGYDDWLPAWRETTTPRRWETY

AVS-2   SVVWFSQQITEWLGMERFQQYVDRFDYGNRDLSGNPGKHDGLTQAWLSSS
AsbB1   SVVWFSQQITEWLGMERFQQYVDRFDYGNRDLSGNPGKHDGLTQAWLSSS
AmpS    SVVWFSQQITEWLGMERFQQYVDRFDYGNRDLSGNPGKHDGLTQAWLSSS

AVS-2   LAISPEEQARFLGKMLSGKLPVSAQTLQYTANILKVSEIDGWQIHGKTGM
AsbB1   LAISPEEQARFLGKMVSGKLPVSAQTLQYTANILKVSEVEGWQIHGKTGM
AmpS    LAISPEEQARFLGKMVSGKLPVSAQTLQYTANILKVSEIDGWQIHGKTGM

AVS-2   GYPKKLDGSLNRAQQIGWFVGWASKPGKQLIFVHTVVQKPGKQFASLKAK
AsbB1   GYPKKLDGSLNRDQQIGWFVGWASKPGKQLIFVHTVVQKPGKQFASIKAK
AmpS    GYPKKLDGSLNRDQQIGWFVGWASKPGKQLIFVHTVVQKPGKQFASLKAK

AVS-2   EEVLAALPAQLKKQ
AsbB1   EEVLAALPAQLKKL
AmpS    EEVLAALPAQLKTQ
```

[a] Amino acid residues conserved in all three proteins are represented in black, whereas, red lettering indicates residues which differ amongst the three proteins

**Table 8.3 Amino acid identity between AVS-2, AsbB1 and AmpS**

Identity values are expressed as a percentage of the 264 amino acid sequence

| Enzyme | AVS-2 | AsbB1 | AmpS |
|---|---|---|---|
| AVS-2 | - | - | - |
| AsbB1 | 95.8 | - | - |
| AmpS | 97.3 | 95.5 | - |

## 8.9 Discussion

This chapter has described the protocols employed in an attempt to clone the carbapenemase AVS-1. The cloning of *Aeromonas* carbapenemases is known to be problematic and stems from the fact that these enzymes normally display a very narrow substrate profile which limits the choice of β-lactams that can used in the cloning selection procedure. Furthermore, the *Aeromonas* carbapenemases do not confer carbapenem resistance in an *E. coli* background (Hayes, 1995, Rossolini *et al.*, 1995; Walsh - personal communication). Purification of the AVS-1 carbapenemase from *A. veronii* bv. *sobria* revealed that this enzyme hydrolysed not only imipenem but also nitrocephin, ampicillin and cephaloridine, thus, cloning strategies were employed that involved the selection of clones with ampicillin and cephaloridine containing media.

Although several clones were selected on media containing ampicillin, none of these clones could hydrolyse imipenem. Further analysis of these ampicillin resistant clones did, however, reveal important information concerning the β-lactamase complement of *A. veronii* bv. *sobria* strain 13. It was initially thought that this strain (along with strain 99) possessed two β-lactamases, an unusual *Aeromonas* carbapenemase designated AVS-1 with a pI of 5.84 and a second clavulanic acid sensitive high pI (8.3) β-lactamase, however, this was found not to be the case. Isoelectric focusing of ten ampicillin resistant clones identified two different β-lactamases, neither of them AVS-1. Eight of these clones (2, 3, 4, 8, 9, 10, 11, 12) harboured the pI 8.3 β-lactamase which could efficiently hydrolyse ampicillin. The

other two clones (1 and 5) produced a β-lactamase with a pI of 4.75, previously thought to be a satellite band of the AVS-1 carbapenemase. This enzyme was named AVS-3 and could hydrolyse both ampicillin and cephaloridine.

Clones containing both AVS-2 and AVS-3 were sent away for DNA sequencing, although only the sequence of AVS-2 could be determined. Sequence analysis of the AVS-2 β-lactamase revealed that this enzyme was closely related to the molecular class D penicillinases AsbB1 and AmpS, identified previously in a strain of *A. jandaei* and *A. veronii* bv. *sobria*, respectively (Rasmussen *et al.*, 1994a; Walsh *et al.*, 1995b).

Previously, up to three different inducible β-lactamases have been described in individual *Aeromonas* isolates including a metallocarbapenemase, a class D penicillinase and a class C cephalosporinase (Walsh *et al.*, 1995a; Rasmussen *et al.*, 1994a; Yang and Bush 1996). Three different β-lactamases have now been identified in *A. veronii* bv. *sobria* 13 that include a pI 5.84 carbapenemase (AVS-1), a pI 8.3 class D penicillinase (AVS-2) and a third unidentified β-lactamase AVS-3. It is possible that this AVS-3 is related to the cephalosporinases such as AsbA1 and CepS previously identified in other *Aeromonas* isolates (Rasmussen *et al.*, 1994a; Walsh *et al.*, 1995b). However, the ability of AVS-3 to hydrolyse ampicillin with greater effiiency than cephaloridine is not a property normally associated with *Aeromonas* cephalosporinases (Rasmussen *et al.*, 1994a; Walsh *et al.*, 1995b). Only future sequencing data on AVS-2 can resolve the classification of this enzyme. Although the cloning strategies described in this chapter have been successful in recovering

two β-lactamases from strain 13 the gene encoding the AVS-1 carbapenemase has not been isolated. Other methods (described in Section 10) are therefore, required to recover $bla_{AVS-1}$.

# 9:RESULTS

## An assessment of the heterogeneity of the metallo-β-lactamases from clinical *Stenotrophomonas maltophilia* isolates

### 9.1 Introduction

The following section describes the application of PCR in the detection of *S. maltophilia* metallo-β-lactamases, followed by DNA sequencing of the PCR products to ascertain the relationship between *S. maltophilia* m-β-l types 1-7 described by Payne *et al.*, (1994a; 1994b). This study also includes the metallo-β-lactamase of *S. maltophilia* 511 characterised biochemically by Felici *et al.*, (1993) that has the same pI (6.8) as the m-β-l type 5 (Payne *et al.*, 1994a). An additional comparison is also made between the m-β-l types 1-7 and the amino acid sequences of the metallo-β-lactamases from strains GN12873 and IID1275 to determine their relatedness.

### 9.2 Bacterial strains

The initial aim was to analyse the m-β-l types 1-7 from seven *S. maltophilia* strains originally isolated from hospitalised patients in the United Kingdom (Payne *et al.*, 1994a). Unfortunately, strain 136 that harbours m-β-l type 6 could not be recovered

and therefore this study concentrates on m-β-l types 1-5 and 7 (Table 9.1).

**Table 9.1 *S. maltophilia* strains that produce m-β-l types 1, 2, 3, 4, 5 and 7**

| Strain number | m-β-l type | pI value |
|---|---|---|
| GEL | 1 | 4.8 |
| 0062 | 2 | 5.5 |
| U152 | 3 | 5.7 |
| J2323 | 4 | 6.0 |
| 37 | 5 | 6.4 |
| A37454 | 7 | 6.8 |

## 9.3 Intragenic PCR primer design and cycling parameters

The nucleotide sequences of the metallo-β-lactamase genes $bla_{L1}$ and *blaS* from *S. maltophilia* IID1275 (Walsh *et al.*, 1994) and *S. maltophilia* GN12873 (Sanschagrin *et al.*, 1998) respectively, were aligned using the Genbank Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast). Intragenic stretches of homology between these sequences were identified and primers were designed within these regions of homology with the aid of Primer 3 software (Section 2.17.1). The chosen primer sequences as featured in $bla_{L1}$ from strain IID1275 (Walsh *et al.*, 1994) are shown in Figure 9.1. A single nucleotide in each primer (highlighted in red) was not conserved in the sequence of *blaS* from strain GN12873 (Sanschagrin *et al.*, 1998). These primers yield a 671 bp PCR product.

The PCR procedure was performed as described in Section 2.17.2, with 1mM $MgCl_2$. A recombinant plasmid (pUB8902), encoding the the metallo-β-lactamase $bla_{L1}$ from *S. maltophilia* IID1275 (Walsh *et al.*, 1994), was extracted and employed as a positive PCR control. Template DNA from test *S. maltophilia* strains was prepared using the Genie genomic DNA extraction. Standard laboratory strains *E. coli* NCTC 10418 and *P. aeruginosa* NCTC 10662 were used as negative PCR controls. PCR amplification conditions are shown in Table 9.2.

## Figure 9.1 Intragenic PCR primers homologous to the known $bla_{L1}$ sequence of *S. maltophilia* IID1275

| Sequence | Position[a] |
|---|---|
| 5′ GTT CTA CCC TGC TCG CCT TC 3′ | 5 |
| 5′ CTC GAT CAG CTG CGG ATA AC 3′ | 687 |

[a] Numbering is according to Walsh *et al.*, (1994).

Cytosine (C) is replaced by thymine, and thymine (T) is replaced by adenine in the sequence of GN12873 (Sanschagrin *et al.*, 1998).

**Table 9.2 PCR cycling parameters for the amplification of *S. maltophilia* metallo-β-lactamases**

| Segment | Temperature (°C) | Time (minutes) | Function | No. of cycles |
|---|---|---|---|---|
| 1 | 94 | 5 | Denaturation | 1 |
| 2 | 94 | 1 | Denaturation | x30 |
| | 60 | 1 | Annealing | |
| | 72 | 1.5 | Extension | |
| 3 | 72 | 10 | Final extension | 1 |

## 9.4 Detection of *S. maltophilia* metallo-β-lactamases by PCR

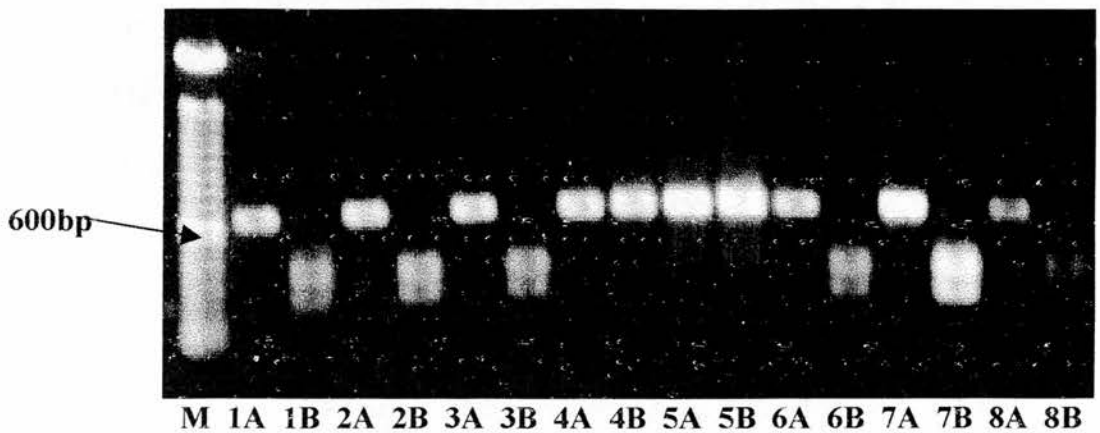
A single PCR product of 671 bp in size was amplified from the seven *S. maltophilia* test strains and from the positive control pUB8902 plasmid DNA. No PCR product was obtained from the negative control strains.

## 9.5 Restriction of the PCR products

The 671 bp nucleotide sequence of $bla_{L1}$ (Walsh *et al.*, 1994), that could be amplified by PCR, was entered into the WebCutter 2.0 restriction site analysis program (http:www.firstmarket.com/cutter/cut2.html) to identify a suitable restriction enzyme that would cleave once within this fragment of DNA. The restriction endonuclease *Hin*fI cuts once within this sequence to give two fragments that were 381 and 290 bp in size. Restriction digests with *Hin*fI were set up with the PCR products amplified from the seven *S. maltophilia* strains. The results of the restriction enzyme analysis

are shown in Figure 9.2. The restriction site is conserved in five of the seven strains, PCR products from strains 511 and 37 were not digested by *Hin*fI.

**Figure 9.2 *S. maltophilia* PCR products restricted with *Hin*fI**




**A**- uncut PCR product, **B**- restricted PCR product, **M**-100bp ladder, **1**- GEL (m-β-l type 1), **2**- 0062 (m-β-l type 2), **3**- U152 (m-β-l type 3), **4**- 511, **5**- 37 (m-β-l type5), **6**- J2323 (m-β-l type 4), **7**- A37454 (m-β-l type 7), **8**- pUB8902 (L1 clone).

## 9.6 Intergenic PCR primers

PCR primers were designed from upstream and downstream of the $bla_{L1}$ gene open reading frame of *S. maltophilia* IID1275 (Walsh *et al.*, 1994) in an attempt to extend the PCR product size to produce a DNA fragment that would contain the entire $bla_{L1}$ gene (Figure 9.3). Amplification conditions were applied as in Section 9.3, but with varying annealing temperatures and $MgCl_2$ concentrations. A single fragment of DNA of the appropriate size (944 bp) could be amplified from the positive control plasmid DNA (pUB8902) employing an annealing temperature of 58°C and 1mM

$MgCl_2$. However, when the reaction was repeated with DNA from the seven test *S. maltophilia* strains (GEL, 0062, U152, J2323, 37, 511 and A37454), no product was obtained. Therefore, under these optimised conditions, this primer set appears to specific for only the metallo-β-lactamase from *S. maltophilia* strain II1275D and the PCR product lengths from the seven test strains could not be extended.

**Figure 9.3 Intragenic PCR primers homologous to the known $bla_{L1}$ sequence of *S. maltophilia* IID1275**

| Sequence | Position[a] |
|---|---|
| 5′ AAG GAG GCC CAT GCT AGT TT 3′ | 464 |
| 5′ CTG CTC TGC TGG ACT CAA CA 3′ | 1407 |

[a] Numbering is according EMBL Data Bank, accession number X7504. Guanine (G) is replaced by cytosine, thymine (T) by guanine and adenine (A) by thymine in the sequence of GN12873 (Sanschagrin *et al.*, 1998).

## 9.7 Sequence analysis of intragenic PCR products

DNA sequencing of the 671 bp intragenic PCR products from the seven *S. maltophilia* strains (GEL, 0062, U152, J2323, 511, 37 and AK37454) was performed on an Applied Biosystems automated sequencer (Haematology Dept., Edinburgh University). Sequences were determined on both DNA strands, to check sequence accuracy. A sequence of 561 bp in length could be determined from each of the PCR products. The resulting DNA sequences were entered into the BLAST database and

aligned against the *bla*$_{L1}$ and *bla*S nucleotide sequences from *S. maltophilia* strain IID1275 and strain GN12873, respectively.

The nucleotide and deduced amino acid sequences of the PCR products from the seven *S. maltophilia* strains are shown in Figures 9.4 – 9.9. The *Hin*fI target site (G↓ANTC) is highlighted by a grey box in the sequences from strains GEL, 0062, U152, J2323 and AK37454 (Figures 9.4, 9.5, 9.6, 9.7, 9.9). In the sequences from strains 37 and 511 (Figure 9.7), thymine at position 391 (numbering according to Walsh *et al.*, 1994) was replaced by adenine, *Hin*fI does not recognise this sequence (GANAC) and therefore, the DNA was not cleaved at this point (Figure 9.2). The DNA fragments from strains 37 and 511 differ by only four nucleotide substitutions (Figure 9.8). These nucleotide differences all occured in the third position of a codon and represent the same amino acid in each sequence. Thus the deduced amino acid sequences from 511 and 37 are identical.

A comparison between the amino acid sequences derived from the 561 bp DNA fragment sequenced from the seven *S. maltophilia* strains and the known amino acid sequences of the metallo-β-lactamases from IID1275 and GN12873 (Walsh *et al.*, 1994; Sanschagrin *et al.*, 1998) are shown in Figure 9.10. Amino acid residues identified from the crystal structure of the metallo-β-lactamase from *S. maltophila* IID1275 (Ullah *et al.*, 1998) as being involved in zinc ion ligation or β-lactam substrate binding are also highlighted in Figure 9.10. Table 9.3 lists the percentage amino acid identity between the 187 amino acid section of the L1-type metallo-β-lactamases sequenced from nine *S. maltophilia* strains.

**Figure 9.4 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* GEL (m-β-l type 1) with $bla_{L1}$ specific primers**

```
68*
GAA GTA CCA CTG CCG CAG CTG CGG GCC TAC ACC GTG GAC GCC TCG TGG CTG
 E   V   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   L
120
CAG CCG ATG GCA CCG GTG CAG ATC GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   I   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACC GCG CTG CTT GTG CAG ACC CCC GAC GGC GCG GTG CTG CTC
 E   D   L   T   A   L   L   V   Q   T   P   D   G   A   V   L   L
222
GAC GGC GGC ATG CCG CAG ATG GCC AGC CAC CTG CTG GAC AAC ATG AAG GCG
 D   G   G   M   P   Q   M   A   S   H   L   L   D   N   M   K   L
273
CGT GGC GTG ACA CCT CGG GAT CTG CGG CTG ATC CTG CTC AGC CAC GCA CAC
 R   G   V   T   P   R   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAT GCC GGA CCG GTG GCG GAG CTG AAG CGC CGT ACG GGC GCC AAA
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   K
375
GTG GCG GCC AAC GCC GAA TCG GCG GTG CTG CTG GCG CGT GGC GGC AGC GAT
 V   A   A   N   A   E   S   A   V   L   L   A   R   G   G   S   D
426
GAC CTG CAC TTT GGC GAT GGC ATC ACC TAC CCG CCT CCG AAT GCA GAC CGC
 D   L   H   F   G   D   G   I   T   Y   P   P   P   N   A   D   R
477
ATC ATC ATG GAT GGT GAA GTG ATC ACG GTG GGC GGC ATC GTG TTC ACC GCG
 I   I   M   D   G   E   V   I   T   V   G   G   I   V   F   T   A
528
CAC TTC ATG GCG GGG CAC ACA CAG GGC AGC ACC GCG TGG ACC TGG ACC GAT
 H   F   M   A   G   H   T   Q   G   S   T   A   W   T   W   T   D
579
ACC CGC ATT GGC AAG CCG GTG CGC ATC GCC TAC GCC GGC AGC CTG AGT GCA
 T   R   I   G   K   P   V   R   I   A   Y   A   G   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
Grey boxed nucleotides indicate *Hin*fI restriction site.

**Figure 9.5 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* 0062 (m-β-l type 2) with $bla_{L1}$ specific primers**

```
68*
GAA GTA CCA CTG CCG CAG CTG CGG GCC TAC ACC GTG GAC GCC TCG TGG CTG
 E   V   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   W
120
CAG CCG ATG GCA CCG CTG CAG ATC GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   I   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACC GCG CTG CTT GTG CAG ACC CCC GAC GGC GCG GTG CTG CTC
 E   D   L   T   A   L   L   V   Q   T   P   D   G   A   V   L   L
222
GAC GGC GGC ATG CCG CAG ATG GCC AGC CAC CTG CTG GAC AAC ATG AAG GCG
 D   G   G   M   P   Q   M   A   S   H   L   L   D   N   M   K   A
273
CGT GGC GTG ACG CCT CGG GAT CTG CGG CTG ATC CTG CTC AGC CAC GCA CAC
 R   G   V   T   P   R   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAT GCC GGA CCG GTG GCG GAG CTG AAG CGC CGT ACG GGC GCC AAA
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   K
375
GTG GCG GCC AAC GCC GAA TCG GCG GTG CTG CTG GCG CGT GGC GGC AGC GAT
 V   A   A   N   A   E   S   A   V   L   L   A   R   G   G   S   D
426
GAC CTG CAC TTC GGC GAT GGC ATC ACC TAC CCG CCT GCC AAT GCA GAC CGC
 D   L   H   F   G   D   G   I   T   F   P   P   A   N   A   D   R
477
ATC GTC ATG GAT GGT GAA GTG ATC ACG GTG GGC GGT ATC GTG TTC ACC GTG
 I   V   M   D   G   E   V   I   T   V   G   G   I   V   F   T   V
528
CAC TTC ATG GCG GGG CAC ACC CCG GGC AGC ACC GTC TGG ACC TGG ACC GAT
 H   F   M   A   G   H   T   P   G   S   T   V   W   T   W   T   D
579
ACC CGC AAT GGC AAG CCC GTT CGC ATC GCC TAC GCC GAC AGC CTG AGT GCA
 T   R   N   G   K   P   V   R   I   A   Y   A   D   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
Grey boxed nucleotides indicate *Hin*fI restriction site.

**Figure 9.6 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* U152 (m-β-l type 3) with $bla_{L1}$ specific primers**

```
68*
GAA GTA CCA CTG CCG CAG CTG CGG GCC TAC ACC GTG GAC GCC TCG TGG CTG
 E   V   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   L
120
CAG CCG ATG GCA CCG CTG CAG ATC GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   I   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACC GCG CTG CTT GTG CAG ACC CCC GAC GGC GCG GTG CTG CTC
 E   D   L   T   A   L   L   V   Q   T   P   D   G   A   V   L   L
222
GAC GGC GGC ATG CCG CAG ATG GCC AGC CAC CTG CTG GAC AAC ATG AAG GCG
 D   G   G   M   P   Q   M   A   S   H   L   L   D   N   M   K   A
273
CGT GGC GTG ACG CCT CGG GAT CTG CGG CTG ATC CTG CTC AGC CAC GCA CAC
 R   G   V   T   P   R   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAT GCC GGA CCG GTG GCG GAG CTG AAG CGC CGT ACG GGC GCC AAA
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   K
375
GTG GCG GCC AAC GCC GAA TCG GCG GTG CTG CTG GCG CGT GGC GGC AGC GAT
 V   A   A   N   A   E   S   A   V   V   L   A   R   G   G   S   D
426
GAC CTG CAC TTC GGC GAT GGC ATC ACC TAC CCG CCT GCC AAT GCA GAC CGC
 D   L   H   F   G   D   G   I   T   Y   P   P   A   N   A   D   R
477
ATC GTC ATG GAT GGT GAA GTG ATC ACG GTG GGC GGC ATC GTG TTC ACC GTT
 I   V   M   D   G   E   V   I   T   V   G   G   I   V   F   T   V
528
CAC TTC ATG GCG GGG CAC ACC CCG GGC AGC ACC GCG TGG ACC TGG ACC GAT
 H   F   M   A   G   H   T   P   G   S   T   I   W   T   W   T   D
579
ACC CGC AAT GGC AAG CCG GTG CGC ATC GCC TAC GCC GAC AGC CTG AGT GCA
 T   R   N   G   K   P   V   R   I   A   Y   A   D   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
Grey boxed nucleotides indicate *Hin*fI restriction site.

**Figure 9.7 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* J2323 (m-β-l type 4) with $bla_{L1}$ specific primers**

```
68*
GAA GTA CCA CTG CCG CAG CTG CGG GCC TAC ACC GTG GAC GCC TCG TGG CTG
 E   V   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   L
120
CAG CCG ATG GCA CCG CTG CAG ATC GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   I   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACC GCG CTG CTT GTG CAG ACC CCC GAC GGC GCG GTG CTG CTC
 E   D   L   T   A   L   L   V   Q   T   P   D   G   A   V   L   L
222
GAC GGC GGC ATG CCG CAG ATG GCC AGC CAC CTG CTG GAC AAC ATG AAG GCG
 D   G   G   M   P   Q   M   A   S   H   L   L   D   N   M   K   A
273
CGT GGC GTG ACG CCT CGG GAT CTG CGG CTG ATC CTG CTC AGC CAC GCA CAC
 R   G   V   T   P   R   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAT GCC GGA CCG GTG GCG GAG CTG AAG CGC CGT ACG GGC GCC AAA
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   K
375
GTG GCG GCC AAC GCC GAA TCG GCG GTG CTG CTG GCG CGT GGC GGC AGC GAT
 V   A   A   N   A   E   S   A   V   L   L   A   R   G   G   S   D
426
GAC CTG CAC TTC GGC GAT GGC ATC ACC TAC CCG CCT GCC AAT GCA GAC CGC
 D   L   H   F   G   D   G   I   T   Y   P   P   A   N   A   D   R
477
ATC GTC ATG GAT GGT GAA GTG ATC ACG GTG GGC GGC ATC TTG TTC ACC GTG
 I   V   M   D   G   E   V   I   T   V   G   G   I   L   F   T   V
528
CAC TTC ATG GCG GGG CAC ACC CCG GGC AGC AAC GCA TGG ACC TGG ACC GAT
 H   F   M   A   G   H   T   P   G   S   T   A   W   T   W   T   D
579
ACC CGC AAT GGC AAG CCG GTG CGC ATC GCC TAC GCC GGC AGC CTG AGT GCA
 T   R   N   G   K   P   V   R   I   A   Y   A   D   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
Grey boxed nucleotides indicate *Hin*fI restriction site.

**Figure 9.8 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* strains 37 (m-β-l type 5) and 511 with $bla_{L1}$ specific primers**

```
68*
GAG GCA CCG CTG CCA CAA CTG CGG GCC TAT ACC GTG GAT GCG TCC TGG CTG
 E   A   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   L
120
CAG CCG ATG GCG CCG CTG CAG GTT GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   V   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACC GCG CTG CTG GTG CAG ACC GCC GAG GGC GCA GTA CTG CTG
 E   D   L   T   A   L   L   V   Q   T   A   E   G   A   V   L   L
222                                                           G
GAT GGC GCA ATG CCA CAG ATG GCC GGT CAC CTG CTG GAC AAC ATG AAA CTG
 D   G   A   M   P   Q   M   A   G   H   L   L   D   N   M   K   L
273                                       C
CGC GGC GTG GCC CCG CAG GAC CTG CGA TTG ATC CTG CTT AGC CAT GCG CAT
 R   G   V   A   P   Q   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAC GCC GGC CCG GTC GCC GAG CTC AAG CGT CGC ACC GGC GCG CAT
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   H
375
GTG GCG GCC AAT GCA GAA ACG GCG GTG CTG CTG GCG CGC GGC GGC AGC AAC
 V   A   A   N   A   E   T   A   V   L   L   A   R   G   G   S   N
426                                                       T
GAC CTG CAC TTT GGC GAC GGC ATC ACC TAT CCG CCG GCC AGC GCC GAC CGC
 D   L   H   F   G   D   G   I   T   Y   P   P   A   S   A   D   R
477               C
ATC ATC ATG GAT GGT GAA GTG GTC ACG GTG GGC GGC ATC GCA TTC ACC GCG
 I   I   M   D   G   E   V   V   T   V   G   G   I   A   F   T   A
528
CAC TTC ATG CCG GGG CAC ACC CCG GGC AGC ACC GCC TGG ACC TGG ACC GAC
 H   F   M   P   G   H   T   P   G   S   T   A   W   T   W   T   D
579
ACC CGC GAT GGG AAG CCG GTG CGC ATC GCC TAC GCC GAC AGC CTG AGT GCA
 T   R   D   G   K   P   V   R   I   A   Y   A   D   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
A - Thymine (T) at position 391 is replaced by adenine (A), therefore, *Hin*fI cannot cleave the DNA at this site.
-Nucleotides in black type represent the sequence from 37, whereas green letters indicate the nucleotide differences that occur in the sequence from strain 511

**Figure 9.9 Nucleotide and deduced amino acid sequence of a 561 bp intragenic DNA fragment amplified by PCR from *S. maltophilia* AK37454 (m-β-l type 7) with $bla_{L1}$ specific primers**

```
68*
GAG GCA CCG CTG CCA CAG CTG CGG GCC TAC ACC GTG GAT GCC TCC TGG CTG
 E   A   P   L   P   Q   L   R   A   Y   T   V   D   A   S   W   L
120
CAG CCG ATG GCG CCG CTG CAG ATT GCC GAC CAC ACC TGG CAG ATC GGC ACC
 Q   P   M   A   P   L   Q   I   A   D   H   T   W   Q   I   G   T
171
GAG GAC CTG ACT GCG CTG CTG GTG CAG ACC GCC GAG GGC GCA GTA CTG CTG
 E   D   L   T   A   L   L   V   Q   T   A   E   G   A   V   L   L
222
GAT GGC GGC ATG CCG CAG ATG GCC AGC CAC CTC ATC AGC AAC ATG AAG GTG
 D   G   G   M   P   Q   M   A   G   H   L   I   S   N   M   K   V
273
CGC GGC GTG GCC CCG CAG GAC CTG CGA TTG ATC CTG CTC AGC CAT GCG CAT
 R   G   V   A   P   Q   D   L   R   L   I   L   L   S   H   A   H
324
GCC GAC CAC GCC GGC CCG GTC GCC GAG CTC AAG CGT CGA ACC GGC GCG CAT
 A   D   H   A   G   P   V   A   E   L   K   R   R   T   G   A   H
375
GTG GCG GCC AAT GCC GAA TCG GCG GTG CTG CTG GCG CGC GGC GGT AGC AAC
 V   A   A   N   A   E   S   A   V   L   L   A   R   G   G   S   N
426
GAC CTG CAC TTT GCC GAC GGC ATA ACG TAT CCG CCG GCC AGC GCC GAC CGG
 D   L   H   F   A   D   G   I   T   Y   P   P   A   S   A   D   R
477
ATC ATC ATG GAT GGT GAA GCG GTC ACG GTG GGC GGC ATC ACA TTC ACC GCG
 I   I   M   D   G   E   A   V   T   G   G   G   I   T   F   T   A
528
CAC TTC ATG CCA GGG CAT ACC CCG GGC AGC ACC GCC TGA ACC TGG ACC GAC
 H   F   M   P   G   H   T   P   G   S   T   A   W   T   W   T   D
579
ACC CGC GAT GGC AAG CCG GTG CGC ATC GCC TAC GCC GAC AGC CTG AGC GCA
 T   R   D   G   K   P   V   R   I   A   Y   A   D   S   L   S   A
```

* Numbering of nucleotides is in accordance with Walsh *et al.*, (1994).
Grey boxed nucleotides indicate *Hin*fI restriction site.

**Figure 9.10 A comparison between the amino acid sequences deduced from a 561 bp DNA fragment sequenced from seven different *S. maltophilia* strains and the known amino acid sequences from *S. maltophilia* strains IID1275 and GN12873[a]**

```
                  *     *                 *                 *
IID1275  EVPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNM
GN12873  EAPLPQLRAYTVDASWLQPMAPLQVADHTWQIGTEDLTALLVQTAEGAVLLDGGMPQMAGHLLDNM
GEL      EVPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNM
0062     EVPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNM
U152     EVPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNM
J2323    EVPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNM
511/37   EAPLPQLRAYTVDASWLQPMAPLQVADHTWQIGTEDLTALLVQTAEGAVLLDGAMPQMAGHLLDNM
AK37454  EAPLPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTALLVQTAEGAVLLDGGMPQMAGHLISNM

                        * * **                          *  *   *
IID1275  KARGVTPRDLRLILLSHAHADHAGPVAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITYPPA
GN12873  KLRGVAPQDLRLILLSHAHADHAGPVAELKRRTGAHVAANAETAVLLARGGSNDLHFGDGITYPPA
GEL      KLRGVTPRDLRLILLSHAHADHAGPVAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITYPPP
0062     KARGVTPRDLRLILLSHAHADHAGPVAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITFPPA
U152     KARGVTPRDLRLILLSHAHADHAGPVAELKRRTGAKVAANAESAVVLARGGSDDLHFGDGITYPPA
J2323    KARGVTPRDLRLILLSHAHADHAGPVAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITYPPA
511/37   KLRGVAPQDLRLILLSHAHADHAGPVAELKRRTGAHVAANAETAVLLARGGSNDLHFGDGITYPPA
AK37454  KVRGVAPQDLRLILLSHAHADHAGPVAELKRRTGAHVAANAESAVLLARGGSNDLHFADGITYPPA

                              *                      **  *
IID1275  NADRIVMDGEVITVGGIVFTAHFMAGHTPGSTAWTWTDTRNGKPVRIAYADSLSA
GN12873  SADRIIMDGEVVTVGGIAFTAHFMPGHTPGSTAWTWTDTRDGKPVRIAYADSLSA
GEL      NADRIIMDGEVITVGGIVFTAHFMAGHTQGSTAWTWTDTRIGKPVRIAYAGSLSA
0062     NADRIVMDGEVITVGGIVFTVHFMAGHTPGSTVWTWTDTRNGKPVRIAYADSLSA
U152     NADRIVMDGEVITVGGIVFTVHFMAGHTPGSTIWTWTDTRNGKPVRIAYADSLSA
J2323    NADRIVMDGEVITVGGILFTVHFMAGHTPGSTAWTWTDTRNGKPVRIAYADSLSA
511/37   SADRIIMDGEVVTVGGIAFTAHFMPGHTPGSTAWTWTDTRDGKPVRIAYADSLSA
AK37454  SADRIIMDGEAVTVGGITFTAHFMPGHTPGSTAWTWTDTRDGKPVRIAYADSLSA
```

[a] Amino acid residues conserved in all of the nine sequences are represented in black, whereas red lettering indicates residues which differ from sequence to sequence.
* Indicates residues identified as being involved in the coordination of zinc ions or substrate binding from the crystal structure of the metallo-β-lactamase from *S. maltophilia* IID1275 (Ullah *et al.*, 1998).

## Table 9.3 Amino acid identity between L1-type β-lactamases sequenced from nine *S. maltophilia* strains

Identity values are expressed as a percentage calculated from an amino acid sequence that is 187 residues in length.

| Strain | IID1275 | GN12873 | GEL (1) | 0062 (2) | U152 (3) | J2323 (4) | 37 (5) | 511 | A37454 (7) |
|---|---|---|---|---|---|---|---|---|---|
| **IID1275** | - | - | - | - | - | - | - | - | - |
| **GN12873** | 91.4 | - | - | - | - | - | - | - | - |
| **GEL (1)**[a] | 96.8 | 91.4 | - | - | - | - | - | - | - |
| **0062 (2)** | 98.4 | 89.3 | 95.2 | - | - | - | - | - | - |
| **U152 (3)** | 98.9 | 88.8 | 95.2 | 98.4 | - | - | - | - | - |
| **J2323 (4)** | 98.9 | 90.4 | 95.7 | 98.4 | 98.9 | - | - | - | - |
| **37 (5)** | 90.4 | 99.5 | 89.8 | 88.8 | 88.8 | 89.8 | - | - | - |
| **511** | 90.4 | 99.5 | 89.8 | 88.8 | 88.8 | 89.8 | 100 | - | - |
| **A37454 (7)** | 89.8 | 95.7 | 88.8 | 88.2 | 88.2 | 89.3 | 95.2 | 95.2 | - |

[a] *S. maltophilia* metallo-β-lactamase types are numbered in parenthesis

## 9.8 Discussion

PCR with the intragenic primer set described (Section 9.3) has proved to be an excellent technique for rapidly identifying the presence of metallo-β-lactamase genes in *S. maltophilia* isolates, and for investigating the variation within the L1 family of enzymes at the molecular level.

It has been shown that the deduced 187 amino acid residues from m-β-types 1-5 and 7 share ≥ 88.2% homology with one another and ≥ 88.3% homology with the metallo-β-lactamases sequenced from strains IID1275 and GN12873 (Table 9.3). Therefore, the *S. maltophilia* metallo-β-lactamases are a family of related enzymes differing by a few amino acids and these differences account for their diversity in pI and subtle variations in substrate profiles. Figure 9.9 shows that the residues proposed to be involved in the co-ordination of the two zinc ions bound at the active site or substrate binding (Ullah *et al.*, 1998) are conserved within the amino acid sequences of the different m-β-l types, m-β-l type 1 is the only exception to this. In the sequence from *S. maltophilia* GEL (m-β-l type 1) the aspartate (D) 184 residue (numbering according to Ullah *et al.*, 1998) identified as a zinc ligand orientating group (Ullah *et al.*, 1998) is replaced by glycine (G). The importance of this amino acid substitution in terms of structure and function could only be determined in the future by constructing a mutant m-β-l type 1 with a single aspartate substitution in place of glycine and then evaluating the kinetics of the mutant enzyme.

It is interesting that the m-β-l type 5 from strain 37 shares 100% identity with the

187 amino acids deduced from metallo-β-lactamase produced by strain 511 (Table 9.3). The metallo-β-lactamases from these two strains have previously been shown to have a similar substrate profile and pI value (6.8) and it has been speculated that these could indeed be the same enzyme. Discrepancies have, however, been found between the inhibitor profiles of these enzymes, for example 2mM nitroacetic acid inhibits the metallo-β-lactamase of strain 511, but does not inhibit m-β-l type 5 (Payne *et al.*, 1994b). It is therefore, still possible that there are amino acid differences between these two metallo-β-lactamases within the remaining residues which make up the complete protein and have not been determined here, and these differences may only be reflected in inhibitor profiles. Only further sequence analysis would determine whether this is, in fact, the case. If, however, m-β-l type 5 from strain 37, (originally identified in the UK (Payne *et al.*, 1994a)) and strain 511 (an Italian isolate (Felici *et al.*, 1993)) do encode metallo-β-lactamases identical in amino acid sequence this leads on to the question – are some *S. maltophilia* metallo-β-lactamase types more prevalent than others? A future study determining the relative abundance of m-β-l types amongst clinically isolated *S. maltophilia* from different geographical areas would offer the answer to this question.

The genes $bla_{L1}$ and *bla*S from *S. maltophilia* IID1275 and GN12873, respectively, are known to translate to an unmodified protein that is 290 amino acids in length (Walsh *et al.*, 1994; Sanschargrin *et al.*, 1998). In this study, an intragenic stretch of 187 amino acids has been deduced by sequencing the PCR products from seven *S. maltophilia* strains, however, for completeness it would be interesting to determine the level of relatedness that exists between the remaining 103 residues. Section 9.6

describes the design of primers outside the metallo-β-lactamase open reading frame, but unfortunately, a PCR product could only be amplified from the positive control (pUB8902). PCR with these intergenic primers appears to be more sensitive to the amplification conditions. Three nucleotide differences also occur between the $bla_{L1}$ and *bla*S right hand intergenic primer sequences (Figure 9.3, start position 1407) and therefore, to achieve success different PCR primers may be required. Nevertheless, in the knowledge that the metallo-β-lactamases encoded by $bla_{L1}$ and *bla*S share 91.4% homology over this 187 amino acid section of the protein and 88. 6% homology over the entire protein (Sanschargrin *et al.*, 1998), it is highly probable that the m-β-l types 1-5 and 7 exhibit a similar degree of heterogeneity throughout the complete protein as they do in the deduced 187 amino acid sequence.

From Table 9.3, the metallo-β-lactamases from nine *S. maltophilia* strains can be divided into two groups. The first group comprises the m-β-l types from strains GN12873, 37, 511 and A37454 which all share ≥95.2% homology with one another but ≤91.4% homology with the second group made up of the enzymes from strains IID1275, GEL, 0062, U152 and J2323. The 187 amino acid sequence determined from the m-β-l types from IID1275, GEL, 0062, U152 and J2323 also share ≥95.2% homology with one another (Table 9.3). Once again, only further sequence analysis would establish if these two groupings were true. It would be interesting to study the relatedness of these nine strains over the rest of the genome and determine whether strains that have more closely related metallo-β-lactamases are also more closely associated throughout the entire genome. *S. maltophilia* is at present the sole member of the genus *Stenotrophomonas* and as molecular-based typing techniques

become more routinely employed it is possible that speciation within the genus *Stenotrophomonas* will undergo changes.

The heterogeneity exhibited by the *S. maltophilia* metallo-β-lactamases is by no means a unique phenomenon. Considerable sequence variation is also known to exist within other β-lactamase types such as at the *ampC* locus within the species *C. diversus* and also *C. freundii* (Jones *et al.*, 1994). Additionally, the chromosomal molecular class A cephalosporinases (L2) that are coordinately expressed with the *S. maltophilia* metallo-β-lactamases also appear to be a heterogeneous group of enzymes (Payne *et al.*, 1994a; Pradhananga *et al.*, 1996). The heterogeneity of the L2 enzymes have not yet been studied at the molecular level.

# 10: GENERAL DISCUSSION

## 10.1 Introduction

The main aims of this thesis have been to examine the role of β-lactamases in carbapenem-resistant strains from Japan and carbapenem-resistant *A. veronii* bv. *sobria* isolated from Vellore, South India. In addition, the molecular heterogeneity of the *S. maltophilia* metallo-β-lactamases has been analysed.

## 10.2 The relevance of serine-based carbapenem-hydrolysing β-lactamases

Assessing the problem of β-lactamases in carbapenem resistance is vitally important for the implementation of therapeutic strategies. In the early 1990s, potent carbapenemase activity had not been associated with a serine β-lactamase and it was thought that metallo-β-lactamases would become the major resistance mechanism to the carbapenems (Livermore, 1993b; Payne, 1993). In this thesis, a survey on carbapenem-resistance mechanisms in 61 Japanese *P. aeruginosa* clinical isolates has demonstrated that an association between a serine-based chromosomal cephalosporinase and an alteration in outer membrane permeability was the most prevalent mechanism of resistance amongst the strains studied (Section 3). Recently the metallo-β-lactamase IMP-1 has been disseminating amongst clinically isolated *P. aeruginosa* in Japan. However, in this study no IMP-1 metallo-β-lactamase was

detected (Section 3.6 – 3.7) which implies that the occurrence of this enzyme is still relatively rare in *P. aeruginosa*. Further studies on *P. aeruginosa* isolates from Kuwait and Turkey have also demonstrated the widespread nature of a carbapenem resistance mechanism which is a combination of a chromosomal class C β-lactamase and the loss of an imipenem-specific porin channel (R. Walker - unpublished data).

In recent papers from Japan emphasis is generally placed on the detection of IMP-1 in carbapenem-resistant isolates, and on occasion these reports have ignored or failed to detail the carbapenem resistance mechanisms of non-IMP-1 isolates. In the original Japanese report (Senda *et al.*, 1996a), 132 carbapenem resistant *P. aeruginosa* strains were examined and 13% were found to carry the $bla_{IMP}$ gene. The carbapenem resistance mechanism in the remaining 87% was not determined and it is highly probable that the resistance mechanism in many of these strains that were not further investigated involved a cephalosporinase and a reduction in outer membrane permeability.

Although it was initially thought that the metallo-β-lactamases would be the most common cause of carbapenem resistance, it is becoming increasingly evident that the rise in the number of serine-based carbapenemases in clinical bacteria is an important issue that must be addressed. The importance of the molecular class C β-lactamase in conferring carbapenem resistance as part of a two-component mechanism has been emphasised in this thesis. The involvement of a class C cephalosporinase in carbapenem resistance has also been identified in *E. cloacae*, *C. freundii*, *P. rettgerii*, *P. mirabilis* and recently *K. pneumoniae* (Section 1.10.1.2). In addition, serine-based

molecular class A and D imipenem-hydrolysing enzymes in *Acinetobacter* species are attracting much attention (Section 1.12.1). Furthermore, it has also been revealed recently that the production of a molecular class A Sme-1 like carbapenemase in *S. marcescens* is not such a rare event as originally thought (Section 1.12.1).

It is important to remember that most common β-lactamases have a serine-based catalytic mechanism and it seems reasonable to assume that some of these enzymes will adapt themselves to include the carbapenems in their substrate profile. This scenario has most recently been illustrated in a report by Dorai-John *et al.*, (1998) on the identification of a novel putative molecular class C enzyme that efficiently hydrolyses imipenem. This is unlike previously reported class C cephalosporinases that can only feebly hydrolyse imipenem, although this is sufficient to confer carbapenem resistance in a less permeable background.

In Section 3.9 it was shown that the imipenem MICs in carbapenem resistant *P. aeruginosa* from Japan were all less than the breakpoint for resistance when imipenem MICs were determined in the presence of BRL42715. BRL42715 is a good inhibitor of molecular class A, C and D β-lactamases in vitro, and it is unfortunate that this compound cannot be applied clinically. A general serine β-lactamase inhibitor in combination with a β-lactam could possibly be important in overcoming the problem of serine based carbapenemases. Currently many molecular class A and D β-lactamases can be targeted using clavulanic acid, sulbactam or tazobactam, there are, however, no molecular class C β-lactamase inhibitors.

## 10.3 The significance of metallo-β-lactamases

Although the importance of serine β-lactamses in carbapenem resistance is beginning to be realised, the metallo-β-lactamases do still present a threat to the efficacy of the carbapenems as postulated by Payne (1993) and Livermore (1993b). The emergence in Japan of the broad-spectrum plasmid mediated β-lactamase IMP-1 in a variety of clinically important bacteria, including *K. pneumoniae* is probably one of the most significant events in terms of metallo-β-lactamase mediated resistance. Recent findings indicate that the isolation of $bla_{IMP}$ containing strains is still increasing (Ito *et al.*, 1997); however, one consolation is that, as yet, this enzyme has still not been reported outside of Japan. Although IMP-1 has been described in common bacterial pathogens, as already illustrated in the introduction of this thesis (1.11.1.5), many of the metallo-β-lactamases that have been identified, to date, are often found in bacteria of considerably less clinical importance.

## 10.4 The significance of *Aeromonas* carbapenemases

*Aeromonas* species, some of which produce metallo-β-lactamases, are considered to be infrequent pathogens although their ability to cause infection has been reported increasingly in the past few years (Wilcox and Jones 1995). The role of metallo-carbapenemases in conferring resistance has been rather unclear, as most species that produce a carbapenem-hydrolysing metallo-β-lactamase remain susceptible to the carbapenems under standard sensitivity testing conditions ($10^4$cfu). This thesis has

reported the identification of two carbapenem resistant *A. veronii* bv. *sobria* strains tested with a conventional inoculum ($10^4$cfu), that were subsequently found to have derepressed expression of a novel carbapenemase (Table 5.1 and Section 5.5). In Table 5.2 it was shown that unless there are at least $10^6$cfu of bacteria present the inducible carbapenemase does not afford *A. veronii* bv. *sobria* with protection against imipenem, thus the number of bacteria present and the amount of metallo-β-lactamase produced is crucial to the manifestation of carbapenem resistance. It has been suggested that at the site of infection bacterial concentrations are likely to be larger than that used in conventional susceptibility testing, although this is perhaps debatable (Rossolini *et al.*, 1995). Currently carbapenem resistant *A. veronii* bv. *sobria* are not a problem clinically, however, if they do pose a problem in the future, possibly as a consequence of greater reliance on the carbapenems, it is most likely that it will be caused by the selection of derepressed mutants. Therefore, care should be taken in the choice of agents, particularly for invasive *Aeromonas* infections because of the potential to select for derepressed mutants that are generally not only hyper-producers of a carbapenemase but also of a penicillinase and cephalosporinase. Agents such as gentamicin, or the fluoroquinolone ciprofloxacin have been previously recommended for the treatment of serious *Aeromonas* infection (Bakken *et al.*, 1988).

## 10.5 The β-lactamases of carbapenem-resistant *A. veronii* bv. *sobria* strain 13

In this thesis, the β-lactamases of *A. veronii* bv. *sobria* strain 13 have been examined in detail by applying several different techniques such as ion exchange chromatography, PCR and gene cloning. It appears that like many *Aeromonas* species *A. veronii* bv. *sobria* 13 produces three chromosomally encoded β-lactamases. These β-lactamases include a novel carbapenemase AVS-1, a penicillinase named AVS-2 and a third β-lactamase AVS-3 that is possibly related to previously reported *Aeromonas* cephalosporinases. The gene cloning and sequence analysis of the AVS-2 penicillinase demonstrated that this enzyme is closely related (≥95.8% amino acid homology) to the molecular class D penicillinases AsbB1 and AmpS identified in a strain of *A. jandaei* and *A. veronii* bv. *sobria*, respectively (Table 8.3) (Rasmussen *et al.*, 1994a; Walsh *et al.*, 1995b). Class D penicillinases appear to be ubiquitous throughout the *Aeromonas* genus and have been shown to be linked to a two component cre-like operon that is involved in the regulation of *Aeromonas* β-lactamases (Alksne and Rasmussen, 1997; Niumsup *et al.*, 1997).

## 10.6 The novel AVS-1 carbapenemase

The novel carbapenemase AVS-1 was found to possess several unusual properties including an atypical pI value and substrate and inhibitor profiles, when compared with previously reported *Aeromonas* carbapenemases (Sections 5 and 6). The

employment of an imipenem substrate based modification after IEF (Section 5.7) proved invaluable in initially identifying the ability of the AVS-1 β-lactamase to hydrolyse nitrocephin efficiently in addition to imipenem, an attribute not previously described for the *Aeromonas* carbapenemases. Purification of the AVS-1 β-lactamase (Section 6) further demonstrated the unusual properties of this enzyme, including its ability to hydrolyse ampicillin and cephaloridine, its resistance to the metallo-β-lactamase inhibitor EDTA (100mM) and its insensitivity to variations in zinc sulphate concentrations. The *Aeromonas* metallo-β-lactamases CphA and ImiS exhibit a very narrow substrate profile, with a marked preference for carbapenems and are inhibited by EDTA (Rossolini *et al.*, 1996). The β-lactamase AVS-1 was also found to be sensitive to the serine β-lactamase inhibitors clavulanic acid, tazobactam and sulbactam, although it has already been speculated that these inhibitors could be acting as competitive substrates (Section 6.9), because these compounds are known to be hydrolysed by some metallo-β-lactamases. Both ImiS and CphA are known to hydrolyse BRL42715, however, hydrolysis of these other serine β-lactamase inhibitors has not been reported (Matagne *et al.*, 1995; Walsh *et al.*, 1996).

The arguments for the AVS-1 carbapenemase being encoded by a metallo-β-lactamase gene identified in both strain 13 and 99 have been put forward in Section 7.6. The metallo-β-lactamase gene, although not sequenced in its entirety (27 nucleotides could not be determined at the 5' end of the gene), was found to be closely related (≥95% amino acid homology) to the previously identified *Aeromonas* metallo-β-lactamases CphA, CphA2 and ImiS (Table 7.2). These results suggested

that if the metallo-β-lactamase gene does encode the AVS-1 enzyme then there are only a few amino acids that could possibly account for the extended substrate spectrum and the unusual inhibitor profile of this AVS-1 carbapenemase.

Only two amino acids (lysine 49 and asparagine 206) were identified from the sequence of the metallo-β-lactamase gene from *A. veronii* bv. *sobria* 13 and 99 as being possibly close to the enzyme active site (Section 7.5) and therefore, these may be the important amino acid substitutions. It should also be pointed out that these residues were identified by aligning the metallo-β-lactamase sequence identified in both strain 13 and 99 with the sequences of the metallo-β-lactamases from *B. fragilis* (CcrA3) and *B. cereus* (β-lactamase II) which function optimally with two zincs, unlike the *Aeromonas* metallo-β-lactamases which are known to function at their maximum with only one zinc. The difference in the requirement for zinc between these enzymes suggests that *Aeromonas* enzymes are radically different from CcrA3 and β-lactamase II, thus residues important in catalysis in CcrA3 and β-lactamase II are not necessarily the same in *Aeromonas* metallo-β-lactamases. The importance of individual residues in the *Aeromonas* metallo-β-lactamase will only be made clearer when the crystal structure of an *Aeromonas* metallo-β-lactamase has been resolved and from site-directed mutagenesis studies.

Although arguments for the probable metallo-β-lactamase origin of the AVS-1 β-lactamase have been reasoned in Section 7, the cloning of this carbapenemase is ultimately required to confirm whether it is truly related to previously identified *Aeromonas* metallo-β-lactamase. The strategies employed in this study in an attempt

to clone AVS-1 have been unsuccessful (Section 8). A possible alternative cloning strategy could involve preparing a genomic DNA library from *A. veronii* bv. *sobria* 13, preparing colony blots of the library and hybridising these blots with a probe consisting of the metallo-β-lactamase encoding PCR product amplified from strain 13. β-Lactamase extracts prepared from any hybridisation positive clones could be analysed by IEF to determine whether they encode a metallo-β-lactamase with the same pI as AVS-1 (that is 5.84).

## 10.7 *Stenotrophomonas maltophilia* metallo-β-lactamases

Although the relevance of *Aeromonas* metallo-β-lactamases in conferring carbapenem resistance has been uncertain, in contrast the *S. maltophilia* metallo-β-lactamases are known undoubtedly to cause imipenem and meropenem resistance. Permeability is thought to play a part in *S. maltophilia* carbapenem resistance, however, the production of the metallo-β-lactamase, whether inducible or derepressed, is known to account for high level resistance (Akova *et al.*, 1991). *S. maltophilia* isolates are known to produce a variety of L1-type metallo-β-lactamases that can be differentiated from one another biochemically. In this study, 561bp intragenic DNA sequences of metallo-β-lactamases from six *S. maltophilia* strains have been determined from PCR products (Section 9). A comparison of the 187 deduced amino acid sequences of the six metallo-β-lactamases with one another and with the same region encoded by the $bla_{L1}$ and $bla_{S}$ metallo-β-lactamase genes from *S. maltophilia* IID1275 and GN12873 has revealed that these enzymes are all closely

related to one another (≥88.8% homology – Table 9.3). These enzymes all differ from one another at a few amino acid positions that have generally not been reported as residues known to be important in enzyme catalysis and these changes account for the differences in pI and substrate and inhibitor profiles (Section 9.7). Metallo-β-lactamase heterogeneity is a feature not only between bacteria of different genera but also between strains of the same species and this is exemplified by *S. maltophilia* metallo-β-lactamases. Work in this thesis also suggests that biochemical heterogeneity can be a feature of the *Aeromonas* metallo-β-lactamases, but at the molecular level, this is probably caused by minor amino acid substitutions.

Investigating metallo-β-lactamase heterogeneity is important clinically for future drug development. The *S. maltophilia* metallo-β-lactamase heterogeneity study described here, is helpful in identifying whether recognised active site residues are conserved, which is essential when designing new antibiotics or inhibitors. Gene sequence data also reveal residues that may be responsible for substrate profile differences and may result in clinical failure in a strain that possesses one type of enzyme but not another.

## 10.8 Overcoming metallo-β-lactamase mediated carbapenem resistance

Although the role of metallo-β-lactamases in conferring resistance, particularly in *Aeromonas* has not always been clear cut, metallo-enzymes such as IMP-1 can pose

a clinical problem and there is a need for new drug strategies against these enzymes. The monobactam aztreonam is unlike most other β-lactams in that it remains stable in the presence of zinc carbapenemases. There are, however, draw backs to using such an agent, specifically the fact that several of the metallo-β-lactamase producing bacteria can additionally produce chromosomal cephalosporinases which, when hyperproduced, can destroy aztreonam.

Designing new agents to overcome metallo-β-lactamase mediated resistance may involve devising more stable carbapenems by altering their side chain substituents. An alternative approach would be to discover or design effective inhibitors against metallo-β-lactamases that can be administered in combination with a β-lactam. The latter, however, may prove to be difficult to achieve because of the heterogeneous nature of the metallo-β-lactamase group as a whole. This potential problem has recently been illustrated by Payne *et al.*, (1997), where a series of mercaptoacetic acid thiol esters were found to be good inhibitors of some metallo-β-lactamases but completely ineffective against others. A continued effort to understand the catalytic mechanisms and substrate binding of metallo-β-lactamases is thus important in the rational design of effective inhibitors. In the mean time until new compounds become available it is imperative that carbapenems, as with all antibiotics, should be employed with extreme caution to keep the selection of carbapenemases at bay, whether they be serine or metallo-based.

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