RESISTANCE TO BETA-LACTAM ANTIBIOTICS IN GRAM-NEGATIVE BACTERIA

by

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1986

Abstract

The prevalence of various ß-lactamase enzyme types produced by a population of cephaloridine resistant Gram negative aerobic bacteria was determined. The majority of the strains (50.8%) produced only a chromosomal ß-lactamase. ß-lactamases typically associated with plasmids were found in 112 strains (47.9%), the most common was TEM-1 (41.9% of the total population). Conjugation experiments demonstrated that only 93 out of 112 ß-lactamases, predicted as plasmid-mediated by biochemical analysis could transfer these ß-lactamase genes.

The *Escherichia coli* TEM-1 ß-lactamases were heterogeneous in their levels of expression and when transferred to *E. coli* $J62\text{-}_2$ variation in expression was again observed; however, these variations did not mirror those observed in the clinical strains. The TEM-1 ß-lactamase did not confer significant cephaloridine resistance in *E. coli* K12 and it was concluded that other host factors must play a major role in cephaloridine resistance in the clinical isolates.

Amongst the survey strains a novel plasmid-mediated ß-lactamase was identifed in a *Klebsiella pneumoniae* strain, carried by plasmid pUK702. The enzyme had a unique combination of biochemical and enzymic properties with broad-spectrum hydrolytic activity; it was similar to the TEM-like enzymes in its inhibition profile and was designated TLE-2. Two enterobacterial strains were isolated which produced the ß-lactamase PSE-4. The biochemical and biophysical properties of the PSE-4 genes were found to be host dependent. This

was the first isolation of this β-lactamase outwith *Pseudomonas aeruginosa*.

The *incP*-1 plasmid, R751, which had been used to mobilise the PSE-4 gene was found to confer a degree of carbenicillin resistance on the *E. coli* K12 J62 host strain. This plasmid also had the effect of increasing the strains' mutation rate, resulting in resistance to a variety of antimicrobial drugs. The mutatory effect of R751 resulted in the expression of SHV-1 β-lactamase by *E. coli* K12 J62 when selection was made for carbenicillin/ampicillin resistant mutants.

Six strains were identified from the cephaloridine resistance survey which transferred β-lactam resistance that was not mediated by a β-lactamase. In three of the six strains changes in the outer membrane proteins of the *E. coli* J62-2 transconjugants were identified.

In parallel, the incidence and mechanisms of ampicillin resistance was investigated in 105 clinical *Haemophilus influenzae* isolates. Six of the 15 ampicillin resistant strains had alterations in the penicillin binding proteins or outer membrane proteins. In one strain no resistance mechanism could be identified.

In conclusion, although the role of β-lactamases is paramount in resistance to β-lactam antibiotics, resistance is multifactorial and several mechanisms, either acting alone or in combination, contribute to the resistance of the cell.

"Scientific discovery is a private event,

and the delight that accompanies it, or

the despair of finding it illusory does not travel"

Sir Peter Medawar

in "Hypothesis and Imagination"

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

Alison J. Reid

22nd August 1986

Acknowledgements

I would like to thank both my supervisors, Dr SGB Amyes and Dr IN Simpson for their help and encouragement during my PhD. I would also like to acknowledge the advice and guidance of Professor JG Collee and Dr PB Harper.

My thanks go to my colleagues in the Bacteriology Research Laboratory, Edinburgh and the Chemotherapy Department, Glaxo Group Research, especially Miss M James, for support and discussions. Dr AA McColm carried out the animal work necessary for this thesis and I am grateful to him for his help and instruction. I would also like to thank Mr D Dirom for his photographic advice and Mrs F Govan for skillfully typing the manuscript.

Finally I would like to thank the Science and Engineering Research Council for the Collaborative Award in Science and Engineering, in partnership with Glaxo Group Research, which enabled me to carry out the work for this thesis.

Abbreviations

ade	adenine
AMP (Ap)	ampicillin
AZT	aztreonam
ß-NAD	ß-Nicotinamide adenine dinucleotide
BHI	brain heart infusion
CAZ	ceftazidime
CED	cephradine
CER	cephaloridine
CFS	cefsulodin
CFU	colony forming units
CFX	cefoxitin
CFZ	cefazolin
Cm	chloramphenicol
CMD	cefamandole
CTN	cefotetan
CTR	ceftriaxone
CTX	cefotaxime
CXM	cefuroxime
d	daltons
DM	Davis and Mingioli
DNA	deoxyribonucleic acid
DSTA	diagnostic sensitivity test agar
EDTA	ethylenediaminetetraacetic acid
Gm	gentamicin
H/No	hospital number

Abbreviations (continued)

Hg	mercuric chloride
his	histidine
IEF	isoelectric focusing
ilv	isoleucine/valine
inc	incompatibility group
IS	insertion sequence
kb	kilobases
km	kanamycin
lac	lactose fermenting
leu	leucine
log	logarithm
met	methionine
MIC	minimum inhibitory concentration
Mr	molecular size
Mx	moxalactam
µCi	micro Curies
Na	nalidixic acid
NB	nutrient broth
Nf	norfloxacin
OD	optical density
OMP	outer membrane protein
p	probability
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
pCMP	para-chloromercuribenzoic acid
pfu	plaque forming units

Abbreviations (continued)

pI	isoelectric point
PIP	piperacillin
pro	proline
psi	pounds per square inch
R	resistance
rec	recombinant
Rf	rifampicin
RNA	ribonucleic acid
RSAI	relative substrate affinity index
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
Su	sulphamethoxazole
Tc	tetracycline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tn	transposon
Tp	trimethoprim
trp	tryptophan
UTI	urinary tract infection
val	valine

Publications

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PREFACE

It is almost 60 years since Fleming described the antibacterial properties of penicillin (Fleming 1929). Subsequently, penicillin was successfully purified and produced in some quantity (Chain *et al* 1940) and the first therapeutic successes followed immediately (Abraham *et al* 1941). So began a new era of medicine and the treatment of infectious disease is now based firmly on antimicrobial chemotherapy.

It was prophetic that in his original paper Fleming also described groups of bacteria resistant to the action of penicillin (Fleming 1929). In 1940 these observations were confirmed, and in a succinct letter to "Nature" Abraham and Chain (1940) described the isolation of an active substance with enzymic properties from *Escherichia coli* resistant to penicillin : the term "penicillinase" was coined. Antimicrobial drug resistance is now a fact of life and its study and exploitation has led to a greater understanding of both bacterial genetics and biochemical pathways.

The effective use and development of antimicrobials are inextricably interwoven with the need to understand old and new bacterial defence mechanisms which are waged against our armoury of antimicrobial compounds. Along with other antimicrobial groups, the β-lactams have been developed along their present lines in order to combat bacterial resistance.

Research into the diversity and mechanisms of such resistance only serves to highlight the fact that although many battles have been won, the war against bacterial infection is far from over.

I. INTRODUCTION

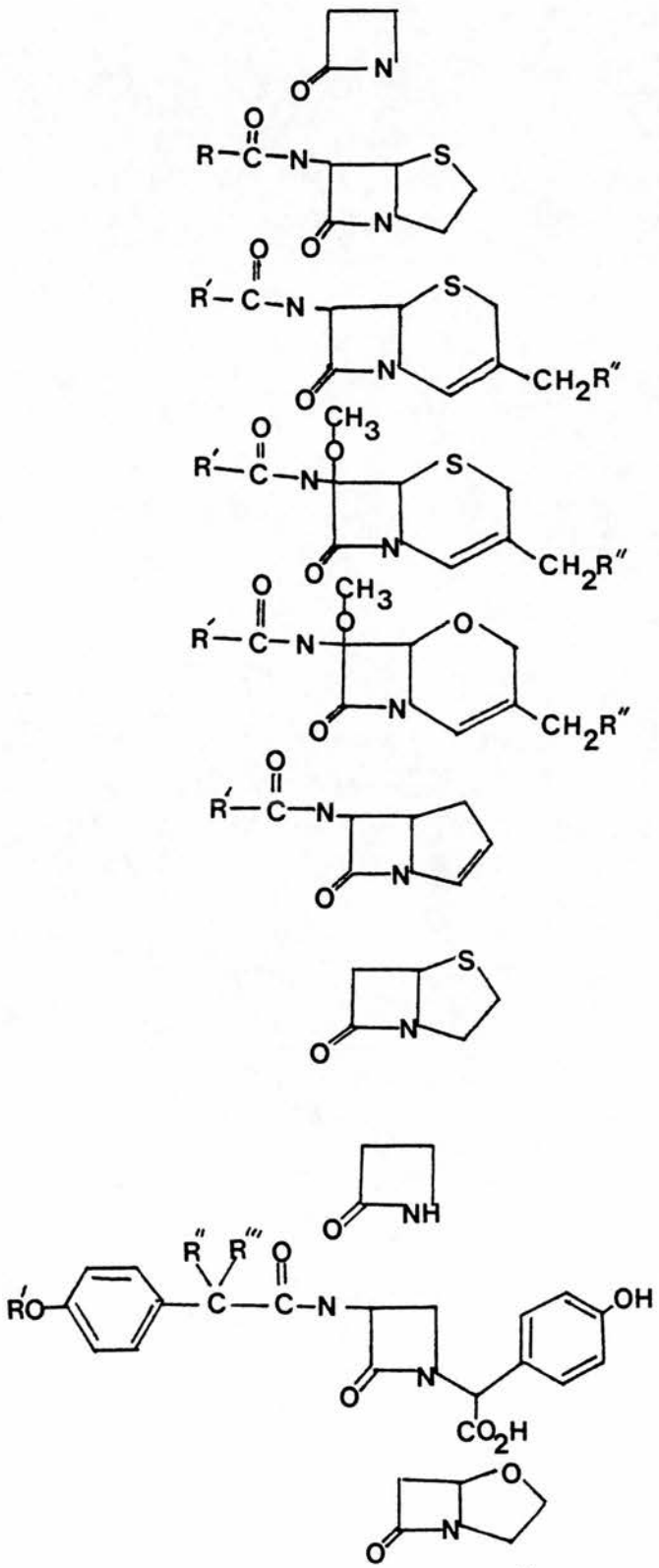
THE β-LACTAMS

1. The β-lactam Family (design concepts)

The ever increasing number of β-lactam antibiotics highlights not only their great clinical importance but also their commercial significance. β-lactam sales (£6,000M) in 1984 comprised 10% of total drug sales and 60% of sales of antibacterial drugs worldwide (Bulletin - Recent Advances in Chemotherapy 1984). The exponential use of this class of antibiotics during the last 15 years, has resulted in a bewildering array of preparations on the commercial market and undergoing clinical trials (Rolinson 1986).

β-lactams (Figure 1) interact with the two main groups of bacterial enzymes. The first group are the lethal target site of these compounds, the cell wall synthesising enzymes (Park and Strominger 1957, Wise and Park 1965, Tipper and Strominger 1965, Izaki et al 1966). The second group, in direct contrast, inactivate β-lactam antibiotics and protect the cell against β-lactam attack - they are the β-lactamase enzymes (Richmond and Sykes 1973; Sykes and Matthew 1976, Medeiros 1984). In view of these observations there are two major criteria which surround β-lactam design. The essential criterion is that the compound retains its ability to acylate a susceptible bacterial target enzyme. A second, increasingly important feature is that the structure of the β-lactam either prevents it from acylating the β-lactamase

Figure 1 THE STRUCTURE OF β-LACTAMS



(a) β-lactam Ring

(b) Penicillin (penam nucleus)

(c) Cephalosporin (cephem nucleus)

(d) 7a-methoxycephalosporin

(e) Oxacephem

(f) Carbapenem

(g) Penem nucleus

(h) Monobactam

(i) Nocardicin

(j) Clavam nucleus

enzyme (so that it is not a substrate) or prevents it, once it has been acylated, from undergoing hydrolysis. In this latter case, the turnover is not complete and the β-lactamase will be inhibited (Fisher 1984).

While the process of drug design continues, an attractive and expedient strategy has been to protect a potent β-lactam (which is β-lactamase susceptible) by the simultaneous challenge with a β-lactam that is a specific β-lactamase antagonist. However, the ultimate aspiration seems to be the β-lactam that retains affinity for membrane peptidases while remaining impervious to the potent hydrolytic capability of the β-lactamase (Fisher 1984).

This quest can be followed through the development of the β-lactam group to date.

1.1 The Penams

Penicillins (Figure 1b) are N-acylated derivatives of 6β-amino penicillanic acid (6-APA) a bicyclic ring system derived from the fusion of azotidinone and thiazolidine rings (Batchelor et al 1959).

The first penicillin introduced into therapeutic use was benzyl penicillin (Penicillin G) as described by Abraham and colleagues (1941), the natural fermentation product of the mould Penicillium chrysogenum (Raper et al 1944).

However, Penicillin G is acid-labile and could not be administered orally as it is degraded by hydrochloric acid in the stomach. The incorporation of various precursors into the fermentation mould was found to produce small modifications in the penicillin structure. This resulted in the production of phenoxymethylpenicillin (penicillin V) which is much more stable at low pH and thus suitable for oral administration (Behrens and Kingkade 1948).

These early penicillins acted primarily against Gram positive cocci. However, they were susceptible to the action of staphylococcal β-lactamases and there was a need for compounds which were effective against the increasing number of penicillinase producing staphylococci (Ridley et al 1970).

A chance observation revealed that certain bacterial amidases were capable of splitting the side chains of various penicillins to give the penicillin nucleus, 6-amino penicillanic acid (Batchelor et al 1959). The addition of synthetic side chains to the 6-APA nucleus led to the birth of the semi-synthetic penicillins.

Methicillin, introduced in 1960, was the first of these new penicillins to go into commercial production. It is

resistant to the action of staphylococcal β-lactamases but it is similar to penicillin G in that it is acid labile (Stewart 1960).

Further chemical modification gave the isoxazolyl penicillins - cloxacillin, oxacillin, dicloxacillin and flucloxacillin (Knudson et al 1962, Naylor et al 1962, Kirby et al 1962, Gravenkemper et al 1965, Sutherland et al 1970) and, since their introduction, the clinical relevance of β-lactamase mediated resistance in staphylococci has largely been eliminated (Hamilton-Miller 1979, Brown and Reading 1983). This group of penicillins however, is less active than penicillin G against many Gram positive cocci as a result of the chemical modifications which confer β-lactamase stability (Sutherland et al 1970).

A more important chemical modification to the 6-APA nucleus was the insertion of an amino group in the α-position of the benzylpenicillin side chain. The resultant structure, ampicillin (6-D(-)α-aminophenylacetamidopenicillanic acid) is active against many Gram negative bacteria (Rolinson and Stevens 1961) which brought this half of the Bacterial Kingdom into the range of the β-lactam antibiotics. Although suitable for oral use, it was poorly absorbed in the gut because of its low solubility. The simple insertion of any hydroxyl group at the para position in ampicillin's benzyl

side-chain resulted in amoxycillin, a compound with considerably improved gut absorption (Sutherland et al 1972). These two broad-spectrum semi-synthetic penicillins still dominate antibacterial drug usage throughout the world.

Pseudomonas aeruginosa remained outside the scope of the penicillins until an α-carboxy group was added to the 6-APA nucleus to form carbenicillin (Knudsen et al 1967) designated disodium α-carboxybenzyl penicillin. This compound has gained activity against P.aeruginosa and some other Gram negative bacilli resistant to ampicillin but these gains are at the expense of activity against almost every ampicillin-sensitive organism.

Modifications of the ampicillin nucleus to give the acylureidopenicillins azlocillin (Stewart and Bodey 1977), mezlocillin (Bodey and Pan 1977) and piperacillin (Verbist 1978) and the carbenicillin nucleus to give ticarcillin (Bodey and Deerhake 1971), did improve the anti-pseudomonal activity of penicillins. However all these penicillins have poor stability to Gram negative β-lactamases (Brown and Reading 1983).

The recently developed temocillin (BRL 17421) is an α-carboxy penicillin with a 6α-methoxy substituent (Slocombe et al 1981). The design of temocillin was an attempt to produce a

penicillin with activity against Gram negative bacteria and β-lactamase stability. However, it has as a consequence, no activity against Gram positive strains. On the other hand, alpalcillin has a broader spectrum against Gram positive bacteria and is relatively β-lactamase stable. It is active against most Gram negative bacteria including P.aeruginosa. However it has less activity against Serratia sp. and Enterobacter sp. (Noguchi et al 1976, Rolinson 1986).

The penicillins have not provided a very versatile template for the development of broad-spectrum antibacterials which are effective against current pathogens and stable against the action of β-lactamase (Fisher 1984). Intrinsically, penicillins lack sufficient flexibility because they possess only one position available for functional group development (C-6). It seems that it may not be possible for penicillins to differentiate between membrane peptidases and β-lactamase enzymes in order to combine a broad spectrum of activity with β-lactamase stability (Fisher 1984).

1.2 The Cephems

Cephalosporins are N-acylated derivatives of 7β-amino-cephalosporanic acid (7-ACA) which is derived from the cephem nucleus (Figure 1C). A cephem is the biyclic ring system obtained by the fusion of an azetidinone ring with a dihydrothiazine ring (Brown and Reading 1983).

The fungus Cephalosporium acremonium which produces cephalosporin C was isolated in Sardinia from cultures taken at a sewer outfall (Brotzu 1946). Florey received a culture of the fungus in Oxford in 1948 and he later fully described the products of this "versatile fungus" from which was isolated not one antibiotic, but seven, amongst which was cephalosporin C (Florey 1955).

The antibacterial activity of cephalosporin C was found to be low but this molecule had a high degree of stability to staphylococcal β-lactamases, which was, at that time, unique amongst antibiotics of this general structure.

The essential difference between penicillins and cephalosporins was that the latter possess two positions on the nucleus (C-3' and C-7) at which function group substitutions could be made. The cephalosporin structure was much more versatile than that of penicillin. Modifications at C-7 and C-3 produce dramatic changes in chemotherapeutic properties (O'Callaghan 1975, 1979, Queener and Neuss 1982, Brown and Reading 1983, Fisher 1984).

The first two cephalosporins introduced into clinical use were cephalothin and cephaloridine in 1964. (Boniece et al 1962, Chauvette et al 1962, Muggleton et al 1964).

Cephalothin retained the 3-acetoxy group of cephalosporin C and consequently it is hydrolysed by esterases in the body to the 3-hydroxymethyl compound (O'Callaghan and Muggleton 1963). Cephaloridine overcame this disadvantage by replacing the acetoxy group at C-3 with a pyridinium group, it thus has the metabolic stability that cephalothin lacks (O'Callaghan 1975,1979). Although cephalosporins are stable at acid pH, cephaloridine and cephalothin are not absorbed when given by mouth and are administered by intra-muscular and intravenous injection respectively (reviewed by O'Callaghan 1979).

These compounds are active against many Gram negative and Gram positive species and are stable to most staphylococcal β-lactamases. They are however susceptible to the β-lactamases of Gram negative bacteria and are inactive against such organisms as P.aeruginosa. Attempts to improve on these properties led to cefazolin which has marginally better intrinsic Gram negative activity but still little increased resistance to β-lactamase action (Nishida et al 1969).

The first oral cephalosporin was cephaloglycin (Wick and Bon iece 1965). It was soon superceded by cephalexin (Wick 1967) which has the same 7-acyl group as cephaloglycin. Cephradine (Gadebusch et al 1972) is structurally very similar to cephalexin and together with other members of this group such as cefaclor (Neu and Fu 1978a) these compounds are thought to owe their oral absorption properties to both the

α-amino group in the 7-acyl substituent and a small uncharged group at position C-3. All have similar antibacterial properties to cefazolin and remain susceptible to the majority of Gram negative β-lactamases.

A clinically significant step was made with the design of cephalosporins more resistant to the action of Gram negative β-lactamases. These compounds vary in structure but their β-lactamase stability is thought to reply on the steric hindrance conferred by bulky side chains at C-7 (O'Callaghan 1975, Fisher 1984).

Cefamandole (Wick and Preston 1972) has good stability to Gram negative β-lactamases conferred by a 7-acyl side group. Cefuroxime (O'Callaghan et al 1976) has an α-methoxyimino group at C-7 which confers β-lactamase stability (O'Callaghan 1979). They have a different spectrum of activity with cefuroxime being very effective against Haemophilus influenzae and Enterobacter spp. Cefamandole and cefuroxime heralded the second generation of cephalosporins (Neu 1982). However their antibacterial spectra still did not encompass such organisms as P.aeruginosa.

Recently clinical trials have been carried out with cefuroxime axetil, the 1-acetoxyethyl ester of cefuroxime (Williams and Harding 1984). This is an oral cephalosporin

with β-lactamase stability and high intrinsic activity, an advance over such oral compounds as cephradine and cephalexin.

The β-lactamase stability conferred by the α-methoxyimino group in the cefuroxime side chain was exploited in the development of further α-methoxyimino cephalosporins which would form the third generation of cephalosporins (Neu 1982). Cefotaxime (Heymes et al 1977), cefmenoxime (Tsuchiya et al 1981), and ceftizoxime (Fu and Neu 1980) are of similar design. In these compounds, the furyl ring in the 7-acylamino side chain of cefuroxime is replaced by the 5-aminothiazol-3-yl group which had been shown to give good anti-bacterial activity in cefotiam (Tsuchiya et al 1978a). The three compounds - cefotaxime, cefmenoxime and ceftizoxime - have a broad antibacterial spectrum and marginal activity against P.aeruginosa.

Compounds fully active against Pseudomonas spp. have only been developed recently. Cefsulodin (Tsuchiya et al 1978b) the extreme of the range of compounds, has antibacterial activity which is limited almost exclusively to Pseudomonas. Cefoperazone (Neu et al 1979a) was developed with similar side-chain modification to the acylureidopenicillin, pipericillin (the C-7 substitution of cefoperazone being the same as the C-6 substitution in pipericillin). Although

cefoperazone has a broad spectrum of activity which includes Pseudomonas spp, it is moderately susceptible to β-lactamases, which limits its effectiveness.

Ceftazidime (O'Callaghan et al 1980) is an extended spectrum third generation compound combining high stability to β-lactamases (Simpson et al 1982) with exceptionally broad antibacterial activity which includes Pseudomonas spp. This was achieved by replacing the methyl group of the α-methoxyimino substituent of cefuroxime with 2,2-dimethyl-acetic acid. The pyridine side-chain at C-3 of the cephem nucleus confers good pharmacokinetics on the compound and ceftazidime is well distributed throughout the body (Neu 1982). Ceftriaxone (Neu et al 1981) is another extended spectrum third generation compound. The C-7 substitution is similar to that in ceftazidime and they have a similar antibacterial spectrum.

1.3 The Cephamycins and Oxacephems

The insertion of a methoxy group in the C-7 position of the 7-ACA nucleus gave rise to the cephamycins (Figure 1d). Cephamycin C, a 7α-methoxycephem is stable to β-lactamases by virtue of the steric hinderance provided by the 7α-methoxy group (Daoust et al 1973). Its marketed analogue, cefoxitin (Onishi et al 1974) has a moderate antibacterial spectrum. Cefotetan (Toda et al 1980a, Phillips et al 1983) and cefmetazole (Benlloch et al 1982) are newer cephamycins

which have a similar stability and antibacterial spectra to cefoxitin but they are two to four times more active. Cefotetan has an added advantage in that it has a greatly increased serum half life (Phillips et al 1983).

The oxacephems (Figure 1e) were made by replacing the sulphur atom of the cephem nucleus with oxygen. The most notable member of this group is latamoxef (moxalactam) which was described by Neu and colleagues (1979b). Latamoxef possess a 7α-methoxy group which confers stability to β-lactamase action (a design feature used in the development of the penam, temocillin). In many respects latamoxef represents an evolutionary endpoint in our understanding of β-lactam design within the cephem framework. Latamoxef contains four regions that, it is thought, together provide an excellent balance of β-lactamase inhibitory properties, as well as a broad spectrum of activity.

1. The 7α-methoxy side group (provides β-lactamase stability).
2. The C-7 side chain carboxylate (improves β-lactamase stability).
3. The ring oxygen (provides intrinsic reactivity as an acylating agent).

4. The C-3' functional group (augments antibacterial spectrum).

(Fisher 1984)

Despite such a logical approach to the development of these compounds, resistance especially amongst such organisms as P.aeruginosa, Enterobacter spp. and Citrobacter spp. has been developing apace.

1.4 The Carbapenems

The carbapenems result from the replacement of the sulphur atom of the penem nucleus by a (CH_2) group. (Figure 1f). This type of β-lactam includes the thienamycin and olivanic acid families. The most promising carbapenem so far developed is N-formimidoyl thienamycin (Kropp et al 1980). However, a unique in vivo complication of the carbopenems is their rapid metabolism by a renal peptidase (Kim and Campbell 1982)and thus specific peptide inhibitors are coadministered with the carbapenem (Norrby et al 1983). The 6α-hydroxyethyl side chain is believed to mimic the 6α-methoxy group of the cephamycins by sterically hindering hydrolysis by B-lactamases. Although generally of excellent activity against Pseudomonas spp., thienamycin is hydrolysed by the P. maltophilia B-lactamase (Saino et al 1982) and it is consistently less active against Proteus spp (Kropp et al 1980).

Although the olivanic acids have good antibacterial activity they are generally less active than thienamycin and are not effective against Pseudomonas spp.

1.5 The Penems

The penem nucleus (Figure 1g) is the unsaturated analogue of the penam ring system. One synthetic example is the compound Sch 29482 (Ganguly et al 1982). This compound combines the features of the penicillin and thienamycin families and it is stable to β-lactamases. It is similar to cefotaxime in antibacterial spectrum, but it is an oral preparation. There is a suggestion that it is unstable as indicated by the fact that it is highly serum bound and antibacterial activity decreases in serum (Loebenberg et al 1982). A major problem is that Sch 29482 is metabolised to ethyl mercaptan (Brown and Reading 1983).

1.6 The Monobactams (Azetidinone Derivatives)

The existence of azetidinone derivatives has been realised since before the discovery of penicillin (Sykes and Bonner 1985). Naturally occurring azetidinones with potentially useful properties have been recently isolated from bacterial species and Nocardia. The monobactams (Sykes et al 1981a) and the nocardicins (Hashimoto et al 1976 , Imada et al 1981) are acylated derivatives of the 3-amino-monobactamic acid (3-AMA) (Figure 1h) and 3 amino nocardicinic acid (3-ANA) respectively (Figure 1i).

Antibiotic activity demands an acylation reactivity not attainable in simple monocyclic β-lactams. The unusual reactivity of bicyclic β-lactams is attributed to the unfavourable geometry which makes the molecule susceptible to nucleophile attack and acylation (Fisher 1984). An electron-withdrawing moiety, the sulphonate, brings this activity to the monobactams. It also provides the negative charge, proximal to the nitrogen, for membrane peptidase (and β-lactamase) recognition (Gordon et al 1982).

A semi-synthetic monobactam-aztreonam (Sykes et al 1982), was chosen following evaluation of structure-activity relationships, for further development and clinical trial evaluation. The antibacterial spectrum of aztreonam is restricted to Gram negative aerobes and its activity against these organisms is similar to that of ceftazidime. Aztreonam is also resistant to hydrolysis by most β-lactamases (Sykes et al 1982).

1.7 β-lactamase Inhibitors (β-lactam Class)

Inhibitors of β-lactamase enzymes fall into the following broad catagories (Wise 1982).

Competitive Inhibitors

(a) Reversible - inhibitor and substrate compete for the active site of the enzyme. No chemical inactivation of the enzyme takes place.

(b) Irreversible - interaction between enzyme and inhibitor is irreversible. The enzyme is inactivated, the reaction is usually progressive as the inhibitor is destroyed - "suicide inhibitors".

Non Competitive Inhibitors

The inhibitor does not comp ete for the active site of the enzyme but binds at another site, inducing a distortion of the molecule which is then incapable of enzymic hydrolysis of susceptible substrates.

Alkoxy Penicillins

Methicillin (Rolinson et al 1960) is a reversible competitive inhibitor for several Gram negative β-lactamases. It can potentiate the effect of benzylpenicillin against P. aeruginosa due to the inhibition of the P.aeruginosa β-lactamase in vivo (Sabath and Abraham 1964).

Isoxazolyl Penicillins

Isoxazolyl penicillins are reversible competitive inhibitors of β-lactamase enzymes (Sutherland and Batchelor 1964) Cloxacillin inhibits enterobacterial β-lactamases (Cole 1979) and it has been used in combination with ampicillin against Haemophilus influenzae present in mixed infections (May and Ingold 1971).

Other penicillins have been shown to have inhibitor properties. Carbenicillin has an inhibitory spectrum similar

to cloxacillin (Cole 1979). There have been reports that ampicillin also has inhibitory activity (Hamilton-Miller and Smith 1964) however it was found by other workers to be relatively inert (Cole 1979).

Cephalosporins

It was realised as early as 1956 that cephalosporin C possessed β-lactamase inhibitory properties (Abraham and Newton 1956).

The nature of the side-chain attached to the 7-amino group and the C-3 substituent are thought to affect the β-lactamase inhibitory properties of cephalosporins. The effect of the substitutions depends on the β-lactamase enzyme under consideration (O'Callaghan and Morris 1972). Cephaloglycin and cefamandole are non-competitive inhibitors of the E. cloacae β-lactamase enzyme (Mahoney et al 1976) whereas the other cephalosporins examined, cephaloridine and cefoxitin, are competitive inhibitors (Cole 1979).

Carbapenems

The carbapenems as well as being potent antibacterials have β-lactamase inhibitory properties. Thienamycin is an irreversible inactivator of β-lactamases from Gram negative bacteria (Toda et al 1980b). Olivanic acids are also progressive and irreversible inhibitors of various β-lactamases depending on the side chain substitutions (Sykes and Bush 1982).

Clavulanic Acid

Clavulanic acid is a potent β-lactamase inhibitor with little antibacterial activity (Reading and Cole 1977). It was isolated from Streptomyces clavuligerus, and has a bycyclic ring nucleus (clavam-Figure 1j). Clavulanic acid resists deacylation and is an irreversible suicide inhibitor of many β-lactamases (Neu and Fu 1978b). The kinetics of clavulanic acid/β-lactamase interaction are progressive and the inhibition is greater with higher concentrations of clavulanate. Irreversible inhibition is thought not to occur during the initial turnover events (Bush and Sykes 1983). Fisher and colleagues (1978) reported on the kinetics of clavulanate's interaction with TEM β-lactamase and found that the response depended on the ratio of inhibitor to enzyme.

Sulbactam, a desaminopenicillin sulphone, resembles clavulanic acid (English et al 1978) and is also a suicide inhibitor with a mechanism of action similar to that of clavulanic acid (Sykes and Bush 1982).

Permeability

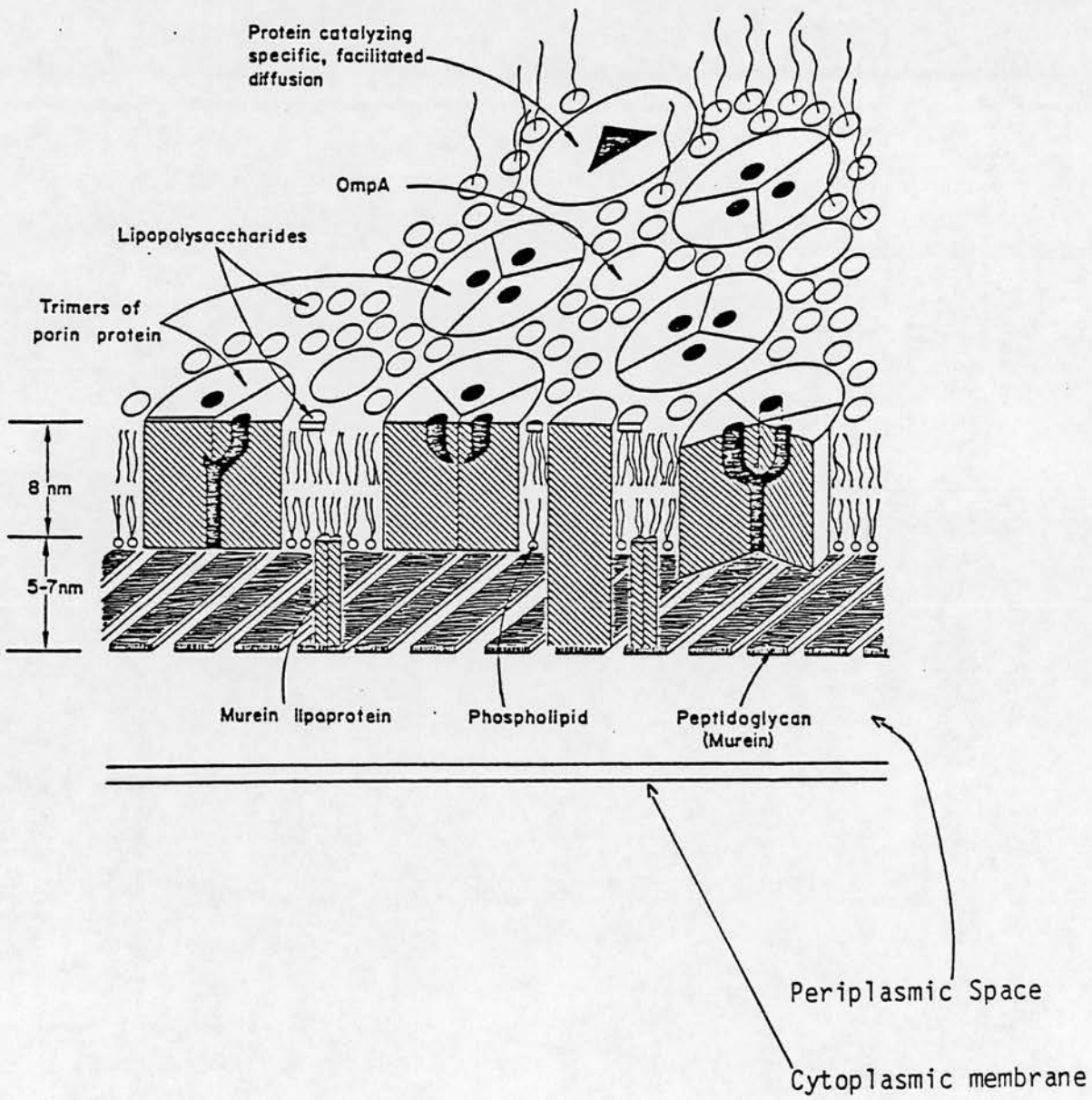
2. The Gram Negative Cell Wall as a Permeability Barrier

From the early days of penicillin therapy it had been thought that the resistance of Gram negative organisms may be attributable to the inability of the compound to penetrate the cell. It was established by Izaki and colleagues (1966) that the Gram negative target site was as sensitive to penicillin as that of the Gram positive. The existence of a barrier to penetration was further demonstrated by the fact that disrupted cell preparations of Gram negative cells hydrolysed β-lactams much faster than intact cells (Smith 1963, Hamilton-Miller 1963). It was also shown that making the cells permeable with ethylenediaminetetraacetic acid (EDTA) increased the hydrolytic activity although no β-lactamase was released into the surrounding medium (Hamilton-Miller 1965). Gram negative β-lactamase enzymes were shown to be predominately located in the periplasmic space (Neu 1968).

It was concluded that Gram negative bacteria are surrounded by an additional membrane layer, not found in Gram positive bacteria (Glaurt and Thornley 1969) which prevented the penetration of β-lactams.

The Gram negative outer membrane is complex. It surrounds a thin layer of peptidoglycan, which imparts rigidity and structure to the cell, which surrounds in its turn, the cytoplasmic membrane (Figure 2). The outer membrane is

Figure 2 The Gram negative Cell Wall
(Taken from Nikaido 1985)



covalently linked to the peptidoglycan by low molecular weight lipoproteins described as Braun proteins in E.coli (Braun 1975). Several other types of outer membrane proteins (OMPs) have been shown to be non-covalently linked with peptidoglycan (Lugtenberg and van Alphen 1983).

Almost half of the mass of the outer membrane is protein (Osborn et al 1972) although some transverse the outer and cytoplasmic membranes, most is thought to be restricted to the outer membrane.

2.1 Major Outer Membrane Proteins

Permeability is governed by the presence of a group of major outer membrane proteins known as the porins (Nikaido 1979, Nikaido and Nakae 1979). Other major proteins include OmpA protein and the murein lipoprotein (Nikaido and Vaara 1985). Neither OmpA nor the murein lipoprotein appear to have pore functions (Nikaido et al 1977, Suzuki et al 1978).

Porin Proteins

The proteins coded for by ompF ompC and phoE genes in Escherichia coli K12 are known as porins (OmpF, OmpC and PhoE). They produce water-filled pores or channels that allow the passage of small hydrophilic molecules across the outer membrane (Nikaido and Nakae 1979).

The role of porins in penetration across the outer membrane has been established by the use of porin mutants (Bavoil *et al* 1977, Beacham *et al* 1977, Lutkenhaus 1977). Reconstitution experiments, where purified porins have been incorporated into proteoliposomes, have resulted in a versatile *in vitro* model of porin function (Nakae 1976a, b).

Bacteria occasionally produce additional porins for example the OmpD porin of *Salmonella typhimurium* LT2 (Nurminem *et al* 1976), the prophage coded protein 2 or Lc of *E.coli* (Pugsley and Schnaitman 1978) and protein K, found amongst unencapsulated strains of *E.coli* (Paakkanen *et al* 1979, Sutcliffe *et al* 1983, Whitfield *et* al 1983). Several other proteins function as more specific diffusion channels.

Porins from both *E.coli* and *S.typhimurium* were found to exist as undenatured trimer proteins when extracted with sodium dodecyl sulphate (SDS) (Rosenbusch 1974, Palva and Randall 1978, Nakae *et al* 1979, Tokunagay *et al* 1979). The estimated diameters of the OmpF and OmpC channels of *E.coli* are 1.16 and 1.04nm respectively (Nikaido and Rosenberg 1983). The reconstitution studies carried out on isolated porin proteins suggest that porin channels in *E.coli* and *S.typhimurium* are non-specific, allowing the passage of any solute provided it is small (less than 600 daltons) and hydrophilic (Nakae and Nikaido 1975, Decad and Nikaido 1976).

Diffusion through pores is a passive process. It can be assumed that if there are no removal or inactiv ation processes taking place, equal concentrations of solutes such as β-lactams will be found inside and outside the cell after equilibrium is reached (Nikaido 1985). Most Gram negative bacteria possess a β-lactamase enzyme (Matthew and Harris 1976) which may destroy a significant proportion of incoming β-lactam molecules. Any assessment of permeability of the outer membrane to β-lactam compounds must take these possible influences into account.

Alternative β-lactam Uptake Pathways

Some β-lactams are lipophilic and may diffuse through the lipid bilayer of the outer membrane. The lipopolysaccharide-protein complexes on the outer membrane however, normally present a hydrophobic barrier (Nikaido 1985). It is possible that such a pathway may become significant in non enteric organisms with significant amounts of phospholipid eg Neisseria gonorrhoeae (Lysko and Morse 1981).

2.2 Assessing Permeability

Crypticity

Hamilton Miller (1963) called the ratio between hydrolysis of B-lactams by intact and disrupted cells the "permeability index" and used this as a measure of permeability. This was later used by Richmond and Curtis (1974) who called the index "crypticity".

The crypticity index is only a valid parameter when different bacterial strains are used containing equal amounts of an identical β-lactamase and they are challenged with a given β-lactam at the same external concentration (Nikaido 1985).

Modifications have been made to the crypticity index in order to make it more quantitative (Sawai et al 1977, Zimmerman and Rosselet 1977). Nikaido et al (1977) have expanded this work to produce a value for β-lactam compounds termed the "permeability coefficient" which is independent of external concentration of β-lactam and the Km and Vmax of the β-lactam hydrolysis.

Use of Mutants in Assessing Permeability

Richmond et al (1976) isolated mutants of E.coli K12 (DC2, DC3) which have been used in permeability studies. Both mutants are more sensitive to the action of β-lactam antibiotics than the parent strain (DC0). The DC2 strain does not appear to be a porin mutant (Unpublished results quoted in Nikaido and Vaara 1985), but it does have changes in lipopolysaccharide (LPS), although it is not clear how these affect permeability (Clark 1984).

The EDTA treatment of Gram negative bacteria produces cells which are similar to the DC mutants (Scudamore et al 1979).

Neither mutation in strains DC2 or DC3 nor EDTA treatment of wild type strains produces uniform changes, increasing permeability to all agents. Removal of LPS enhances diffusion of hydrophobic agents (Leive 1974) and data from Scudamore and colleagues (1979) confirm that misleading results are obtained for hydrophilic agents.

Binding to Penicillin Binding Proteins as a Measure of Permeability

The labelling of penicillin binding proteins (PBPs) in intact and disrupted cells can be compared to evaluate permeability. If the unlabelled β-lactam inhibits well in disrupted cells but poorly in intact cells it is assumed it penetrates poorly (Zimmerman 1980, Rodriguez-Tebar et al 1982). This technique is useful for β-lactamase stable compounds unsuitable for conventional crypticity assays.

Measuring Permeability with Reconstituted Liposome Vesicles

Reconstituted liposomes containing only a few porin channels were first used to assess permeability by Nakae (1976b). The liposome swelling assay has been used widely to assess influx of solutes. The swelling rate of the liposome is limited by the rate of influx of the solute, rather than by the rate of

diffusion of water. It is presumed that the solute is crossing the lipsome membrane through the porin channels (Nikaido and Rosenberg 1981, 1983).

2.3 Diffusion of β-lactams Through the Outer Membrane

The physicochemical properties of the solute determines its rate of diffusion. These are hydrophobicity, size and charge, all of which interact with each other.

Hydrophobicity

The efficacy of a β-lactam is thought to be reduced if the compound is hydrophobic (Biagi et al 1970).

Zimmerman and Rosselet (1977) demonstrated that hydrophobicity resulted in a slowing down of diffusion of β-lactams. There was also a direct correlation between hydrophilicity and penetration rate. Hydrophobicity has a similar effect on penetration through OmpF and OmpC porins in intact cells. However using proteoliposomes, hydrophobicity affects penetration through the narrow channel of OmpC more severely than the wider OmpF porin (Nikaido et al 1983).

Most penicillins are too hydrophobic to be tested with a proteoliposome system; therefore direct comparison is difficult and analogues have been used (Yoshimura and Nikaido

1984). These results have shown that the β-lactam nucleus affects penetration to a greater extent that the hydrophobicity of peripheral substituents.

Effect of β-lactam Size

Liposome swelling assays have shown that β-lactam size plays an important role in determining penetration through porins (Nikaido and Rosenberg 1981). β-lactams penetrate faster through the larger OmpF channel of E.coli (Nikaido and Rosenberg 1983). There are exceptions, and a few β-lactams penetrate very well in spite of their size (Nikaido and Rosenberg 1983).

Effect of Charge

Net negative charges severely impede penetration of a β-lactam through the outer membrane eg cephacetrile and cefsulodin. This phenomenon may relate to the presence of a Donnan potentional across the outer membrane (negative inside) (Stock et al 1977). Diffusion of zwitterionic compounds such as imipenem, cefaclor, ampicillin and cephaloridine is mainly affected by their size. However they penetrate much more rapidly than compounds carrying a net negative charge, as zwitterionic compounds are probably surrounded by hydration shells which impart hydrophilicity to the molecule (Nikaido 1985).

2.4 Other Bacterial Porins

Most porins share the properties of the OmpF channel of E.coli (Hancock 1984, Nikaido and Vaara 1985, Nikaido 1985). Generally porins favour cations over anions, the exception being N.gonorrhoeae which was reported to be anion-selective (Greco et al 1980). However Young et al (1983) showed that the porin was cation-selective and not anion-selective as previously reported.

The large porin channels of P.aeruginosa and N.gonorrhoeae are less affected by the solute's size, charge or hydrophobicity state. Haemophilus influenzae has been shown to produce a porin with a molecular weight exclusion limit which lies between the value for the Enterobacteriaceae and the pseudomonads (Vachon et al 1985). H. influenzae porin protein I has been shown not to act as a barrier to diffusion and the organism has no effective permeability barrier (Coulton et al 1983, Vachon et al 1985). P.aeruginosa on the other hand produces large porins (Hancock et al 1979) but is well documented as being impermeable to many solutes (Brown

1975, Bryan 1979). It is thought that only a small fraction of porin molecules in P.aeruginosa produce open channels at any one time (Benz and Hancock 1981, Yoshimura et al 1983). This phenomenon combines with the organism's inducible chromosomal β-lactamase to make it resistant to most β-lactams.

Cefsulodin is active against P.aeruginosa, despite its large size and two negative, one positive charge differential. It probably achieves its affect by its relative stability to β-lactamase enzymes and its affinity for sensitive target enzymes in Pseudomonas (Tsuchiya et al 1978b). Cefsulodin is however, inactive against E.coli probably because of these very features.

2.5 Opening and Closing Porins

Porin channels may be opened and closed in response to stimuli and in adverse conditions, perhaps to stop uptake of antibiotics. An electrical potential can close porin channels (Schindler and Rosenbusch 1978, 1981). However, Nikaido and coworkers (reviewed by Nikaido and Vaara 1985) feel that the electrical potentials present under physiological conditions would be insufficient to regulate porin channels.

The β-lactam Target Sites

3. Cell Wall Synthesis - The Target Site of β-lactam Antibiotics

Gardner (1940) was the first to observe the physiological effects of penicillin on growing cell cultures. He observed that at low concentrations of penicillin the cells did not lyse but were converted to filaments. Duguid (1946) indicated that penicillin may interfere with the synthesis of an unknown structure of bacterial cell surfaces. The first biochemical clue was provided by Park and Johnson (1949) and Park (1952) who discovered that several novel uridine nucleotides accumulate in the cytoplasm of penicillin treated Staphylococcus aureus. It was not until 1957 that Park and Strominger observed the composition of these nucleotides was similar to those of the recently discovered cell wall, this suggested that they may be cell wall precursors. It was demonstrated that in a hypertonic medium penicillin produces viable spheroplasts of E.coli and not lysis (Lederberg 1956, 1957, Hahn and Ciak 1957). Penicillin was thus shown to interfere with cell wall biosynthesis.

Since the 1950s the complex mechanism of cell wall synthesis has been elucidated (reviewed by Rogers 1970, Waxman and Strominger 1982).

3.1 The Target Site

From the early studies, it was recognised that radioactive

penicillin was bound to a (protein) target in membranes of bacterial cells (reviewed by Cooper 1956). The primary target was the terminal step in cell wall biosynthesis, catalysed by an enzyme (peptidoglycan transpeptidase*) (Wise and Park 1965, Tipper and Strominger 1965, Izaki et al 1966). Subsequently it was found that several proteins in the membrane bound penicillin specifically. Another enzyme, D,D-alanine carboxypeptidase* was shown to be inhibited by penicillins but it was not regarded as a lethal target site (Blumberg and Strominger 1971, 1972, Suginaka et al 1972).

The existence of multiple penicillin binding proteins (PBPs) in the membrane of all bacteria studied so far, suggests that there are multiple targets of penicillin action. It is now clear that different β-lactams exert their bactericidal effects by interacting with different subsets of PBPs (Spratt 1975, 1977, 1983, Spratt and Pardee 1975).

3.2 Peptidoglycan Biosynthesis

Peptidoglycan biosynthesis has been extensively reviewed (for example see Ghuysen 1977, Rogers et al 1980).

During the final stage of biosynthesis, a free amino group on the N-acetylmuramyl pentapeptide of one glycan stand, displaces the terminal D-alanine from a pentapeptide of the

* E.C. number not available.

second glycan stand in a transpeptidation reaction. It is this cross-linking step which is the penicillin-sensitive reaction of cell wall biosynthesis (Wise and Park 1965, Tipper and Strominger 1965).

In addition to the transpeptidase reactions, two other related penicillin sensitive reactions have been described (Figure 3). D,D-alanine carboxypeptidase effects cleavage of the terminal D-alanine from pentapeptide side chains of cell wall precursors (Izaki et al 1966, 1968, Araki et al 1966). The D,D-alanine carboxypeptidase activity in E.coli membranes is significantly more sensitive to penicillin than is the transpeptidase activity (Blumberg and Strominger 1974). The other penicillin sensitive reaction is endopeptidase* catalysis which leads to hydrolysis of the interpeptide cross-link formed in a transpeptidation reaction. The sequence of transpeptidase followed by endopeptidase reactions produces a product indistinguishable from that of the D,D-alanine carboxypeptidase reaction (Figure 3) (Waxman and Strominger 1982).

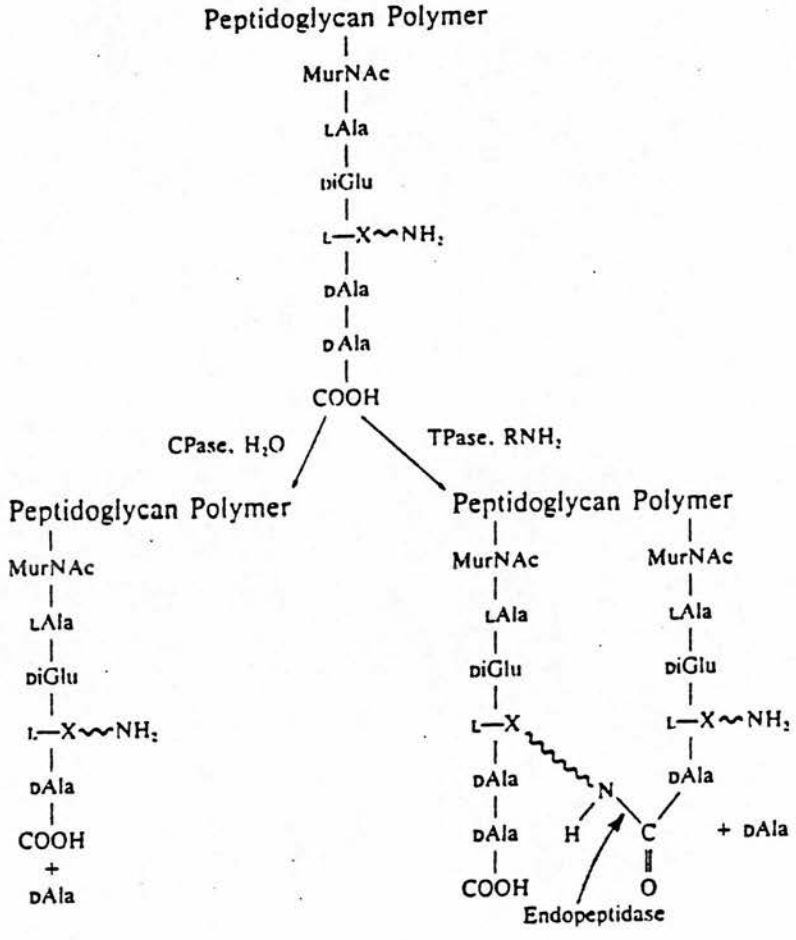
3.3 The Properties of the Penicillin-Sensitive Enzymes

Penicillin binding proteins are numbered in order of decreasing molecular size which ranges from 120,000 to around

* E.C. Number not Available

Figure 3 Penicillin Sensitive transpeptidase (TPase), D-alanine carboxypeptidase (CPase), and endopeptidase reactions.

(From Waxman and Strominger 1983)



CPase = Carboxypeptidase
TPase = Transpeptidase

27,000 daltons (Makover et al 1981, Waxman and Strominger 1982). They vary in relative abundance, for instance, PBP2, in E.coli binds only around 1% of available radiolabelled penicillin (Spratt 1977). It is estimated this corresponds to between 15 and 20 molecules of PBP2 per cell.

The PBPs themselves can be divided into two categories, high molecular weight PBPs (60-92,000 in E.coli) and low molecular weight PBPs (40-49,000 in E.coli). Their role in E.coli has been elucidated in two main ways (Spratt 1980a). B-lactams have at least three morphological effects on E.coli (filimentation, rapid cell lysis and growth into spherical cells). The degree of response can be correlated with binding to particular PBPs (Spratt and Pardee 1975, Spratt 1975). The consequences of PBP inactivations has been determined by the use of mutants which lack PBPs or produce only thermolabile forms (Spratt 1975, 1980b, Spratt and 1977, Suzuki et al 1978, Broome-Smith and Spratt 1982).

Studies have shown that high molecular weight PBPs are essential for cell viability (PBPs 1, 2 and 3 in E.coli). In contrast, mutants that lack low molecular weight PBPs (4,5 and 6 in E.coli) are viable and do not show significant morphological abnormalties (Spratt 1977, 1983).

The High Molecular Weight PBPs

Spratt (1975) was the first to deduce a likely physiological role for each high molecular weight PBP of E.coli (Table 1) (PBPs1A, 1B. 2 and 3). Generally the high molecular weight PBPs are more sensitive to the action of β-lactams, especially the cephalosporins (Waxman and Strominger 1982).

In E.coli, PBPs1A and 1B are enzymes of cell elongation. Rapid lysis of E.coli occurs when both 1A and 1B are inhibited (Spratt 1975, 1977, Tamaki et al 1977, Suzuki et al 1978). β-lactams such as cephaloridine have a high affinity for PBPs1A and 1B (Spratt 1975). Mutants which lack either PBP1A or 1B are viable (Spratt and Jobanputra 1977, Suzuki et al 1978). PBP 1A mutants have a much slower rate of lysis and PBP1A is thought to have a role in peptidoglycan metabolism. Mutants of PBP1B have decreased peptidoglycan biosynthetic activity and they over-produce PBPs1A and 2 to compensate (Tamaki et al 1977). The mutants are extremely susceptible to the action of cephalosporins (Suzuki et al 1978) owing to the high sensitivity of PBP 1A which becomes indispensible. Mutation in both PBPs1A and 1B is lethal to the cell (Spratt and Jobanputra 1977)

E.coli PBP2 has a role in cell shape. Mecillinam is highly selective for PBP2 and produces large osmotically stable

Table 1. Properties of the Penicillin Binding Proteins of Escherichia coli (taken from Spratt 1983).

PBP	Molecular Size (daltons)	Abundance* (% total number of PBPs)	Consequences of Inactivation of PBP	Enzymic Activity of PBP
1A	92 000	6	Rapid cell lysis occurs if both PBP1A and PBP1B are inactivated	Both PBP1A and PBP1B are transglycosylase/ transpeptidases involved in cell elongation
1B	90 000	2		
2	66 000	0.7	Growth as spherical cells	A transpeptidase (and possibly also a transglycosylase) that may initiate peptidoglycan insertion at new growth sites
3	60 000	2	Inhibition of cell division leading to filamentous growth	A transglycosylase/ transpeptidase required specifically for formation of the cross wall at cell division
4	49 000	4	No obvious growth defect	A D-alanine carboxypeptidase that may function *in vivo* as a secondary transpeptidase in the maturation of peptidoglycan
5	42 000	65	No obvious growth defect	A D-alanine carboxypeptidase
6	40 000	21	No obvious growth defect	A D-alanine carboxypeptidase

* from Spratt (1977)

round cells which eventually lyse after several hours of growth (Tybring and Melchior 1975). The PBP3 of E.coli is important in cell division. Cephalexin, ampicillin and cefuroxime bind to this PBP and induce filamentation of E.coli by specifically inhibiting cell division without affecting cell lysis (Spratt 1975, 1980a). It is thought that PBP3 may be synthesised or inactivated upon completion of DNA replication (Spratt 1977).

Enzymic Activity of High Molecular Weight PBPs

Difficulties have been encountered in detection of the enzymic activity of purified high molecular weight PBPs (Table 1) and most progress has been made with PBP1B of E.coli. It appears to perform a coupled transpeptidase/ transglycosylation reaction in vitro with a lipid intermediate (Tamura et al 1980, Suzuki et al 1980). As the synthesis and cross linking of peptidoglycan needs to be strictly controlled, it makes sense to combine transglycosylase and transpeptidase activities in a single enzyme (Spratt 1983).

There is preliminary evidence that PBP2 is a peptidoglycan transpeptidase but it is not known if it also has transglycosylase activity (Ishino et al 1982). PBPs1A and PBP3 have also been reported to be bifunctional transglycosylase/ transpeptidases (Ishino et al 1980, Ishino and Matsuhashi

1981). These high molecular weight PBPs do not appear to act as D,D-alanine carboxypeptidases (Matsuhashi et al 1982). However, each presumably has a specific function: PBP1B and possibly 1A may be involved in cell elongation peptidoglycan biosynthesis; PBP3 may synthesise the peptidoglycan of the septum at cell division and PBP2 may be responsible for the initiation of synthesis at new sites of cell wall growth (Spratt 1983).

The Low Molecular Weight PBPs

The low molecular weight PBPs in E.coli (PBPs4,5 and 6) are generally less sensitive to β-lactams, especially cephalosporins. They are the major PBPs in the inner membrane and comprise 50 to 90% of the total (Waxman and Strominger 1982). Strains with deletions of the PBP5 and PBP6 gene have been engineered and have shown that these PBPs are non-essential and their loss has no effect on cell morphology (Spratt 1980b, Broome-Smith and Spratt 1982). Only point mutants of PBP4 have been described and the effect of total loss of PBP4 has not been established. Loss of PBPs 4,5 and 6 in one strain has not been examined and the effect is unknown (Spratt 1983). The properties of these PBPs are summarised in Table 1.

Enzymic Activity of Low Molecular Weight PBPs

PBPs4,5 and 6 of E.coli all catalyse D,D-alanine carboxypeptidase reactions. Under appropriate conditions the

low molecular weight PBPs will also catalyse a model transpeptidase reaction, and PBP4 has been shown to have endopeptidase activity (Tamura et al 1976, Spratt and Strominger 1976, Amanuma and Strominger 1980).

PBP4 is thought to catalyse the secondary transpeptidation which produces the characteristic mature cell wall. (de Pedro Schwarz 1981). Mutants that are deficient in this enzyme are unable to carry out this secondary phase of crosslinking but show no obvious morphological defects (Spratt 1983).

3.4 Relationships Amongst PBPs

There is good evidence that the PBPs of a given organism are independent proteins and are not related by a preproduct. Each of the PBPs of E.coli has been mapped to a different position on the bacterial chromosome (Suziki et al 1978). There is also comparatively little amino acid homology among the different PBPs of E.coli (Broome-Smith et al 1985). Such results do not preclude evolutionary relationships or any structural similarity between PBPs.

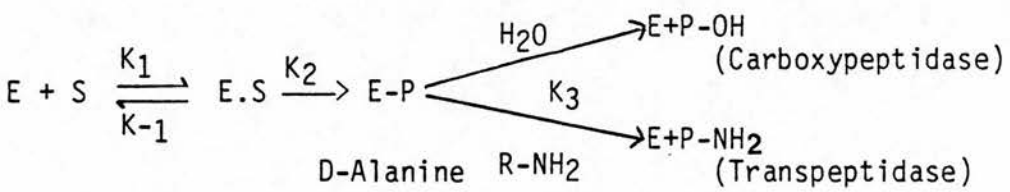
3.5 Is Lysis the Direct Result of Inactivation of Essential PBPs?

There is now substantial evidence that induction of cell death and lysis are both secondary responses to inhibition of cell wall assembly. This evidence has been reviewed by Shockman et al (1979) and Tomasz (1979a, 1979b).

In several organisms, the lytic effects of β-lactams can be eliminated by the inactivation of peptidoglycan hydrolases also know as autolysins*. This effect is described as "tolerance" and it is not accompanied by a change in growth sensitivity to β-lactams which indicates that the primary targets (the PBPs) remain unaltered.

3.6 The Action of Carboxypeptidase and Transpeptidase in Vitro

The activity of carboxypeptidase in vitro is monitored by the release of free D-alanine which results from nucleophile attack at the carbonyl carbon of the penultimate D-alanine of a cell wall pentapeptide. The nucleophile may be from water (OH). However if it is an amino compound (R-NH2) a transpeptidation reaction takes place, i.e. transpeptidase activity (Tamura et al 1976, Waxman et al 1980).



E, Enzyme
S, Substrate
P, Product

Figure 4 Schematic Presentation of PBP Action
(Taken from Waxman and Strominger (1982))

There is competition between the pathways leading to carboxypeptidase and transpeptidase products. Endopeptidases

* E.C. number not available

hydrolyse the D-alanine, D-alanine peptide dimers formed via transpeptidase reactions (Tamura et al 1976).

Penicillin Binding to PBPs

The kinetics of binding β-lactam antibiotics is similar to the processing of R- D Alanine - D Alanine substrates by carboxypeptidases.

$$E + I \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} E.I. \xrightarrow{K_2} E\text{-}I \xrightarrow[(Slow)]{K_3} E + \text{degraded } \beta\text{-lactam}$$

E, Enzyme
I, Inhibitor (β-lactam)
E.I, Non covalent, reversible complex
E-I, Covalent penicilloyl PBP

Figure 5 Inhibition of PBPs by β-lactam Antibiotics

(Taken from Blumberg and Strominger (1971), Frère et al 1975a)

The K_3 (E-I ⟶ E + degraded β-lactam) is low and therefore there is an accumulation of stable penicilloyl enzyme complex (E-I). Binding of β-lactams is characterised by first order kinetics (Frère et al 1975a). Once bound to the active site a suitable β-lactam side chain may interact with a specific enzyme grouping so as to correctly position and/or catalytically inactivate the β-lactam for subsequent enzyme acylation (Ghuysen et al 1980). The kinetics of β-lactams suggest a classic competitive inhibition (Umbreit and Strominger 1973, Yocum et al 1974, Frère et al 1975b).

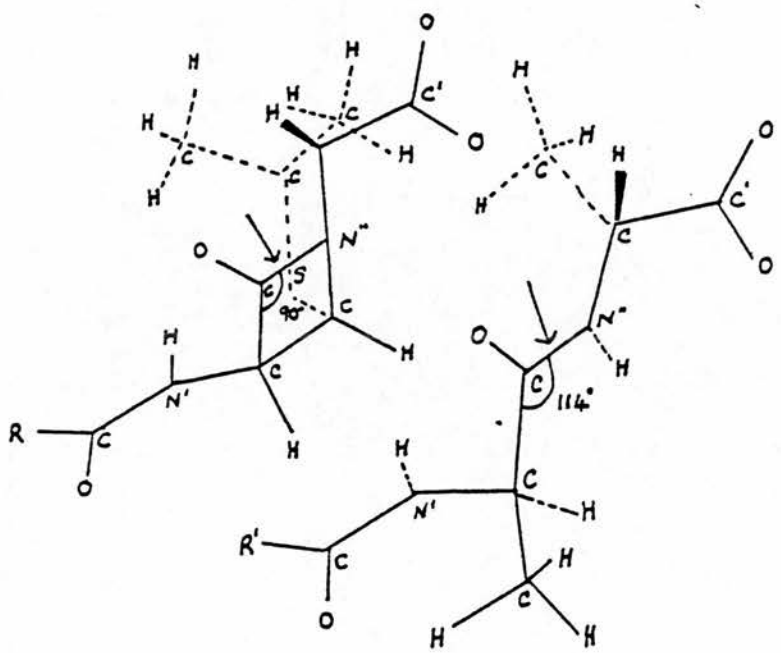
In the absence of added nucleophiles, degradation of penicilloyl-PBP complexes can occur by either of two pathways. The bound penicilloyl moiety can be enzymically hydrolysed to yield penicilloic acid eg PBPs 5 and 6 of E.coli. (Tamura et al 1976). The second possibility is fragmentation of the bound penicilloyl moiety at a very slow rate (Marquet et al 1979). The choice of pathways between hydrolysis and fragmentation depends on the β-lactam in question, the reaction conditions and the nature of any exogenous nucleophile (Ghuysen et al 1980).

3.7 The Action of β-lactams - The Substrate Analogue Hypothesis

Tipper and Strominger (1965) proposed that penicillin acts as a structural analogue of the acyl-D-alanyl-D-alanine terminus of nascent polypeptide strands (Figure 6). Penicillin was predicted to bind to and acylate the enzyme with its highly reactive β-lactam bond positioned at the active site (Yocum et al 1979). In order to be effective as a substrate analogue, β-lactams must be sufficiently similar to the natural substrate to permit recognition by cell wall enzymes and inhibit their enzymic action (Waxman and Strominger 1982, 1983).

There are contradictions to the substrate analogue theory when the β-lactam structure is examined closely and compared to that of acyl-D-alanine (reviewed by Waxman and Strominger 1983).

Figure 6 Stereomodels illustrating the proposed similarity between acyl-D-alanine (lower structure) and penicillin (upper structure).
Arrows indicate bonds cleaved during substrate catalysis or during the reaction leading to inactivation by penicillin.
(Taken from Waxman and Strominger 1983)



Lee (1971) has argued that several anomalies can be explained by the possible structural analogy of penicillin and a transition state structure formed during cleavage of the D-Alanine D-Alanine peptide bond.

It is believed that a transition state enzyme bound substrate has a compressed and twisted peptide dihedral angle. There is concomitant loss of double bond character and weakening of the peptide bond. β-lactams have a bicyclic ring system which imparts significant single bond character to the corresponding β-lactam bond which favours β-lactam binding to the enzyme (Waxman and Strominger 1982, 1983). Other features, including side-chain substitutions and portions of the β-lactam nucleus with no analogy in the dipeptide probably decrease enzyme recognition. Overall, substrate and β-lactam are thought to bind with similar affinity (Ghuysen et al 1979).

3.8 Homology to β-lactamase Enzymes

It has been suggested that β-lactamase enzymes may have evolved from the penicillin sensitive enzymes of cell wall synthesis (Tipper and Strominger 1965). Several PBPs have been found to have β-lactamase activity (Spratt 1980b, Waxman and Strominger 1983). Several β-lactamases and PBPs have been shown to use an acyl-enzyme reaction mechanism via an active site serine residue (Frère et al 1976, Knott-Hunziger et al 1979, Waxman and Strominger 1980).

Some homology between the low molecular weight PBPs of bacilli and β-lactamases has been determined (Knott-Hunziger et al 1979, Waxman and Strominger 1983). If PBPs and β-lactamases have a common evolutionary history, this should emerge from further comparison of their amino acid sequences.

4. Genetics of Resistance to β-lactam Antibiotics

4.1 Chromosomal Resistance

Chromosomal mutants can be obtained with relative ease by sub-culture in the presence of an antimicrobial agent. Spontaneous mutants arise at a frequency between one in 10^6 and one in 10^8 per cell division (Lederberg and Lederberg 1952).

Step-wise mutations can lead to antibiotic resistance and the strain retains its apparent virulence. In N.gonorrohoeae chromosomal resistance to penicillin can result from the acquisition by transformants of the pen A locus which alters the affinity of PBP2. Further transformation of mtr and pen B loci alter the outer membrane permeability of the organism and further contribute to penicillin resistance (Guymon et al 1978). Alterations of other genes, perhaps pem and tem, have resulted in reduced affinity of PBP1 for penicillin and highly penicillin resistant gonococci (Barbour 1981, Dougherty et al 1980). Single-step chromosomal mutants can occur, such as the carbenicillin-resistant mutants in

P.aeruginosa, which have reduced affinity of all PBPs (Godfrey and Bryan 1982).

A degree of intrinsic β-lactam resistance in some bacteria can be attributed to the presence of a basal level of chromosomal β-lactamase. The enzyme may be inducible (Sykes and Matthew 1976) and mutations in the induction regulatory system in organisms such as Pseudomonas, Enterobacter, Serratia and Citrobacter can lead to high levels of constitutive β-lactamase production (Gootz et al 1982).

Hyperproduction of chromosomal β-lactamase has been observed in E.coli. Expression of the E.coli K12 amp C, structural gene is normally low which is thought to result from the presence of a weak promoter and a transcriptional attenuator in the ampC leader (Jaurin et al 1981). E.coli mutants have been isolated which hyperproduce chromosomal β-lactamase. Up-promoter mutations have been shown to increase expression seven to 21 fold (Jaurin et al 1981). Expression could also be increased up to 21 fold in one-step by (1) the insertion of an insertion sequence (IS) into the ampC promotor (Jaurin and Normark 1983) (2) ampC attenuator mutation (Jaurin et al 1981) (3) gene amplification (Edlund et al 1979).

Naturally occuring ampC hyperproducing strains have been found with 24 to 48 fold increases in level of production (Bergstrom and Normark 1979). They all had an identical

449 base pair sequence which encodes a very strong B-lactamase promotor. This promotor is identical to that found in Shigella sonnei except for a single base pair change. It appears that naturally occuring chromosomal β-lactam resistance may have evolved by horizontal transfer of ampC DNA from Shigella species (Olsson et al 1983).

4.2 Plasmid - Determined Resistance

Transferable resistance to antimicrobial agents was first discovered amongst strains of S.flexneri isolated in Japan (by Akiba et al 1959 and Ochai et al 1960), who recognised the R (resistance) factor as being analogous to the F (fertility) factor of E.coli K12 (Cavalli-Sforza et al 1953, Hayes 1953).

It has been generally observed that the introduction of a new antimicrobial agent is generally (although not always) followed by the identification of plasmid - mediated resistance mechanisms (Anderson 1968, Datta 1984). Ampicillin (Rolinson and Stevens 1961) was introduced into clinical use in 1962 and the first R plasmids encoding resistance to this compound were detected soon after (Anderson and Datta 1965).

It has been established that conjugative plasmids were present in enteric bacteria isolated from the "pre-antibiotic" era (1917-1954). The plasmids are similar to

the R plasmids of today except for the lack of antibiotic resistance determinants (Hughes and Datta 1983, Datta and Hughes 1983). There was therefore, a reservoir of transferable plasmids present before the advent of antimicrobial selection pressure.

Plasmids, and other resistance genes can be transferred between bacteria by conjugation, transformation and transduction (reviewed by Broda 1979 and Saunders 1984). All plasmids are dependent on host replication functions, although different plasmids vary in individual respects (Norstrom et al 1984). Plasmids replicate independently of the chromosome.

Those of more than 35 kilobases tend to maintain low numbers per cell (one to four copies), whereas smaller plasmids are usually present in high copy number (Broda 1979, Nordström et al 1984).

Replication is governed by regulating the number of initiation events at the origin. Mutations in the regulatory genes can result in runaway, uncontrolled plasmid replication (Tomizawa et al 1981, Light and Molin 1981).

Plasmid Classification

Plasmids can be classified into incompatibility (Inc) groups. Plasmids of the same Inc group fail to coexist stably in the same host cell, [illegible] have closely related replication regulation systems and have a large degree of DNA homology (Grindley et al 1973, Falkow et al 1974, Smith et al 1974, Roussel and Chabbert 1978).

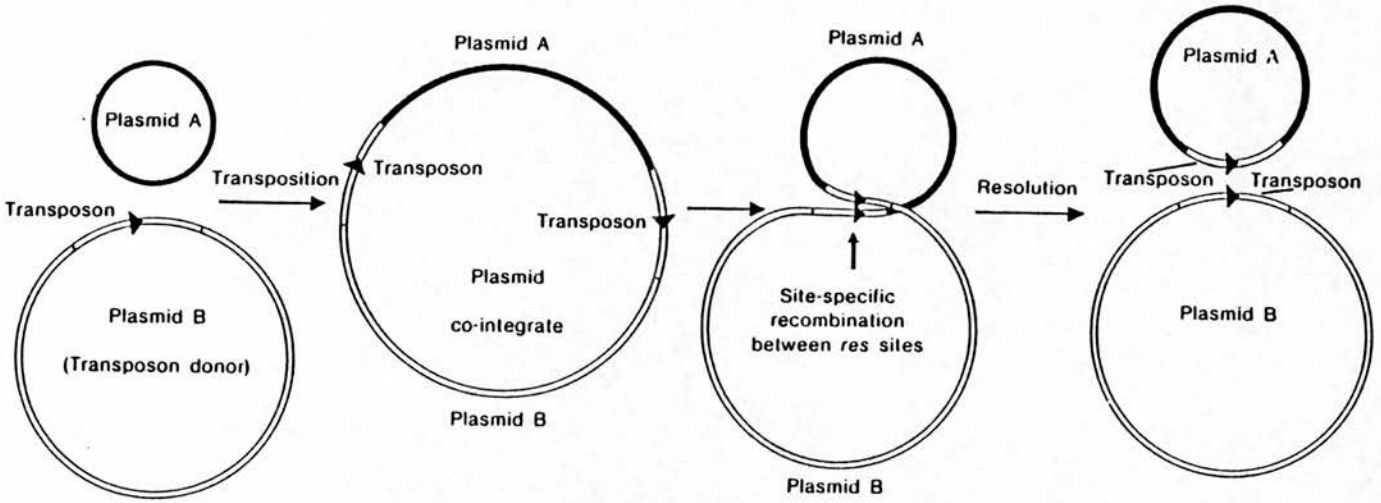
Incompatibility grouping is generally impractical for large surveys of resistance plasmids. Difficulties may also be encountered with natural plasmids which carry two or more different Inc group determinants (Grant et al 1980, Taylor et al 1981, Nugent et al 1982, Bird and Pittard 1983).

Plasmids have been classified by size, the resistance markers present and their individual restriction endonuclease digest pattern (O'Brien et al 1982, Tietze and Tschape 1983).

4.3 Transposons

Transposons are units of DNA capable of transferring (transposing) themselves from one replicon to another. They are unable to replicate independently and must be maintained as part of a functional replicon (Figure 7) (reviewed by Kleckner 1981). Transposition itself occurs independently of the normal host homologous recombination mechanism i.e. the process is recA-independent (Hedges and Jacob 1974, Kopecko and Cohen 1975).

Figure 7 Formation and Resolution of Plasmid Cointegrates During Transposition of Tn3
(Taken from Saunders 1984)



The diagram indicates the orientation of the DNA sequence of each copy of the transposon and position of *res* site.

There are three types of transposon. The first group are the composite transposons which consist of one or more resistance genes bounded by IS sequences 700 to 1500 base pairs in length, usually in reverse orientation (Ross *et al* 1979 Rosner and Guyer 1980,). The second group of transposons are the Tn3-like group. They have three sites and two functional groups essential for transposition; *tnpA* determines transposase and *tnpR* a bifunctional protein which regulates the expression of transposase and resolves intermediate cointegrate formations in the transposition process (Chou *et al* 1979, Gill *et al* 1979). The three essential sites are the two inverted repeat sequences (38 base pairs long) at the ends of the unit and the internal site (*res* or IRS - the internal resolution site) necessary for cointegrate resolution (reviewed by Kleckner 1981).

The third class of transposon is exemplied by Tn916. This group are collectively termed the conjugative transposons, units which apparently transfer by a conjugation process in the absence of detectable plasmid DNA (Franke and Clewell 1981).

The Transposition Event

Much of the work on the mechanism of transposition has been carried out with Tn3. The inverted repeat sequences are thought to act as recognition sequences for the transposase.

This enzyme cuts the target DNA molecules and joins them to form a cointegrate. In Tn3, the enzyme resolvase, resolves the cointegrate at a specific site (res), it also regulates its own synthesis and the synthesis of the transposase (reviewed by Kleckner 1981, Saunders 1984). Transposition cointegrates are resolved by host resolvases in some cases.

The phenomenon of transposition immunity (Robinson et al 1977) prevents intermolecular transposition into a replicon which already contains a copy of the transposon. Clustering of resistance determinants, perhaps in non-essential sites, is also observed. Transposons may thus show a regional specificity for insertion into DNA molecules. Transposon 3 inserts into regions rich in AT base pairs (Heffron et al 1975a) whereas Tn7 inserts into many plasmid sites but into a single chromosomal site in E.coli and many other bacteria (Lichtenstein and Brenner 1982).

Transcription of bla encoded by Transposons

Differences in the absolute levels of β-lactamase produced by various transposons has been explained by the efficiency of the transcription of the bla gene itself. This is a similar phenomenon to that described with E.coli amp C chromosomal mutations (Olson et al 1983) described earlier. Some bla genes have more efficient promoters and the evolutionary success of certain β-lactamases such as TEM-1 may have resulted from mutations of a promoter region, resulting

in enzyme hyperproduction rather than mutations in the structural region which would produce a qualitative alteration (Yamamoto et al 1982).

4.4 Gene Amplification

Amplification by Tandem Duplication

Tandem duplication is a method of elevating the numbers of gene copies - the gene dosage effect (Anderson and Roth 1977). Both chromosomal (Edlund et al 1979) and plasmid - borne determinants (Yagi and Clewell 1976, Schmitt et al 1979, Schoffl and Puhler 1979, Wiebauer et al 1981) can be amplified in this way.

Increased Plasmid Copy Number

Mutations in plasmid replication control genes can result in a large number of resistance plasmid (Nordström et al 1972, Tomizawa et al 1981, Light and Molin 1981). Plasmid copy mutants have been used to relate gene dosage effects to antimicrobial drug susceptibility (Uhlin and Nordström 1977).

4.5 Acquisition of Multiple Copies of a Transposon

Multiple transposition is distinct from tandem duplication and it has been observed for such transposons as TnA (Tn2660) in the R6K plasmid (Holmans and Clowes 1979). Transposition immunity (Robinson et al 1977) seems to apply to inter-molecular transposition and has little effect on intra-molecular transposition (Holmans and Clowes 1979, Wallace et al 1981).

4.6 Resistance Epidemiology

The promiscuity of transferable resistance genes has led to wide spread antimicrobial drug resistance. The clonal expansion of a bacterium carrying a resistance plasmid when selection pressure is applied has also made a large epidemiological impact (Rowe et al 1980, Hughes et al 1980). "Endemic plasmids" have also spread widely within bacterial populations and carried drug resistance into many bacterial genera (Ingram et al 1973, Witchitz and Chabbert 1972, Datta 1984).

Transposons have complicated and magnified the problems of the 1960s. About half of the transferable β-lactamases have been found to be mediated by transposons (table 2) (Yamamoto et al 1981, Medeiros et al 1982, Sinclair and Holloway 1982, Phillipon et al 1983, Yamamoto et al 1983, Kratz et al 1983, Jacoby personal communication). The evolutionary success of TnA encoding the TEM β-lactamase is well documented and is due to the promiscuity of the TEM transposons (Tn 1, 2, 3, 401, 801, 802, 901, 902, 1701, 2601, 2602, 2660) (Kleckner 1981). The spread of TEM-1 through the bacterial population will be discussed in a later section.

5. β-lactam Drug Resistance

There are at least eight possible biochemical mechanisms of drug resistance (Goldstein et al 1974).

1 - decreased intracellular drug level i.e. impermeability

2 - the destruction of the drug

3 - the decreased conversion of a drug to a more active form

4 - the increased concentration of a metabolite antagonising the drug action

5 - an increase in the level of target enzyme

6 - decreased requirements for the product of the drug sensitive reaction (hypothetical)

7 - a mechanism for by-passing or repairing the drug sensitive reaction

8 - decreased affinity of the receptor for the drug

Five of these mechanisms (1, 2, 5, 7, 8) are probably involved in β-lactam resistance and can be broadly divided into enzymic detoxification/destruction mechanisms (2) and mechanisms which do not involve enzymic destruction (1, 5, 7, 8).

5.1 Enzymic Destruction of β-lactam Antibiotics

β-lactam antibiotics are substrates for a number of hydrolytic enzymes. The enzyme described originally by Abraham and Chain (1940) as a "penicillinase" was isolated from E.coli a type of strain subsequently found to produce the enzyme penicillin amidase (penicillin amido hydrolase, E.C. 3.5.1.11.) (Batchelor et al 1961).. This enzyme has also been called an amino acid acylase and is quite distinct from the enzyme classically associated with the name penicillinase (Pollock 1971). Bacterial amidase enzymes act principally against penicillins, removing the acyl side chain to form aminopenicillanic acid (Batchelor et al 1961, Hamilton-Miller 1966). Although their contribution to bacterial resistance is unclear, their enzymic action has been exploited in the production of semi-synthetic penicillins (Batchelor et al 1959).

A second type of enzymic degradation involves the removal by acyl esterases* of the acetyl group attached at C-3 on the cephalosporin C nucleus. Certain semi-synthetic cephalosporins with an acetoxy methyl function at C-3, ie cephalothin and cefotaxime are also susceptible (Sykes and Smith 1979, Sykes 1982). The product of the reaction is a compound of reduced antibacterial activity.

* E.C. number not available

β-lactamases are the most important enzymic resistance mechanism, although not the only β-lactam degradative enzymes (Pollock 1971, Sykes and Matthew 1976, Sykes and Smith 1979, Sykes 1982). Originally they had been called penicillinases and they were given the name "penicillin amido β-lactam hydrolase" E.C. 3.5.2.6. (Pollock 1971). However, with the advent of new substrates such as the broad spectrum penicillins and the cephalosporins, enzyme characterisation improved and the cephalosporinase enzymes were recognised (Jago et al 1963, Fleming et al 1963, Ayliffe 1964, Hamilton-Miller et al 1965). In 1972, the enzyme commission included cephalosporinase (E.C. 3.4.2.8) as official nomenclature, describing penicillinase as β-lactamase I and cephalosporinase as β-lactamase II (Hamilton-Miller 1979). Recently however, these enzymes have been grouped under one heading (E.C. 3.5.2.6) as the "β-lactamase enzymes" (Enzyme Nomenclature 1984).

5.1.1 Classification of β-lactamase Enzymes Produced by Gram Negative Bacteria

Gram negative bacteria produce a wide variety of β-lactamase enzymes (Richmond and Sykes 1973, Sykes and Matthew 1976, Medeiros 1984). The enzymes may be inducible or constitutively produced and they are generally periplasmic or cell bound (Richmond and Sykes 1973). There are many classification schemes for β-lactamase enzymes. The earliest

relied on the substrate profile or specificity of the enzyme as the primary parameter and included additional factors such as enzyme inhibition profiles and cross-reaction to antisera for finer discrimination (Jack and Richmond 1970).

Richmond and Sykes (1973) elaborated this technique and described five broad enzyme classes based on substrate profile and inhibition studies.

Class I enzymes are the chromosomal cephalosporinases of Gram-negative bacteria which are competitively inhibited by cloxacillin and carbenicillin.

Class II enzymes are broad spectrum penicillinases which are competitively inhibited by cloxacillin but not carbenicillin.

Class III enzymes are broad spectrum and are competitively inhibited by cloxacillin but not carbenicillin or *para*-chloromecuribenzoic acid (*p*CMB). They comprise the plasmid-mediated TEM-type β-lactamases.

Class IV enzymes are broad spectrum chromosomal enzymes and are sensitive to *p*CMB.

Class V is a heterogenous grouping of penicillinase enzymes which are resistant to inhibition of *p*CMB and cloxacillin. They include the oxacillin and carbenicillin hydrolysing plasmid mediated β-lactamase enzymes.

(Richmond and Sykes 1973)

Sykes and Matthew (1976) later described how enzyme substrate profile, inhibitor studies, immunological studies, molecular weight determination and the genetic location of β-lactamase genes could all be used as a means of classification. These parameters were combined with the isoelectric point (pI) of the β-lactamase enzyme to create a classification index.

The use of analytical isoelectric focusing (IEF) developed by Matthew et al (1975) enables the identification and classification of β-lactamases in large numbers of clinical bacteria (Simpson et al 1980, Roy et al 1983). Each transferable β-lactamase has a unique pI and characteristic banding pattern on IEF (Matthew and Harris 1976). IEF however, does not reveal any similarities between β-lactamase enzymes.

The use of substrate profile for enzyme classification has been criticised as collecting information about the Vmax of the enzyme for the substrate but little about the affinity (Km). In the classification of new β-lactamase enzymes a direct comparison of hydrolysis of substrates is probably best made using relative efficiency of hydrolysis as described by Sykes et al (1981b).

The efficiency of hydrolysis = $\frac{\text{Vmax per unit enzyme}}{\text{Km}}$

The relative efficiency may be normalised with respect to that of penicillin G (Sykes et al 1981b).

The most recent classification system has evolved from the methodologies of amino acid and nucleotide sequencing. Three evolutionary distinct classes of β-lactamase enzyme have been recognised (Ambler 1980, Bergström et al 1982): Class A have an active site serine residue and are around 29,000 daltons in size. They are principally penicillinase enzymes with significant amino acid sequence homology. The group includes the TEM-type β-lactamases and the penicillinases of S.aureus and B.licheniformis. Class B is restricted to the 23,000 dalton metallo-thio cephalosporinase of B.cereus type II. Class C β-lactamase enzymes are large proteins (around 39,000 daltons) and they include the chromosomal cephalosporinases of Gram negative bacteria. They show little sequence homology with Class A enzymes although they both have an active site serine residue (Bergström et al 1982, Jaurin and Grundstrom 1981, Knott-Hunziger et al 1982). It is thought that the similarity of the active sites of class A and class C enzymes may result from convergent solution (Knott-Hunziger et al 1982).

5.1.2 β-lactamase Enzymes Mediated By Chromosomal Genes

Chromosomal β-lactamase enzymes are generally specific for species and, sometimes, for subspecies (Matthew and Harris 1976). The chromosomal enzymes of Richmond and Sykes (1973) class I are often inducible.

Recent studies have shown that many of the newly introduced β-lactams are capable of inducing high levels of β-lactamase production in a variety of Enterobacteriacae (Minami et al 1980, Sanders et al 1982, Sykes and Bush 1982, Gootz and Sanders 1983). There have also been reports of induction by penicillins (Minami et al 1983). Occasionally, new forms of the β-lactamase enzyme are seen after induction and it is unclear whether these are new enzymes or a modified form of the native enzyme (Sanders et al 1982).

Chromosomal β-lactamase enzymes have now been found which hydrolyse most classes of β-lactam antibiotic. Even the extremely stable carbapenem thienamycin is hydrolysed by an enzyme produced by P.maltophilia (Saino et al 1982).

5.1.3 β-lactamase Enzymes Mediated By Plasmids and Transposons

The transferable β-lactamase enzymes are produced constitutively and generally in greater quantities than the chromosomal β-lactamases (Matthew 1979).

Twenty eight plasmid mediated β-lactamases have been described so far (Table 2). They can be broadly subdivided into four classes, the broad spectrum enzymes, the oxacillinases, the carbenicillinases and the cephalosporinase enzymes (Matthew 1979, Medeiros 1984). Within the classes there can be a degree of immunological cross-reactivity (Matthew 1979, Holland and Dale 1985).

Some transferable β-lactamase enzymes hydrolyse the newer β-lactam antibiotics. Several of the OXA and PSE enzymes have been shown to have hydrolytic activity against "β-lactamase stable" compounds, albeit small (Simpson et al 1982, Medeiros 1984).

Knothe et al (1983) have reported a transferable β-lactamase which conferred resistance to second generation cephalosporins and cefotaxime. The enzyme, named SHV-2, is thought to have evolved from SHV-1 by mutations in at least two base pairs, subtly cha nging the β-lactamase enzyme's hydrolytic properties (Kliebe et al 1985).

5.1.4 Distribution of Plasmid-Mediated β-lactamase Enzymes in Clinical Bacteria

The location of β-lactamase genes on transferable elements has led to ubiquitous spread of some types of β-lactamases throughout the world. This has been particularly evident with the TEM-1 and TEM-2 β-lactamase enzymes.

Table 2 The Transferable β-lactamase Enzymes

	Relative Rates of Hydrolysis*							Inhibitors			Molecular Size (k daltons)	Isoelectric Point	Transposon Mediated	Reference
	AMP	CARB	OXA	METH	CLOX	CER	CTX	CLOX	pCMB	CLAV				
Broad spectrum Enzymes														
TEM-1	106	10	5	0	0	76	<1	+	-	+	22	5.4	+	Hedges et al (1974)
TEM-2	107	10	5	0	0	74	0	+	-	+	23.5	5.6	+	Hedges et al (1974)
SHV-1	212	8	0	<2	<2	56	0	+	+/-	+	17	7.6	+	Petrocheilou et al (1977)
SHV-2	145	ND	0	ND	ND	32	4	+	+/-	+	17	7.6	+	Kliebe et al (1985)
HMS-1	253	14	<2	<2	<2	183	ND	+	+	-	21	5.2		Matthew et al (1979)
TLE-1	67	13	4	5	6	52	6	+	ND	+	19.8	5.55		Medeiros et al (1985)
TLE-2	140	13	ND	<0.1	<0.1	NM	ND	+	+	+	19	6.5		This Thesis
ROB-1	186	25	6	ND	ND	24	ND	ND	ND	ND	ND	8.1		Rubin et al (1981)
LCR-1	145	4	ND	20	3	55	ND	+	-	+	44	6.5(5.85)		Simpson et al (1983)
NPS-1	223	18	40	<0.1	ND	3	<1	-	-	ND	25	6.5		Livermore and Jones (1986)
BRO-1	103	78	ND	131	5	23	ND	+	ND	+	ND	5.6		Eliasson and Kamme (1985)
Oxacillinases														
OXA-1	382	30	197	332	190	30	22	+	+/-	ND	23.3	7.4	+	Dale and Smith (1974)
OXA-2	179	15	646	23	200	37	1	-	-	-	44.6	7.45,7.7	+	Dale and Smith (1974)
OXA-3	178	10	336	29	350	44	0	-	-	ND	41.2	7.1		Dale and Smith (1974)
OXA-4	438	39	220	711	64	194	63	+/-	ND	-	23	7.5	+	Medeiros et al (1985)
OXA-5	188	40	210	109	258	89	49	-	ND	-	27	7.62	+	Medeiros et al (1985)
OXA-6	596	46	1048	585	301	149	28	+	ND	-	40	7.8	+	Medeiros et al (1985)
OXA-7	545	48	702	424	494	136	31	-	ND	-	25	7.65		Medeiros et al (1985)
Carbenicillinases														
PSE-1	90	97	<2	<2	<2	18	27	-	+	-	28.5	5.7	+	Matthew and Sykes (1977)
PSE-2	267	121	317	803	371	32	16	ND	+	-	12.4	6.1		Matthew (1978)
PSE-3	101	253	ND	ND	3	10	<1	ND	-	-	12	6.9		Sawada et al (1974)
PSE-4	88	150	8	16	<2	40	1	ND	-	-	32	5.3	+	Furth (1975)
CARB-4	130	79	1	<1	<1	18	ND	-	+	-	22	4.3	+	Philippon et al (1986)
SAR-1	63	122	<0.1	<0.1	<0.1	21	<0.1	+	-	+	33.7	4.9		Reid and Amyes (1986)
AER-1	38	98	0.9	0.3	0	26	20	ND	ND	ND	22	5.9	+	Hedges et al (1985)
N-3	113	113	ND	ND	<2	12	ND	+	ND	ND	24	6.0		Takahashi et al (1983)
N-29	124	128	ND	ND	<2	6	ND	+	ND	ND	22	6.9		Takahashi et al (1983)
Cephalosporinase														
CEP-2	ND	48	ND	ND	ND	108	ND	-	-	ND	36.2	8.1		Levesque et al (1982)

*Benzyl penicillin 100%

Abbreviations

AMP, Ampicillin
CARB, Carbenicillin
OXA, Oxacillin
METH, Methicillin
CLOX, Cloxacillin
ND, Not done
CER, Cephaloridine
CTX, Cefotaxime
CLOX, Cloxacillin
pCMB, p-chlormericuribenzoic acid
CLAV, Clavulanic acid
NM, Not measurable

This enzyme which is common amongst the Enterobacteri aceae, has been found on plasmids from most incompatibility groups thus facilitating its spread (Hedges et al 1974, Heffron et al 1975b). Ampicillin is widely used in the treatment of H.influenzae infections and in 1974 ampicillin resistant H.influenzae were identified (Thomas et al 1974). The strains produced TEM-1 plasmid-borne β-lactamase (Elwell et al 1975). One H.influenzae strain was found to carry one-third of the Tn3 sequence on a plasmid (DeGraaff et al 1976). It has been proposed that this transposon (Tn2) may have evolved from Tn3 by deletion and subsequent acquisition by cryptic plasmids indigenous to H.influenzae (Laufs and Kaulfers 1977, Kaulfers et al 1978).

Treatment of gonococcal infections also relied heavily on penicillin and TEM-1 producing N.gonorrhoeae were isolated in 1976 (Percival et al 1976, Ashford et al 1976). Here too, all or part of Tn3 is found in TEM-1 producing strains. It has been shown that two β-lactamase plasmids from gonococci show homology with an R factor from H.influenzae (Elwell et al 1977. It is possible that ampicillin resistance plasmids were acquired by gonococci from H.influenzae or that both acquired similar plasmids from an enterobacterial source.

More recently there has been a report that a strain of N.meningitidis has been isolated which produces the TEM-1

β-lactamase. However this claim is unsubstantiated (Dillon et al 1983).

Transferable ampicillin resistance in Vibrio cholerae emerged in the 1970s (Rahal et al 1973, Hedges et al 1977, Threlfall et al 1980) and the TEM-1 β-lactamase enzyme was identified (Hedges et al 1977). A novel β-lactamase has since been identified in V.cholerae (SAR-1) (Reid and Amyes 1986) a parallel to the discovery of ROB-1 in H.influenzae (Rubin et al 1981). These two genera only produce TEM-1 and their respective novel β-lactamase.

The remaining plasmid encoded β-lactamase enzymes have different distributions amongst the bacterial genera (Matthew 1979, Simpson et al 1980, Roy et al 1983). In contrast to TEM-1 another broad spectrum enzyme, HMS-1, is extremely rare having been found only once in an isolate of P.mirabilis (Matthew et al 1979). The distribution of the enzymes within bacterial genera also differs. In E.coli TEM-1 is the most prevalent in the United Kingdom and Spain (Simpson et al 1980, Roy et al 1983), whilst in P.aeruginosa in France, PSE-1 occurs most frequently (Phillipon et al, personal communication in Medeiros 1984). In the United Kingdom PSE-4 is the most common transferable β-lactamase found in P.aeruginosa (Williams et al 1984).

5.1.5 Mode of Action of β-lactamase Enzymes

The TEM-1 β-lactamase is the best studied enzyme in mechanistic terms and a general reaction scheme has been outlined:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} E.S. \xrightarrow{K_2} E\text{-}S \xrightarrow{K_3} E + P$$

E, Enzyme
S, Substrate
P, Hydrolysed Substrate

Figure 8 Action of β-lactamase Enzymes
(Taken from Bush and Sykes 1984)

For the β-lactamase enzymes examined so far the first step has been found to equilibrate rapidly (Bush and Sykes 1984). The formation of a covalent acyl enzyme intermediate from the E.S. complex has been shown for the TEM β-lactamase (Fisher et al 1980) and some chromosomal β-lactamases (Knott-Hunzi ger et al 1980, Anderson and Pratt 1981, Cartwright and Fink 1982). The deacylation of E-S yields free enzyme and a β-lactam with an open β-lactam ring. For most substrates for the deacylation step is rate determining (Frère 1981).

After the opening of the β-lactam ring, the penicillins further break down to penicillanic acid (Newton et al 1967). The cephalosporins may also undergo spontaneous degradation and fragmentation because of the conjugated ring system and the substituent at the C-3 position of the dihydrothiazine

ring renders the hydrolysed molecule unstable (Hamilton-Miller et al 1970, Sykes and Smith 1979).

β-lactamase enzymes are "floppy" enzymes and lack rigid tertiary structure. They satisfy the requirements of the induced fit model of enzyme action (Koshland 1970).

Acyl intermediates of class C enzymes have also been found with P.aeruginosa and E.coli K12 (Knott-Hunziger et al 1980, 1982). The labelled serine residues from these β-lactamase enzymes were found in peptides which differed from those of the class A series (Knott-Hunziger et al 1982).

5.1.6 Contribution of the β-lactamase Enzyme to β-lactam Resistance

The extrapolation of β-lactamase enzyme in vitro properties to the in vivo function of the enzyme is difficult. The in vitro testing of antimicrobial susceptibility and the measurement of the enzyme activity all require careful standardisation (Pollock 1971).

The levels of β-lactam resistance of Gram negative bacilli often do not correlate with the rates at which these antibiotics are hydrolysed by the cell-free β-lactamase enzyme (Percival et al 1963, Richmond and Sykes 1973, Medeiros et al 1974). The permeability of a cell and the susceptibility of its target site(s) both interact with the β-lactamase to determine the cell's resistance to β-lactam antibiotics. If a β-lactamase is to compete successfully

with the PBP target site for the incoming β-lactam antibiotic then it should have a higher affinity for the β-lactam (Bush and Sykes 1984). Many PBPs however are extremely susceptible to β-lactams at levels of 0.5μM or less (Curtis et al 1979, Georgopapadakou and Liu 1980) and thus the β-lactamase would not be effective as the sole resistance mechanism.

5.1.7 Non-Hydrolytic "Trapping" of β-lactam Antibiotics By β-lactamase Enzymes

Treatment failures have occurred during therapy with a variety of β-lactamase stable β-lactam antibiotics. These failures result from the emergence of resistant strains including E.cloacae, Citrobacter sp., P.aeruginosa and S.marcescens (Sugarman and Pesanti 1980, Preheim et al 1982, Olsson et al 1983). All the strains had a derepressed constitutive chromosomal β-lactamase enzyme which result from mutations in the regulatory genes controlling β-lactamase expression (Gootz et al 1982). The strains were also found to be resistant to a variety of third generation β-lactam antibiotics (Nikaido 1985).

Class I enzymes (Richmond and Sykes 1973) were found to have a high affinity for virtually all the newer cephalosporins and it was suggested that the β-lactamase enzyme was unable to hydrolyse the β-lactam but bound to it and "trapped" the compound thereby preventing it from reaching its target site (Yokota and Azuma 1980, Then and Angehrn 1982, Sanders 1983).

This argument has been opposed in the belief that the amount of enzyme needed to trap the incoming β-lactam would necessarily be large (Seeberg et al 1983). However, most of the β-lactams penetrate the outer membrane slowly and the amount of enzyme present in constitutively derepressed cells is relatively high (Nikaido 1985). The bacteria remained sensitive to thienamycin probably because it penetrates the Gram negative outer membrane very efficiently and has high affinity for PBP2 which is present in small numbers in the cell (Spratt 1975, MSD Product Monograph 1985).

The most stable β-lactams may, in fact, be slowly hydrolysed by some β-lactamase enzymes (Livermore 1985). Changes in the methods of assay have reduced the amount of substrate present and increased the amount of enzyme. Therefore it is possible that "periplasmic detoxification" is efficient in vivo (Bush et al 1985, Livermore 1985, Sanders and Sanders 1986).

Transfer of the E.cloacae β-lactamase (bla) genes into the standard genetic background of E.coli K12 resulted in the strain becoming resistant to β-lactamase stable cephalosporins and demonstrated that resistance was not a strain property because E.coli K12 is permeable to third generation cephalosporins (Seeberg et al 1983). This result supported the "trapping" theory.

Livermore (1985) contests this theory and cites the covalent linkage of clavulanic acid with the TEM β-lactamase (Charnas et al 1978). The minimum inhibitory concentrations of clavulanate are not changed with the introduction of plasmids coding for TEM into the E.coli strain. as would be expected if "trapping" were taking place. Livermore (1985) argues that the results of Seeberg et al (1983) could be explained if slow hydrolysis of the incoming β-lactam antibiotics were taking place, and thus "trapping" is not a valid hypothesis.

It is possible that both trapping and slow hydrolysis occur in vivo and their roles may be complementary as derepression of the β-lactamase results in large amounts of the enzyme in the periplasmic space.

The mechanics of β-lactamase/β-lactam interaction are summarised in Figure 8.

Good substrates will be found as products of the reaction (P). Slowly hydrolysed substrates will remain primarily in the E.S complex, the condition postulated by the "trapping" theory. Some non-substrate drugs such as moxalactam may fulfill the trapping conditions entirely by remaining as E.S (Bush et al 1982, Gwynn and Rolinson 1983 and Sanders and Sanders 1986).

β-lactamase stability which once seemed an important attribute for β-lactam antibiotics is perhaps now less clear-cut. Future drug design rationale appears to require a compound which is stable to β-lactamase enzymes, but which is smaller in size and penetrates the cell wall rapidly eg thienamycin (Kropp et al,1980 Nikaido 1985).

5.2 Resistance Mechanisms to β-lactam Antibiotics Which Do Not Involve Enzymic Destruction

There are a variety of possible resistance mechanisms to β-lactam antibiotics which do not involve enzymic degradation. These include permeability changes, altering the amount of target enzyme, and decreasing the affinity of the target for the β-lactam (Goldstein et al 1974).

5.2.1 Reductions in Outer Membrane Permeability

It has been demonstrated that the loss of porin proteins results in β-lactam resistance in E.coli (Bavoil et al 1977, Harder et al 1981), in Salmonella (Nikaido et al 1977) P.aeruginosa (Nicas and Hancock 1983), P.mirabilis and E.cloacae (Sawai et al 1982). Changes in outer membrane proteins can also result in resistance to other types of antimicrobial agent eg tetracyclines (Foulds 1976) and chloramphenicol (Burns et al 1985).

The genetic basis of permeability resistance in E.coli K12 is the alteration in production of OmpF and/or OmpC porin at the OmpB regulatory gene locus (Jaffe et al 1982). Mutants lacking more permeable OmpF porin are more resistant to hydrophobic or dianionic compounds which have difficulty in penetrating the narrower OmpC Channel (Harder et al 1981). Resistance to latamoxef has also been shown to result from changes in E.coli outer membrane proteins (Komatsu et al 1981). It appears retrospectively, that the observed change was a decrease in the amount of OmpF protein specifically.

Porin protein production may be repressed or induced by varying salt or sucrose concentrations in growth medium (van Alphen and Lugtenberg 1977). Resistance in clinical isolates may therefore result from phenotypic rather than mutational alterations in porin composition.

β-lactam resistance resulting from permeability changes has been regarded as generally being chromosomally mediated. Curtis and colleagues however described a mutant of the RP1 plasmid (RP1 amp-1) which specified ampicillin resistance in the apparent absence of detectable β-lactamase activity (Curtis et al 1973, Curtis and Richmond 1974) The locus determining the resistance was called irp (intrinsic resistance to penicillins). The RP1 plasmid was found to

protect P.aeruginosa from the lytic action of EDTA and polymyxin B, as well as from the effects of cold shock (Kenward et al 1978). Shipley and Olsen (1974) had described how the tetracycline resistance loci of RP1 may change the permeability of bacteria carrying the plasmid. They postulated that a product of RP1 may insert into the cell membrane blocking the normal channels or altering the electrostatic properties of the cell surface (Shipley and Olsen 1974). Such a mechanism may affect the penetration of β-lactams as well as tetracyclines. Other plasmids - R1818 and RTEM were also found to affect the gross physical properties of the P.mirabilis cell envelope. In this case increasing the sensitivity of the cells to deoxycholate (Hesslewood and Smith 1974).

The RP1 amp-1 description was picked up by some workers as a plasmid-encoded permeability barrier to β-lactams (Ikeuchi and Osada 1981). It is believed however that Curtis and colleagues (1973, 1974) overlooked a basal level of β-lactamase production by RP1 amp-1 which was responsble for the residual ampicillin resistance (Crowlesmith and Howe 1980a,b).

Other resistance plasmids have also been reported to have an effect on the outer membrane of E. coli and produce β-lactam resistance (Iyer et al 1978, Rossouw and Rowbury 1984). In one study, the plasmid only produced resistance in one type of strain - E. coli B/r and not in the E. coli K12 strains (Iyer et al 1978).

Irvine et al (1981) have also reported that hypersensitivity to β-lactam antibiotics can also result from changes in outer membrane proteins.

5.2.2 Lipopolysaccharide Changes Associated with Resistance to β-lactams

Changes in lipopolysaccharide (LPS) have been most fully studied in S.typhimurium (Sanderson et al 1974, Koplow and Goldfine 1974, Ames et al 1974, Nikaido 1976, Prehm et al 1976, Janzer et al 1981). Alterations in the LPS could potentially produce changes in sensitivity to antibiotics. Although LPS mutants show increased susceptibility (Tamaki et al 1971 Coleman and Leive 1979), mutations correlating with β-lactam resistance have been reported in P.aeruginosa (Godfrey et al 1984). Two of the mutants differentiated between β-lactams on the basis of relative hydrophobicity (hydrophilic β-lactams were less effective). As no outer membrane protein changes were seen in these two strains, it was postulated that LPS may have an interaction with the porin channels themselves and it is therefore difficult to separate changes in LPS from the possible affects on the porins (Angus et al 1982).

Changes in whole cell lipid content may also alter β-lactam susceptibility (Dunnick and O'Leary 1970, Legaskis et al 1978, Lysko and Morse 1981). Increases in the relative concentrations of lipid have been reported to produce

resistance to methicillin, cloxacillin and penicillin G in S.aureus, S.faecalis and B.subtilis (Hugo and Stetton 1966). β-lactam resistance in S.marsescens was also attributed to an increase in extractable lipid and phospholipid (Miller et al 1973).

5.2.3 Alterations in Penicillin Binding Proteins

Decrease in the amount of PBP or the affinity of PBP(s) for β-lactam antibiotics can result in a significant degree of resistance to these compounds (Park et al 1971). Spratt (1978) isolated E.coli mutants with reduced binding to PBP2. Such strains are resistant to mecillinam and thienamycin which have been shown to have a high affinity for PBP2. (Spratt 1978, M.S.D. Product Monograph 1985). PBP mutants have also been isolated from other species (Curtis et al 1978, Barbour 1981, Hartman and Tomasz 1981, Kleppe et al 1982).

Such mutants have also been found amongst clinical strains. In cystic fibrosis patients β-lactam resistant P.aeruginosa emerged with modified PBPs (Godfrey et al 1981). Changes in PBPs associated with ampicillin resistance have also been reported in H.influenzae (Parr and Bryan 1984, Mendelman et al 1984). PBP alterations have also been reported in Gram positive cocci (Hakenbeck et al 1980, Georgepapadakou et al 1982).

β-lactam resistance resulting from alterations in PBPs are most common in bacteria which produce little β-lactamase, or have only recently acquired plasmid-mediated β-lactamases e.g. S.pneumoniae (Hakenbeck et al 1980, Fontana et al 1983), N.gonorrhoeae (Dougherty et al 1980) and H.influenzae (Parr and Bryan 1984, Mendelman et al 1984). β-lactamase stable antibiotics have been used to treat S.aureus infections for some time and alterations in PBPs are an important resistance mechanism (Georgopapadokou et al 1982, Hartman and Tomasz 1984). The use of β-lactamase stable β-lactams in the treatment of Gram negative bacterial infections may result in a concomitant increase in PBP mutants amongst this population.

Many of the newer compounds show a high affinity for PBP3 in E. coli and therefore low level resistance could arise by mutation in this one PBP (Spratt 1983). However, other β-lactams bind to more than one essential PBP and, therefore, resistance would require a decrease in the affinity of each of these essential PBPs. Such mutations, which produce a generalised reduction in PBP binding have been reported (Mackenzie et al 1980, Godfrey and Bryan 1982). In S.aureus it is thought that further mutations must occur to allow the organism to survive with only one enzymically active PBP (Spratt 1983).

Multiple mutational events can confer higher levels of B-lactam resistance eg N.gonorrhoeae (Dougherty et al 1980).

Hyperproduction of the susceptible PBP target site may also be a B-lactam resistance mechanism. High level resistance in P.aeruginosa has been reported associated with an increase in the levels of production of PBP6, a non-essential PBP (Godfrey et al 1981).

As with permeability mutations, PBP alterations may also result in increased susceptibility to β-lactam antibiotics. The mutation in this case results in an enzyme with increased binding affinity for the β-lactam (Rodriguez and Saz 1975, Noguchi et al 1980).

5.2.4 Appearance of Novel PBPs (A New Insusceptible Target Site)

Enterococci and S.aureus may synthesize a new PBP which can take over the functions of all the other PBPs. This novel PBP then becomes essential for growth. The enzyme has a lower affinity for B-lactams, especially methicillin and the cephalosporins, and the cell becomes β-lactam resistance by virtue of this insusceptible target site (Fontana 1985).

This low affinity PBP (PBP2') is inducible and an increase in the amount of protein correlates with an increase in resistance in S.pneumoniae (Fontana et al 1983) and S.aureus (Hartman and Tomasz 1984). In S.aureus the expression of methicillin resistance in influenced greatly by growth conditions. The low-affinity PBP being best expressed at 30°C and low pH (Sabath 1977). β-lactam susceptible cells do not have any detectable low-affinity PBP even under the above

conditions (Brown and Reynolds 1980, Hartman and Tomasz 1984).

This inducible low-affinity PBP is not essential for normal growth and therefore a new approach may be required in order to direct β-lactams at non-essential low-affinity targets. It is thought that PBP2' may be a detour enzyme of PBP2 and/or PBP3 (Ubukata et al 1985) such a resistance mechanism may also develop in Gram negative bacteria.

Transferable PBP resistance mutations have not been reported however it is possible that the flanking of a chromosomal gene by insertion sequences may result in such an event (Spratt 1983).

5.2.5 Tolerance to β-lactam Antibiotics

Tolerant organisms appear to be "defective" in their production or the action of cellular autolytic enzymes (Horne and Tomasz 1977, Kitano and Tomasz 1979a,b Bergeron and Lavoie 1985). E.coli PBP1, has been implicated in triggering the autolytic system (Kitano and Tomasz 1979b) and therefore changes in PBPs may affect tolerance as well as direct susceptibility.

There have been reports of treatment failures, resulting, from tolerant organisms, which support the clinical significance of this resistance mechanism (Broughton et al 1976, Feldman 1976, Walker et al 1976, Brennan and Durack 1983).

The Aims of This Thesis

1. To identify the principal resistance mechanisms to β-lactam antibiotics in a population of aerobic, clinical, Gram negative bacteria.

2. To identify the β-lactamases present within this population.

3. The characterization of novel, transferable β-lactam resistance mechanisms.

4. To establish the principal mechanisms of resistance in an ampicillin resistant *H. influenzae* population.

5. To determine the role of the plasmid-mediated β-lactamases in β-lactam resistance.

II. MATERIALS AND METHODS

1. Bacterial Strains

The Gram negative aerobic clinical strains used in this thesis were all collected during the period 1983/84. All strains were maintained at -70°C in nutrient broth with 10% glycol v/v. Details of the individual strains are given in the appendix.

The standard bacterial strains used in this thesis are listed in Table 3, and standard bacterial plasmids and bacteriophages are detailed in Table 4. The standard β-lactamase producing strains are listed in Table 5.

2. Information Storage and Retrieval

Information concerning each bacterial strain was recorded on a computer data form and stored on the database program dBASE III (Ashton-Tate, Milton Keynes). The database system was used for the identification and removal from the survey of repeat isolates from individual patients.

3. Materials

3.1 Antimicrobial Agents

The antimicrobial agents and their suppliers are listed in Table 6. Nitrocefin (87/312) was provided by Glaxo Group Research Ltd. All were supplied sterile. β-lactams were prepared by aseptically adding sterile sodium phosphate

Table 3

Standard Bacterial Strains

Bacterial Strain	Markers	Reference
E. coli K12 J62	*pro*⁻*his*⁻*trp*⁻*lac*⁻	Bachmann (1972)
E. coli K12 J62-$_1$	*pro*⁻*his*⁻*trp*⁻*lac*⁻Na^R	Bachmann (1972)
E. coli K12 J62-$_2$	*pro*⁻*his*⁻*trp*⁻*lac*⁻Rf^R	Bachmann (1972)
E. coli K12 J53	*pro*⁻*met*⁻	Bachmann (1972)
E. coli 114	prototroph	Amyes (1974)
E. coli K12 PB1150	*his*⁻*recA* Sm^R Na^R	Given by Professor N. Datta
E. coli NCTC 10418	prototroph	
P. aeruginosa PA08	*met*⁻*ilv*⁻	Isaac and Holloway (1968)
P. aeruginosa PA0381	*leu*⁻ Sm^R	Stanisich and Holloway (1969)
P. aeruginosa PA01670	*ade*⁻*leu*⁻ Rf^R	Mills and Holloway (1976)
P. aeruginosa Pu21	*ilv*⁻*leu*⁻ Rf^{R}Sm^R	Jacoby (1974)
P. aeruginosa NCTC 10662	prototroph	
H. influenzae 1184E	prototroph	Glaxo Group Research Culture

Table 4

Standard Bacterial Plasmids and Phage

Plasmid designation	Markers	(kb)	Reference
R1	Ap Cm Km Sm Su incF$_{II}$	90	Meynell and Datta (1966)
RP4	Ap Km Tc incP-1	52	Datta et al (1971)
R6K	Ap Sm incX	38	Kontamichalou et al (1970)
R1010-6	Ap (tra$^-$) incN	54	Nugent and Hedges (1979)
Sa	Cm Km Sm Su Sp incW	33	Watanabe et al (1968)
R751	Tp incP-1	48	Jobanputra and Datta (1974)
X$^+$		61	Shar ma et al (1984)
R18	Ap Km Tc incP-1	60	Stanisich (1974)
R18-18[a]	Km Tc incP-1	57	Stanisich et al (1976)
Phage			
PR4	Specific for incP, N and W		Stanisich (1974)
PRR1	Specific for incP		Stanisich (1974)

[a]Carbenicillin/ampicillin sensitive derivative of R18 (materials and methods)

Table 5

Standard β-lactamase Producing Strains

Bacterial Strains	Plasmid or transposon	β-lactamase produced	Reference
E. coli J53	R6K (Tn2660)	TEM-1	Hedges et al (1974)
E. coli J53	RP4 (Tn1)	TEM-2	Hedges et al (1974)
E. coli J53	R1010-6	SHV-1	Petrocheilou et al (1977)
E. coli 7604	pMG204b	TLE-1	Medeiros et al (1985)
P. mirabilis	R997	HMS-1	Matthew et al (1979)
P. aeruginosa Pu21	pMG76	LCR-1	Simpson et al (1983)
P. aeruginosa Pu21	302	NPS-1	Livermore and Jones (1986)
A. hydrophila[a]	Tn798	AER-1	Hedges et al (1985)
B. catarrhalis[b]		BRO-1	Eliasson and Kamme (1985)
H. influenzae	R_{ROB}	ROB-1	Rubin et al (1981)
P. mirabilis N-3	pCS203	N-3	Takahashi et al (1983)
P. mirabilis N-29	pCS229	N-29	Takahashi et al (1983)
E. coli J53	R455 (Tn2603)	OXA-1	Dale and Smith (1974)
E. coli J53	R46-T^S	OXA-2	Dale and Smith (1974)
E. coli J53	R57b	OXA-3	Medeiros et al (1985)
E. coli 7529	pMG203	OXA-4	Medeiros et al (1985)
P. aeruginosa	pMG54	OXA-5	Medeiros et al (1985)
P. aeruginosa	pMG329	OXA-6	Medeiros et al (1985)
E. coli	pMG202	OXA-7	Medeiros et al (1985)

P. aeruginosa/...

Table 5 (continued)

Standard ß-lactamase Producing Strains

Bacterial Strains	Plasmid or transposon	ß-lactamase produced	Reference
P. aeruginosa Pu21	RPL11	PSE-1	Matthew and Sykes (1977)
P. aeruginosa Pu21	R151	PSE-2	Matthew (1978)
P. aeruginosa Pu21	Rms149	PSE-3	Sawada *et al* (1974)
P. aeruginosa Dalgleish	pMG19	PSE-4	Furth (1975)
E. coli D31	R22Ka	CEP-1	Bobrowski *et al* (1976)
Alcaligenes sp.	pLQ3	CEP-2	Levesque *et al* (1982)
K. pneumoniae K1		K1	Marshall *et al* (1972)
K. pneumoniae K14		K14	Matthew and Harris (1976)
E. cloacae Hennessey		Enzyme A	Hennessey (1967)
C. diversus 2046E		2064E	Simpson and Plested (1983)
E. cloacae P99		P99	Fleming *et al* (1963)

[a] A = *Aeromonas*

[b] B = *Branhamella*

Table 6

Antimicrobial Agents

Compound	Supplier
Chemotherapeutic Agents	
Ampicillin	Beecham Research Laboratories, Middlesex
Aztreonam	E.R. Squibb and Sons, Middlesex
Benzyl penicillin	Glaxo Laboratories Ltd., Greenford, Middlesex
Carbenicillin	Beecham Research Laboratories, Middlesex
Cefamandole	Dista Products Ltd., Basingstoke
Cefazolin	Eli Lilly and Co. Ltd., Basingstoke
Cefotaxime	Roussel Laboratories Ltd., Middlesex
Cefotetan	I.C.I. Ltd., Macclesfield
Cefoxitin	Merck, Sharp and Dohme Ltd., Herts
Cefsulodin	Ciba-Geigy Laboratories, Horsham
Ceftazidime	Glaxo Laboratories Ltd., Greenford, Middlesex
Ceftriaxone	Roche Products Ltd., Herts
Cefuroxime	Glaxo Laboratories Ltd., Greenford, Middlesex
Cephaloridine	Glaxo Laboratories Ltd., Greenford, Middlesex
Cephradine	E.R. Squibb and Sons, Middlesex
Chloramphenicol	Parke-Davis, Pontypool, Gwent
Clavulanic Acid	Glaxo Group Research Ltd., Greenford, Middlesex
Cloxacillin	Beecham Research Laboratories, Middlesex

Colistin/...

Table 6 (continued)

Antimicrobial Agents

Compound	Supplier
Chemotherapeutic Agents	
Colistin	Pharmax Ltd., Bexley, Kent
Gentamicin	Roussel Laboratories Ltd., Middlesex
Kanamycin	Bristol Laboratories, Middlesex
Methicillin	Beecham Research Laboratories, Middlesex
Moxalactam	Eli Lilly and Co. Ltd., Basingstoke
Nalidixic Acid	Sterling Winthrop Laboratories, Surrey
Norfloxacin	Merck, Sharp and Dohme Ltd., Herts
Oxytetracycline hydrochloride	Glaxo Laboratories Ltd., Greenford, Middlesex
Piperacillin	Lederle Laboratories Division, Hants
Rifampicin	Le Petite, Milan, Italy
Spectinomycin	Upjohn Ltd., Crawley, Sussex
Streptomycin	Glaxo Laboratories Ltd., Greenford, Middlesex
Sulphamethoxazole	Wellcome Foundation Ltd., Kent
Trimethoprim	Wellcome Foundation Ltd., Kent
Non Chemotherapeutic Agents	
Nitrocefin	Glaxo Group Research Ltd., Greenford, Middlesex
Mercuric chloride	Sigma Chemical Co., Ltd., London

All antimicrobial concentrations are expressed in terms of the base.

buffer (pH 7.0, 50mM). Preparations of sulphamethoxazole, nalidixic acid and norfloxacin were made by dissolving the compound in N/10 NaOH and then the solution was made up aseptically with sterile distilled water. Solutions of rifampicin and chloramphenicol were made by first dissolving these drugs in a minimum volume of absolute ethanol and then the solution was made up with distilled water. Nitrocefin was dissolved with the aid of dimethyl sulphoxide (DMSO) (AnalaR, BDH Chemicals Ltd., Poole) and made up in sodium phosphate buffer (pH 7.0, 50mM). All other compounds listed were dissolved directly in sterile distilled water. Compounds, with the exception of nitrocefin were made up freshly as required; nitrocefin stock solution ($500mgL^{-1}$) could be stored for up to one week at $4^{o}C$ in the dark.

3.2 Buffers

Sodium phosphate, sodium acetate and Tris-HCl buffers were prepared as described in Data for Biochemical Research (Oxford University Press, 1974). Other specialist buffers are described in the appropriate methods section.

4. Media

4.1 Complex Media

The complex media used were as follows. Nutrient Broth No. 2 (CM67), Isosensitest Broth (CM473), Brain Heart Infusion Broth (CM225), Diagnostic Sensitivity Test Agar (CM216),

Columbia agar base (CM331), MacConkey agar (CM76), all Oxoid, Basingstoke, Hants; Pseudomonas isolation agar (Difco, Detroit, Michigan), 20mls of glycerol per litre was added to this agar before autoclaving.

4.2 Minimal Medium

Double strength minimal salts medium (DM) was prepared as described by Davis and Mingioli (1950) (Table 7).

Table 7

Preparation of Double Strength Davis and Mingioli Basal Medium

K_2HPO_4	14.0g
KH_2PO_4	6.0g
Tri-sodium citrate	0.9g
$MgSO_4.7H_2O$	0.2g
$(NH_4)_2SO_4$	2.0g

The compounds were dissolved in 1 litre of distilled water in the order given and then 50ml quantities of base were distributed and autoclaved at 15psi for 15 minutes.

Media was diluted to single strength before use. Supplement solutions for incorportion in the minimal medium are given in Table 8.

Table 8

Supplement Solutions

Solution	Source	Strength prepared	Final concentration	Mode of sterilisation
D-glucose	BDH	200mgml^{-1}	2.8mgml^{-1}	Autoclaving
L-adenine	BDH	5mgml^{-1}	50mgL^{-1}	Steaming
L-histidine	BDH	5mgml^{-1}	50mgL^{-1}	Steaming
L-isoleucine	BDH	5mgml^{-1}	50mgL^{-1}	Steaming
L-leucine	BDH	5mgml^{-1}	50mgL^{-1}	Steaming
L-methionine	Sigma	5mgml^{-1}	50mgL^{-1}	Steaming
L-proline	BDH	5mgml^{-1}	50mgL^{-1}	Steaming
L-tryptophan	Sigma	2.5mgml^{-1}	50mgL^{-1}	Steaming
L-valine	BDH	5mgml^{-1}	50mgL^{-1}	Steaming

4.3 Preparation of Plates

DM minimal medium plates

Minimal plates were prepared by adding appropriate supplements (Table 8) aseptically to 50mls double-strength DM base. Antimicrobial drugs were added aseptically where necessary to give the required concentration. Finally, 1.4mls of a 20% glucose solution were added and the volume made up to 60mls by the addition of sterile distilled water. This was mixed and added to 40mls of molten bacteriological agar no. 1 (3.75g) (Oxoid). After rolling gently to mix, the plates were poured and once set, dried inverted at 55°C for 20 minutes. All plates were used within one week of manufacture.

Plates containing complex media

Complex media were made up according to the manufacturer's instruction and were autoclaved at 15psi for 15 minutes before the plates were poured. Plates containing antimicrobial agents were made by adding the required concentration of drug solution to molten Oxoid Diagnostic Sensitivity Test Agar (DSTA) cooled to 55°C. Plates contained approximately 15mls of agar, after setting, all the plates were dried, inverted at 55°C for 20-30 minutes.

Preparation of chocolate blood agar plates

Chocolate blood agar plates were prepared by adding defibrinated horse blood (SR50; Oxoid, Basingstoke, Hants.) to molten Columbia agar base to a final concentration of 5% v/v and maintaining at 95°C for 5 minutes until the medium assumed a uniform "chocolate" colour.

Preparation of lysed blood DSTA plates

Lysed horse blood DSTA plates (LBD) were prepared as follows. Molten DSTA was made up according to the manufacturer's instructions, autoclaving as before, and cooling to 55°C. Saponin (1%, v/v, BDH) was added to lyse the defibrinated horse blood (SR50, Oxoid) the lysed horse blood was added to the agar to a final concentration of 0.25% and β-nicotinamide adenine dinucleotide (β-NAD) (Sigma) was added at a concentration of 10mgL^{-1}. Solutions of antimicrobial agents were added to the appropriate concentrations, as required, before plates were poured.

5. Methods

5.1 Identification of Bacterial Species

The API 20E microtube system (API System, S.A., France) was used for the identification of all strains except *H. influenzae*. Pseudomonas isolation agar (PIA) was used to confirm the identification of *Pseudomonas* species. *H. influenzae* were defined as Gram negative bacilli

requiring both haemin (factor X) (Sigma) and β-NAD (factor V) (Sigma) for growth. These growth requirements were confirmed by disk testing (Mast Laboratories, Liverpool) on Nutrient agar plates (Columbia agar base) with disks containing either factor X or V or both.

5.2 Biotyping and Serotyping of H. influenza Strains

Biotyping (Kilian 1976, Gratten 1983) and serotyping (Ingram et al 1979) were kindly carried out by Mr M. Croughan of Edinburgh's City Hospital.

5.3 Viable Counts

Serial dilutions of bacterial cultures were made using 1 in 10 and 1 in 100 dilutions in DM base. Suspensions, appropriately diluted were vortexed (Rotary Mixer, Gallenkamp) and 0.1ml amounts spread with a sterile glass spreader on to solid medium.

All plates were incubated inverted at 37°C for 18 hours or in the case of DM medium, for 66 hours.

5.4 Selection of Cephaloridine Resistant and Ampicillin Resistant Strains

A 10^{-4} dilution of an overnight, shaken, nutrient broth culture of each organism was made by serial dilution in single strength DM base with no supplements. A Denley A400

Multipoint inoculator was then used to deliver a 2µl amount of this suspension on to the surface of DSTA plates containing either ampicillin or cephaloridine at 8mgL^{-1} (Garrod et al 1981). The inoculum suspension contained approximately 10^6CFU ml^{-1}.

5.5 Sensitivity Testing Against Antimicrobial Agents

Sensitivity to compounds, other than cephaloridine and ampicillin, were determined. The compounds in Table 6 were incorporated into the agar at a concentration of 10mgL^{-1}. The exceptions were sulphamethoxazole and spectinomycin used at 100mgL^{-1}; mercuric chloride used at 10^{-5}M; norfloxacin used at 0.1mgL^{-1} and rifampicin used at 20mgL^{-1}.

Each plate was inoculated with the two control organisms E. coli NCTC 10418 and P. aeruginosa 10662. A DSTA plate containing no antimicrobial agents was also inoculated as a positive control. Plates were incubated, inverted for 18 hours at 37°C. Growth at the point of inoculation defined resistance to the included compound.

Ampicillin resistant H. influenzae were selected by a modification of the above method. Cultures were grown on chocolate blood agar plates which were incubated for 18 hours at 37°C in 5% CO_2. Two colonies from this plate were inoculated into 4.5mls of BHI broth supplemented with β-NAD (10mgL^{-1}) and 10mgL^{-1} haemin (Sigma) and incubated with shaking for 18 hours at 37°C. Broth cultures were diluted

10^{-4} by serial dilution in DM base providing a suspension of approximately 10^5CFU ml^{-1}.

This suspension was inoculated on to the surface of LBD plates containing the appropriate antimicrobial agent. H. influenzae were screened for resistance to $1mgL^{-1}$ ampicillin, other compounds were used at the same concentrations as before. The control organism was used as H. influenzae 1184E. Plates were incubated for 18 hours at 37°C in 5%CO_2; resistance was defined as before.

5.6 Minimum Inhibitory Concentration (MIC) Determination on Solid Media

A 10^{-4} serial dilution of an overnight, nutrient broth culture was made in single strength DM (approximately 10^6CFU ml^{-1}) and 2µl of this was inoculated as before on to the surface of DSTA plates containing anti microbial agents. Drugs were incorporated into the plates in two-fold serial dilutions and the MIC was expressed as the first concentration permitting no visible growth. Controls were included as before to ensure the reproducibility of sensitivity testing results.

MICs for H. influenzae strains were determined similarly with the modifications given in the previous section. The MICs of ampicillin for the H. influenzae strains were also carried out in the presence of a fixed concentration of clavulanic acid ($8mgL^{-1}$).

5.7 Replica Plating

Overnight nutrient broth cultures were serially diluted 1 in 10^7 in DM base lacking glucose. 0.1ml aliquots of this suspension were spread with a sterile glass spreader on nutrient agar plates (or DSTA plates containing an antimicrobial agent where appropriate) and incubated inverted for 12 hours at 37°C. These plates, containing around 150 colonies, were then replica plated (Lederberg and Lederberg 1952). The plates were inverted over a square of sterile velvet held on a cylindrical rubber block (8cm diameter) by a perspex collar. The agar surface was pressed gently against the pile and then removed. Replica plates, marked for subsequent orientation, were inverted in turn over the fabric and pressed against it so as to pick up a sample of each colony adhering to the pile. Plates were then incubated for 12 hours at 37°C.

5.8 Testing for Loss of Resistance Markers

Single colonies were streaked on to DSTA plates containing one of each of the original strains' antimicrobial drug resistance markers, and a DSTA plate without antimicrobial drugs as a positive control. Plates were incubated at 37°C overnight. No growth along the streak line on any of the selective plates indicated loss of that particular marker.

5.9 Plasmid Transfer

Plasmid transfer experiments were performed by inoculating donor cells from a fresh MacConkey agar plate into 4.5mls of Nutrient broth no. 2 (Oxoid) and recipient cells similarly from a single colony into another 4.5mls of nutrient broth. Cultures were incubated shaking (Gallenkamp Orbital Shaker) at 37°C overnight. After a viable count of a 10^{-7} dilution on MacConkey agar was performed, 0.1ml of the donor culture and 1ml of the recipient culture were mixed in 4.5mls of fresh pre-warmed nutrient broth. The mating mixture was incubated statically at 37°C for the required time which varied from 1 to 18 hours (Smith 1969) as specified. At the end of this time the mixture was mixed on a Whirlemixer to separate donor and recipient cells which were then collected by centrifugation at 4,000rpm for 20 minutes (Heraeus Christ Bactifuge). The pellet was resuspended in 5.6mls of DM base without supplements (equivalent to the volume of the original mating mixture) and further serially diluted to 10^{-4} in DM base. A 0.1ml amount of each dilution was spread on the surface of selective DM agar plates which would allow only for the growth of a plasmid-containing recipient strain. Controls were made by spreading 0.1ml amounts of washed donor and recipient cells on to the same selective plates. The plates were incubated for between 48 - 66 hours. Resultant transconjugant colonies were purified by streaking out on identical DM selective plates. Frequency

of plasmid transfer was expressed per viable donor cell as described by Amyes (1974).

5.10 Plasmid Mobilisation

A 0.1ml amount of an overnight nutrient broth culture of the donor strain containing a standard R plasmid was added to 1ml of recipient culture in 4.5mls of pre-warmed nutrient broth. The recipient strains possessed plasmids or chromosomal genes which were not able to promote their own transfer. The mating mixture was incubated statically at 37°C overnight. The mixture was then spread on to DM agar plates selective for R plasmid containing recipients. Plates were incubated for 48 hours at 37°C and the resultant colonies purified on the same selective plates. R plasmid transfer from one of these transconjugants to *E. coli* $J62\text{-}_2$ was performed as described above. The resultant transconjugants were tested for possession of the gene from the original culture by plasmid isolation and testing for markers present in the original recipient.

Mobilisation experiments were also carried out with the transfer factor X^+ (Sharma *et al* 1984) as described by Young *et al* (1986).

5.11 Creating a Carbenicillin/Ampicillin Sensitive Derivative of R18 by Mutagenesis and Replica Plating

A 0.5ml amount of an overnight nutrient broth culture of *P. aeruginosa* PAO1670 containing the plasmid R18 was taken and added to 4.5mls of pre-warmed nutrient broth and shaken at 37°C for 1.5 hours to get the culture into logarithmic (log) growth. A 100µl amount of methane sulfonic acid, ethyl ester (EMS) (Sigma) was added to the culture, which was then vortexed. The culture was incubated at 37°C for 1 hour statically. The culture was then serially diluted to 10^{-2} in warm nutrient broth and the diluted culture was shaken overnight at 37°C. A 10^{-6} dilution of this culture was plated on to DSTA plates containing kanamycin ($20mgL^{-1}$) (an R18 selectable marker). The plates were incubated overnight and the resultant colonies were replica plated as previously described, on to DSTA plates containing kanamycin, tetracycline ($20mgL^{-1}$), or carbenicillin ($200mgL^{-1}$) and a DSTA plate. All carbenicillin sensitive, and kanamycin, tetracycline resistant colonies were isolated and purified on tetracycline ($20mgL^{-1}$). The loss of carbenicillin resistance (TEM-2) was confirmed by isoelectric focusing of a β-lactamase extract and resistance testing on carbenicillin ($60mgL^{-1}$). The resultant carbenicillin sensitive plasmid was designated R18-18.

5.12 Isolation of Plasmid DNA

Three small scale methods were used for the isolation of plasmid DNA.

5.12.1 The method of Birnboim and Doly (1979).

Without modification.

5.12.2 The method of Kado and Lui (1981).

Cells were grown shaking in 4.5mls of nutrient broth overnight and pelleted by centrifugation (4,500rpm, 20 minutes, Christ Bactifuge). Optimum lysis was obtained by holding cells at 60°C for 20 minutes. No further modifications were made.

5.12.3 The method of Takahashi and Nagano (1984).

Cells were grown shaking in 4.5mls nutrient broth overnight at 37°C and harvested by centrifugation (4,000rpm, 15 minutes, Christ Bactifuge). The method suggested by the authors was then followed exactly.

All solutions of plasmid DNA not analysed directly by agarose gel electrophoresis were stored at -20°C until required.

5.13 Preparation of DNA for Restriction Endonuclease Digest

Plasmid DNA for restriction endonuclease digest was extracted as described by Takahashi and Nagano (1984).

To 100µl of plasmid DNA solution were added a further 350µl of TE (10mM Tris-acetate, 2mM disodium EDTA, pH 8.0) buffer and 50µl of acetate buffer (1M sodium acetate, 10mM Tris-acetate, 2mM disodium EDTA, pH 8.0), and this solution was mixed. The DNA solution was ethanol precipitated with 2 volumes of cold (-20°C) 95% ethanol for 5 minutes and collected by centrifugation (2874g, 5 minutes, 4°C, MSE Microcentaur) then the pellet was dried in a vacuum dessicator (60 minutes). The pellet was dissolved in 60µl of a DNAse free RNAse solution (50mgL^{-1}) (Sigma) and incubated at 37°C for 30 minutes.

Ten microlitres of the plasmid DNA solution was removed to a sterile Eppendorf tube. To this was added 2µl of the appropriate restriction enzyme buffer (10 times strength, supplied by NBL Enzymes Ltd., Northumberland), 7µl of sterile distilled water and 5-10U of the required restriction endonuclease. The tube was incubated at 37°C for 3 hours and the reaction was terminated by cooling to 4°C and adding 10µl of loading buffer (0.1% SDS, 0.05% bromophenol blue, 25% sucrose and 5mM sodium acetate (Sigma)). The sample was analysed by agarose gel electrophoresis using as molecular weight markers, DNA digested with HindIII (Murray and Murray 1975).

5.14 Agarose Gel Electrophoresis

Horizontal slab gel electrophoresis was performed by the method of Meyers et al (1976). A 30µl volume of plasmid DNA was mixed with 10µl of loading buffer and placed into the wells of a horizontal slab gel (14 by 25 x 0.5cm) consisting of 0.5% agarose (Sigma) in Tris-acetate buffer (40mM Tris-acetate, 2mM disodium EDTA, pH 7.9). Similarly, the 30µl of restricted DNA samples (which included loading buffer, see above) were loaded into a gel of 0.7% agarose.

The samples were electrophoresed initially at 200V to allow the DNA to enter the gel and then plasmid DNA was electrophoresed overnight at 70V and restricted DNA at 40V overnight. The gels were run submerged in Tris-acetate buffer (40mM Tris-acetate, 2mM disodium EDTA, pH 7.9). The gels were stained with a 0.75mgL^{-1} solution of ethidium bromide in the Tris-acetate buffer for 30 minutes. DNA was visualised over a long-wave ultra-violet light source (Ultra-violet Products Inc., Cambridge). The distance travelled by DNA through the gel is inversely proportional to the logarithm of its molecular size.

Photographic negatives of DNA agarose gels were prepared on Kodak Plus-X pan film using a 5 x 4 inch plate camera fitted with a Wratten 22 orange filter.

5.15 Incompatibility Group Testing Using Phage PR4

Phage PR4 specifically lyses bacterial cells containing plasmids of inc P , N or W. To determine whether a plasmid of these inc groups was present in a strain, 0.1ml log phase culture was spread on to the surface of a nutrient agar plate and allowed to dry. A phage suspension (10^9pfu ml^{-1}) was spotted on to the surface of the plate and the plate was incubated overnight at 37°C. Lysis indicated that the strain contained a plasmid of inc P, N or W.

5.16 Enrichment of a Bacterial Culture for Strains Which Had Lost an incP Plasmid

Phage PRR1 specifically lyses bacterial cells containing incP plasmids. The phage (100µl of a 10^9pfu ml^{-1} suspension) was added to a log phase nutrient broth culture of the incP plasmid containing strain, and gently mixed. The culture was then incubated statically overnight at 37°C. The presence of the phage PRR1 enriches the culture for strains which have spontaneously lost the incP plasmid.

The culture was serially diluted to 10^{-6} in DM base and 0.1ml was plated on to MacConkey agar plates. Individual colonies were then tested for the loss of a selectable plasmid marker as previously described. Loss of the plasmid was confirmed by DNA agarose gel electrophoresis.

5.17 Nitrocefin Spot Test for β-lactamase Activity

Single colonies of bacteria were spotted with a $50mgL^{-1}$ solution of nitrocefin (O'Callaghan *et al* 1972) for β-lactamase activity. The intact substrate is yellow but becomes red when the β-lactam bond is broken. A colour change of the nitrocefin from yellow to red within 5 minutes was taken as positive reaction.

5.18 β-lactamase Preparation

5.18.1 Large scale preparation of crude cell extracts

β-lactamase extracts were prepared as described by Simpson *et al* (1980). Overnight cultures were made in 1 litre volumes of nutrient broth, incubating overnight with shaking. Cells were harvested at 5,000g for 15 minutes at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments). The pellet was resuspended in 15mls of 20mM, pH 7.0 sodium phosphate buffer. Cells were then disrupted by ultrasonication with constant cooling (8μm, 1 minute x 3, MSE Soniprep 150, MSE Instruments, Crawley). Cell debris was removed by centrifugation at 38,000g for 15 at 4°C (Sorvall). The supernatant crude enzyme preparation could then be stored at -20°C until required.

In order to prepare a store of standard β-lactamase enzymes 100ml cultures were used, resuspending cells in 5mls of

phosphate buffer. Crude standard enzymes were stored in 1ml aliquots for sequential use.

5.18.2 Small-scale preparation of crude cell extracts

Small amounts of ß-lactamase enzyme were prepared according to the method of Livermore and colleagues (1984). Cell cultures were grown on 2ml nutrient agar slopes, these were incubated for 18 hours at 37°C. Cells were removed from the surface of the agar slope by addition of 1ml of sodium phosphate buffer (20mM, pH 7.0) and thoroughly resuspended. The cells were then disrupted at 4°C by ultrasonication (8µm, 30 seconds, Soniprep). Cell lysate was cleared using an MSE Microcentaur centrifuge (11,500g, 10 minutes, 4°C). Cleared lysate was stored at -20°C until required.

5.19 ß-lactamase Enzyme Induction

A single colony from a fresh agar plate was inoculated into 4.5mls of nutrient broth and incubated shaking overnight at 37°C. One ml of this culture was added to 100mls of pre-warmed nutrient broth and shaken for 2 hours at 37°C. Cefoxitin, a well documented inducer of ß-lactamase production (Minami et al 1980) was then added to the growing culture at one-quarter of the MIC value for that particular strain and incubation was continued for a further 16 hours. ß-lactamase enzymes were extracted as previously described for large-scale preparation of crude enzyme extracts.

5.20 Assessment of β-lactamase Activity of Crude Enzyme Preparations

Samples of 25µl of crude β-lactamase extract were added to 75µl of nitrocefin solution ($50mgL^{-1}$) in a microtitre tray (Cooke Microtitre System, Sterilin). The reaction time, defined as a colour change from yellow to red, was taken as an indication of the β-lactamase activity of the enzyme preparation (Simpson et al 1980).

5.21 Preparative Isoelectric Focusing (IEF)

β-lactamase crude extracts were purified by preparative IEF. The procedure and apparatus is fully described by Winter et al (1975).

A gel slurry was prepared consisting of crude enzyme and 5mls of ampholines (LKB, Bromma, Sweden, various pH ranges), made up to 100mls with distilled water. Five grams of Ultradex (LKB, Bromma, Sweden) was slowly dissolved in this enzyme/ampholine solution which was then weighed and poured into the flat bed electrofocusing tray. Wicks, soaked in ampholines (pH 3.5-10.0), were first placed at either end of the tray and the gel tray was placed on a level surface and 25% of the initial gel slurry weight was evaporated off by placing the gel next to an electric fan.

The prepared gel was then electrofocused at 8 watts, 1500V for 18 hours (LKB, 10-2000V Power Pack). After focusing,

two strips of paper soaked in nitrocefin solution ($50mgL^{-1}$) were placed at either side of the gel to visualise the focused β-lactamase activity. The gel was then fractionated with a 20 zone grid (LKB) and the fractions collected with a spatula. Each was transferred to a small column (LKB) and 2 mls of sodium phosphate (20mM, pH 7.0) buffer was applied to each, enough to resuspend the gel bed and elute the enzyme fraction.

The enzyme fractions could be stored at -20°C until required and their purity confirmed by analytical IEF (see over). Often a second round of preparative IEF was carried out on a set of pooled fractions using an appropriate narrow pH ampholine range.

5.22 Analytical Isoelectric Focusing

The method used for analytical isoelectric focusing (IEF) of β-lactamases is described by Matthew et al (1975). The crude β-lactamase extracts were focussed on a glass plate supporting a thin layer of polyacrylamide gel containing carrier ampholines. The composition of the gel mixture is described in Table 9.

Table 9

Composition of Polyacrylamide Gel for Isoelectric Focusing (IEF)

Material	Supplier	Volume used	Final Concentration
5% tetramethyl-ethylenediamine (TEMED) in distilled water	Sigma	0.2 ml	$0.25 mgL^{-1}$
40% ampholines w/v (various pH ranges)	LKB	2.0 ml	2% w/v
Acrylamide (100g) plus methylene bisacrylamide (2.7g) in water (300mls)	BDH	9 ml	acrylamide $70 mgL^{-1}$ bisacrylamide $2 gL^{-1}$
Distilled water		25 ml	
Riboflavin ($20 mgL^{-1}$)	Sigma	4 ml	$2 mgL^{-1}$

Details of the preparation of the IEF gels are given in Matthew et al (1975).

Samples of the β-lactamase enzymes (up to 100µl) were applied near the anode on the surface of the gel. The loading of β-lactamase on each track was equalised with respect to enzyme activity (see previously). Isoelectric focusing was carried out at 4°C at 1 watt (maximum voltage 500V) for around 18 hours (LKB 10-2000V Power Pack). The pH of the gel was read at the end of each run using a miniature flat-ended combined glass electrode (Pye-Unicam 403, 30M8E07) at 0.5cm intervals from the cathode to the anode.

After pH measurements, the gel was stained for β-lactamase activity and photographed.

5.22.1 Staining polyacrylamide gels for β-lactamase activity

β-lactamase enzyme activity was detected with nitrocefin. A sheet of Whatman No. 54 paper which had been dipped in a solution of nitrocefin ($500 mg L^{-1}$) was laid over the surface of the gel, taking care to avoid trapping air bubbles. The focused bands of β-lactamase activity appear red on a yellow background.

5.22.2 Photography of Analytical IEF Gels

When a focused gel was treated with nitrocefin the β-lactamase bands appeared gradually and by the time the weakest bands were visible, the strongest bands had diffused. Serial photographs were taken to obtain a complete record. Kodak Ortholith film (35mm) was used with transmitted light and a Wratten 58 green filter.

5.22.3 Identification of β-lactamase enzymes using analytical IEF

Twelve samples of β-lactamase enzyme could be loaded on each plate. To give a visual check on focusing 10µl of myoglobin ($10 mg\ ml^{-1}$) (Sigma) was loaded at the edge of each gel. On primary identification runs all the available tracks were occupied by test β-lactamase extracts. Subsequently, β-lactamases with similar isoelectric points (pI's) and

patterns of satellite bands were checked for identity with standard enzymes (Table 5) being run in adjacent tracks.

5.23 Assays of β-lactamase Activity

5.23.1 Macroiodometric Assay System

The macroiodometric assay system was first described by Perret (1954) and the revised method (Sykes and Matthew 1979) was followed during this thesis.

5.23.2 Spectrophotometric Assay of β-lactamase Activity

A detectable shift in the ultraviolet absorption spectrum of penicillins and cephalosporins occurs upon hydrolysis of the cyclic amide bond in the β-lactam ring. The wavelength at which maximum decrease in absorption occurs following hydrolysis is termed λ max. This is determined by comparing spectra of intact and fully hydrolysed substrate. The rate of substrate hydrolysis is assessed by measuring the rate of decrease in optical density (OD) at λ max.

The basic method employed for spectrophotometric assay was that of O'Callaghan and colleagues (1969). A Pye-Unicam SP1800 uv/vis spectrophotometer with a thermostatically controlled cell carrier was used to measure OD. Penicillin and cephalosporin substrates were prepared at 10^{-2}M and 10^{-3}M respectively in 20mM sodium phosphate buffer (pH 7.0).

Test and blank cuvettes were prepared and equilibrated at 37°C. The test cuvette contained 0.3ml substrate and 2.6mls sodium phosphate buffer (20mM, pH 7.0); the blank cuvette contained 2.9mls buffer. β-lactamase enzyme preparation (0.1ml) was added to each cuvette and monitoring of the decrease in OD at λmax was begun immediately. The initial linear part of the reaction curve was used to obtain a value for change in OD minute^{-1}.

calculation: $$R = \frac{\triangle OD \times N \times \text{enzyme dilution}}{OD_1 \times \text{time}}$$

where R = μmoles of substrate hydrolysed minute^{-1}ml^{-1}enzyme

$\triangle$OD = change in optical density

N = μmoles substrate in cuvette
(0.3 for cephalosporins, 3.0 for penicillins)

OD_1 = optical density of intact substrate

Protein concentrations of crude β-lactamase extracts were determined as described in a further section and the specific activity of each enzyme preparation was expressed per mg protein.

5.23.3 Determination of Michaelis Menton kinetics

The Km and Vmax values were obtained by measuring the rate of hydrolysis at limiting substrate concentrations and plotting the reciprocal of the substrate concentration against the reciprocal of the rate by the Lineweaver-Burk method.

The Vmax values were normalised with respect to benzyl penicillin, i.e.:

$$\text{relative Vmax} = \frac{(\text{Vmax per µl of enzyme}) \text{ of substrate}}{(\text{Vmax per µl of enzyme}) \text{ of benzyl penicillin}} \times 100$$

The efficiency of hydrolysis of the enzyme was obtained by the same parameter as Pollock's "physiological efficiency" as outlined by Sykes et al (1981b) where efficiency of hydrolysis = $\frac{\text{Vmax per µl of enzyme}}{\text{Km}}$

The relative efficiency of hydrolysis was also normalised with respect to benzyl penicillin, i.e.:

$$\text{Relative efficiency} = \frac{\text{Efficiency of hydrolysis of substrate}}{\text{Efficiency of hydrolysis of benzyl penicillin}} \times 100$$

5.24 Inhibition of ß-lactamase Activity

The effect of ß-lactamase inhibitors was examined by the spectrophotometric assay system described above (O'Callaghan et al 1969). Inhibitor studies were performed with nitrocefin (0.1mM) or benzyl penicillin (1mM) as substrate. The potential inhibitor was added at the start of the assay at various concentrations ranging from 10mM to 10^{-2}M in the case of the cephalosporins. The effect of the inhibitors was expressed as the concentration required for 50% inhibition (ID_{50}) of enzyme activity.

5.25 Relative Substrate Affinity Index Values

The relative substrate affinity index (RSAI) values of β-lactamase enzymes against a range of β-lactams were determined (James 1983). The RSAI value is the ratio of the ID_{50} of nitrocefin cleavage to the nitrocefin concentration used in the assay ($50mgL^{-1}$). This technique was semi-automated using a Tikertek Multiscan (Flow Laboratories, Middlesex). Ten test β-lactams could be simultaneously assayed against each enzyme as potential inhibitors of nitrocefin hydrolysis. The adaptations were described by Eliasson and Kamme (1985). Each assay was carried out in duplicate.

5.26 Gel Filtration

A Sephadex G75 column was prepared as follows. Sephadex G75 (Pharmacia, Uppsala, Sweden) was swollen in a 90°C water bath for 3 hours in sodium phosphate buffer (20mM, pH 7.0). The slurry was allowed to cool and then poured carefully into an LKB (Bromma, Sweden) gel filtration column (50 x $1.5cm^2$). When the column was full, the top was connected and the flow started in a downward direction using an LKB peristaltic pump. The flow rate was adjusted to between 6 and 8mls per hour and the column was washed with sodium phosphate buffer (20mM, pH 7.0) for 48 hours. The samples for separation, usually 1ml were applied slowly through the sample applicator (LKB) and eluted with phosphate buffer.

Fractions were collected using an LKB ultrorac fraction collector. Elution was continued until a volume of buffer equivalent to the total column volume had passed through (75mls). The column was washed with sodium phosphate buffer for 12 hours between each run. The fractions containing the β-lactamase activity were determined by testing samples with a nitrocefin solution ($50mgL^{-1}$).

5.27 β-lactamase Enzyme Molecular Weight Determinations

(by the method of Andrews 1964)

The proteins bovine serum albumin (BSA), ovalbumin, β-lactoglobulin, α-chymotrypsinogen and cytochrome C (all Sigma) were all dissolved at $5mgml^{-1}$ and 2mls were applied to the Sephadex G-75 column. The position of the peaks of the protein markers was established by measuring the OD of the fractions by the method of Waddell (1956). The position of cytochrome C was confirmed by measuring the absorbance at 410nm. β-lactamase samples could then be applied to the calibrated column and its molecular size determined from the standard curve.

5.28 Protein Estimations

Protein estimations were carried out according to the method of Waddell (1956) except for penicillin binding proteins (PBPs) where the Lowry assay system was used (Lowry et al 1951). Both methods have been fully described and compared by Hesslewood (1973).

5.29 Raising Antisera to β-lactamase Enzymes

Antisera to the purified β-lactamases TEM-1, SHV-1, N-29 and TLE-2 were raised in female New Zealand White rabbits. Dr A. McColm kindly carried out the animal experiments and the preparation of the antisera. Three rabbits were immunised with each β-lactamase and sera from unimmunised animals was used as a control.

5.30 Auchterlony Gel Diffusion Tests

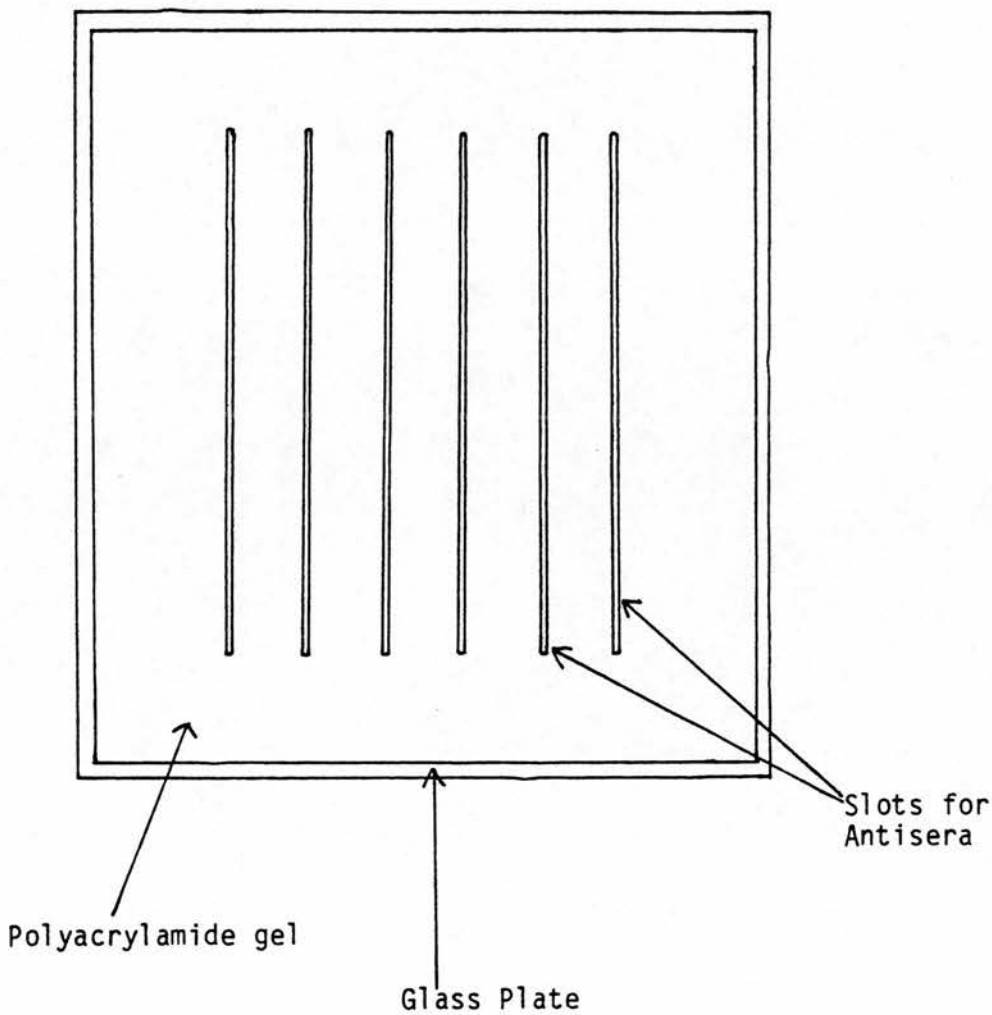
Auchterlony gel diffusion tests were carried out with (2.0%) agarose gels (LKB, Agarose-M), made in 0.05M barbitol buffer (Sigma) containing 0.2% sodium azide. Ten microlitre volumes of reactants were allowed to diffuse against each other for 24 hours in a damp atmosphere (37°C). Gels were washed for 72 hours at 4°C in 0.85% saline and then stained for 1 hour in naphthol blue black (Sigma) solution (0.5% w/v, 5% glacial acetic acid) before destaining with methanol:water:acetic acid (45%, 45%, 10% v/v). Precipitin arcs could then be visualised.

5.31 Immuno Analytical Isoelectric Focusing

Immuno-IEF was carried out according to the method of Matthew *et al* (1975). Polyacrylamide gels were prepared as for analytical IEF but cast with longditudinal slots to allow the application of antisera (Figure 9). β-lactamase was applied to the gel which was then focused as before.

Figure 9

Polyacrylamide gels used for immuno analytical isoelectric focusing.



Antisera was applied to fill the slots (around 100µl) and the gel was incubated at 37°C for 2-3 days to allow for the formation of precipitin arcs next to the focused ß-lactamase. The gel was stained with nitrocefin solution ($50mgL^{-1}$) which revealed the position of the ß-lactamase and the precipitin arcs.

5.32 Preparation of Outer Membrane Proteins

Outer membrane proteins were prepared by harvesting (6,000g, 10 minutes, Sorvall) cells from 100mls of an overnight culture. This was a nutrient broth culture except in the case of H. influenzae where it was BHI supplemented with ß-NAD ($10mgL^{-1}$) and haemin ($10mgL^{-1}$) as before. The cells were washed once in sodium phosphate buffer (50mM, pH 7.0) and the pellet was resuspended in 10mls of the same buffer. The cells were then disrupted by sonication (8µm, 2 minutes, Soniprep), and large cell debris was removed from the lysate by centrifugation (2,500g, 10 minutes, 4°C, Sorvall). The supernatant containing the OMPs was decanted, n-lauryl sarcosine (Sigma) was added to give a final concentration of 2% w/v and this was incubated at room temperature for 30 minutes. Sarcosyl-insoluble OMPs were sedimented by centrifugation (40,000g, 60 minutes, 4°C, Sorvall). The clear OMP pellet was then resuspended in 1ml of sodium phosphate buffer (20mM, pH 7.0) and the protein concentration was measured and adjusted to $5mgml^{-1}$ in the same buffer. Samples (50µl) were subjected to SDS-

polyacrylamide gel electrophoresis (PAGE). Molecular weight markers (BDH) were included on each gel (range 12,300-78,000).

5.33 Preparation of Penicillin Binding Proteins (PBPs)

The preparation and assays of PBPs were carried out as described by Spratt (1977) with the following modifications. Cells were routinely grown in 500ml batch cultures in nutrient broth (or supplemented BHI for *H. influenzae*). Each flask was inoculated with 5mls of an overnight culture and shaken at 37°C until the culture was in late log phase (3.5-4 hours). Cells were harvested (10,000g, 5 minutes, 4°C, Sorvall) and resuspended in 40mls of cold sodium phosphate buffer (20mM, pH 7.0) and then ultrasonicated (8μm, 3 minutes, Soniprep). Unbroken bacteria were removed by centrifugation (8,000g, 10 minutes, 4°C, Sorvall) resuspended in 20mls sodium phosphate buffer (20mM, pH 7.0) and sonicated and centrifuged as before. The cell envelopes contained in the combined supernatants were pelleted by ultracentrifugation (100,000g, 30 minutes, 4°C, MSE Europa) and washed by resuspension in 10mls of phosphate buffer and recentrifuged.

The membrane pellet was resuspended in 4mls of sodium phosphate buffer (20mM, pH 7.0) and adjusted to give a final protein concentration of 10mgml^{-1}). Membranes were stored at -70°C until required.

5.33.1 Penicillin Binding Protein Assay

(^{14}C) Benzyl penicillin was purchased from Amersham International Ltd. For the standard assay of H. influenzae PBPs the procedure followed was that described by Spratt (1977). The final concentration of radiolabelled benzyl penicillin used in the assay was 35mgL^{-1} to ensure saturation of all PBPs. The binding of non-radioactive β-lactams was studied by measuring their competition for the binding of (^{14}C) benzyl penicillin to each of the proteins.

Eleven serial dilutions of the test antibiotic were prepared in sodium phosphate buffer (20mM, pH 7.0) at 20 times the final concentration required in the assay, and 10µl of each dilution was added to a separate Eppendorf tube, and equilibrated at 30°C. Two tubes containing 10µl of buffer were used as controls. The assay was started by the addition of 200µls of pre-warmed membrane and each tube was vortexed and then incubated for 10 minutes. After this time 20µl of (^{14}C) benzyl penicillin (50µCi ml^{-1}; 54mCi mM^{-1}) was added to each tube which was vortexed and incubated for a further 10 minutes. The reaction was terminated by the addition of 5µl of non-radioactive benzyl penicillin (120mgL^{-1}) followed by 20µl of 20% (w/v) sarcosyl, the tubes vortexed and allowed to stand at room temperature for 20 minutes.

The sarcosyl-insoluble outer membrane and peptidoglycan were sedimented by ultracentrifugation (100,000g, 30 minutes, 10°C, MSE Europa). The sarcosyl soluble supernatants are carefully removed from the tubes and either prepared for SDS PAGE electrophoresis as detailed later, or stored at -20°C until required. High molecular weight standards were used (Biorad, Watford, range 45,000-200,000). Fluorography was carried out as described by Spratt (1977).

5.34 SDS PAGE of Outer Membrane Proteins and Penicillin Binding Proteins

The apparatus and discontinous buffer system used for the vertical slab gel SDS PAGE was essentially that of Laemmli and Favre (1973). The slab gel electrophoresis equipment used was supplied by Raven Scientific, Suffolk.

The polyacrylamide gel solutions were as follows. Stock solution I contained 30g Acrylamide plus 3g methylene bisacrylamide. This was adjusted to 100mls with distilled water and kept in the dark at 4°C. Running gel comprised stock solution I (16.6mls), 12.5mls Tris H-Cl (1.5M, pH 8.8), 0.5mls SDS (10% w/v), 0.5mls ammonium persulphate (10mg ml^{-1}) and distilled water (19.8mls). Stacking gel solution contained stock solution I (2.0mls), 0.1ml of 10% (w/v) SDS, 2.5mls Tris H-Cl (0.5M, pH 6.8), 50µl ammonium persulphate (10mg ml^{-1}) and distilled water (5.3mls). Polymerisation was started by the addition of N,N,N',N'-tetramethylenediamine to a final concentration of 0.2%

(v/v). The electrode buffer contained 0.025M Tris, 0.19M glycine and 0.1% SDS (pH 8.3).

The 50µl protein samples were prepared in 0.0625M Tris-HCl (pH 6.8) containing SDS (2% w/v), glycerol (10%), bromophenol blue (0.001% w/v) and 2-mercaptoethanol (5%) (Sigma) final concentrations. Samples were boiled for 5 minutes before application to the polyacrylamide gel.

Electrophoresis was performed at constant current, 40mA through the stacking gel, 60-70mA through the separating gel. Gels were immersed in stain for 1 hour (Coomassie Blue R (Sigma), 0.1% (w/v), methanol 50% (v/v), glacial acetic acid 10% (v/v)) and then destained (methanol 5% (v/v), glacial acetic acid 10% (v/v)).

5.35 Densitometry

A scanning microdensitometer (Joyce Loebbel, Sweden) with peak integration was used to analyse PAGE gels.

III. RESULTS

SURVEY AND COLLECTION OF CEPHALORIDINE RESISTANT STRAINS

1. A Survey of Cephaloridine Resistance Amongst Gram Negative Bacteria from Clinical Isolates

A population of Gram negative bacteria was isolated from clinical specimens in Dundee, Edinburgh and Glasgow during the period January to March 1984. This population was examined to determine the extent of resistance to cephaloridine and ampicillin.

The 549 clinical strains collected were a random population, the majority (441) being isolated from urinary tract infections (UTI). Three hundred and 74 strains were obtained from Edinburgh hospitals, 93 from Dundee hospitals and 82 from Glasgow hospitals.

A minimum inhibitory concentration (MIC) value of greater than $8mgL^{-1}$ of cephaloridine and ampicillin was used to select resistant strains. With these breakpoints, 164 cephaloridine resistant strains and 282 ampicillin resistant strains were isolated from the total population of 549 (29.9% and 51.4% respectively). In order to determine the levels of resistance to ampicillin and cephaloridine in each centre (Edinburgh, Dundee, Glasgow), the sensitivity data were compared (Table 10).

The data indicate that a high proportion of Gram negative bacteria isolated in Glasgow were resistant to ampicillin

Table 10

Resistance to Cephaloridine and Ampicillin Amongst Clinical Isolates from Dundee, Edinburgh and Glasgow

Strain Resistance Profile	Dundee	Edinburgh (Percentage in Parentheses)	Glasgow	Total
CER^S AMP^S*	51 (54.8)	186 (49.7)	23 (28.0)	260 (47.4)
CER^R AMP^S	0 (0)	7 (1.9)	0 (0)	7 (1.3)
CER^R AMP^R+	21 (22.6)	99 (26.5)	37 (45.1)	157 (28.6)
CER^S AMP^R	21 (22.6)	82 (21.9)	22 (26.8)	125 (22.8)
All Strains	93	374	82	549

* CER^S AMP^S MIC of cephaloridine (CER) or ampicillin (AMP) of $8mgL^{-1}$ or less

+ CER^R AMP^R MIC of cephaloridine (CER) or ampicillin (AMP) of greater than $8mgL^{-1}$

and cephaloridine (45.1%). A smaller proportion of strains from Dundee (22.6%) and Edinburgh (26.5%) were resistant to both β-lactams. Ampicillin resistance amongst Glasgow isolates was very common, 71.9% of strains isolated were resistant to 8mgL^{-1}. In Dundee and Edinburgh, 45.2% and 48.4% of strains were resistant to ampicillin. When strains resistant to ampicillin, but sensitive to cephaloridine were examined, similar proportions were found in all three centres.

A high proportion of strains from Glasgow were resistant to cephaloridine (45.1%), while a smaller proportion of strains from Dundee and Edinburgh (22.6% and 28.4% respectively) were cephaloridine resistant. The majority of cephaloridine resistant strains were also ampicillin resistant, however seven Edinburgh strains (1.9%) were cephaloridine resistant but ampicillin sensitive. It was apparent from this survey that a resistance mechanism(s) to cephaloridine does not necessarily confer resistance to ampicillin and vice versa.

Strains from urinary tract infections (UTI) were examined to determine the degree of resistance to ampicillin and cephaloridine amongst one type of isolate (Table 11).

Only the Edinburgh strains in Table 10 comprised UTI and non-UTI isolates. A slightly higher proportion of the UTI isolates from Edinburgh (25.2%) were resistant to ampicillin but sensitive to cephaloridine than had been found amongst all types of isolate from this centre (21.9%). This

Table 11

Resistance to Cephaloridine and Ampicillin in Urinary Tract Infections (UTI)

Strain Resistance Profile	Dundee	Edinburgh (Percentage in Parentheses)	Glasgow	Total
CERS AMPS*	51 (54.8)	130 (48.9)	23 (28.0)	204 (46.2)
CERR AMPS	0 (0)	7 (2.6)	0 (0)	7 (1.6)
CERR AMPR+	21 (22.6)	62 (23.3)	37 (45.1)	120 (27.2)
CERS AMPR	21 (22.6)	67 (25.2)	22 (26.8)	110 (24.9)
All Strains	93	266	82	441

* CER^S, AMP^S MIC of cephaloridine (CER) or ampicillin (AMP) of $8mgL^{-1}$ or less

+ CER^R, AMP^R MIC of cephaloridine (CER) or ampicillin (AMP) of greater than $8mgL^{-1}$

however, did not affect the percentage of ampicillin resistant strains found amongst UTI isolates from Edinburgh as there was a corresponding slight decrease in the number of cephaloridine and ampicillin resistant strains found.

When UTI isolates were compared, Edinburgh and Dundee had similar proportions of ampicillin resistant organisms (48.5% and 45.2% respectively), whereas a significantly larger proportion of strains isolated in Glasgow (71.9%) were resistant to ampicillin ($P<0.001$, $\chi^2 = 16.3$), 2 degrees of freedom). Glasgow was also found to have a larger proportion of UTI strains which were resistant to cephaloridine (45.1%), significantly greater than the numbers in Dundee (22.6%) and Edinburgh (25.9%) ($P<0.01$, $\chi^2 = 13.76$, 2 degrees of freedom).

Thus it appears from this survey that resistance to ampicillin and cephaloridine is a greater problem in Glasgow than in either Edinburgh or Dundee.

In total, from all types of isolate, 164 cephaloridine resistant strains were collected from initial sensitivity studies (106 from Edinburgh, 21 from Dundee and 37 from Glasgow). A further 70 Gram negative bacterial strains resistant to cephaloridine were collected from the three centres in April 1983 on the same criteria as previously described (eight from Edinburgh, 38 from Dundee and 24 from Glasgow). All but one was also ampicillin resistant. In

total the number of cephaloridine resistant strains under examination was 234. These strains are detailed in the appendix. The 70 isolates collected separately are given the prefix "A".

1.1 Identification of Cephaloridine Resistant Bacterial Strains

The genus and species of the 234 cephaloridine resistant strains were confirmed by identification with the API 20E system. A total of 10 genera were identified comprising 20 species (Table 12). In all three centres E. coli was the prevalent species (37.3%, Dundee; 41.2%, Edinburgh; 57.4%, Glasgow). Amongst cephaloridine resistant UTI strains E. coli were found in a higher proportion of isolates (41.9%, Dundee; 58.3%, Edinburgh; 59.3%, Glasgow).

1.2 Sensitivity Testing of Cephaloridine Resistant Bacteria

The cephaloridine resistant strains were tested for sensitivity to a selection of antimicrobial agents. Data for each of the individual strains are presented in the appendix.

To summarise these data, sensitivity to a β-lactam was expressed as the MIC of a β-lactam for 90% of the bacterial population under study, the MIC_{90} (Figures 10 a, and b, and Table 13). Pseudomonas spp. (26 strains) were not included in the cumulative figures, however the MIC_{90} of cefsulodin, a narrow spectrum anti-pseudomonal compound, for

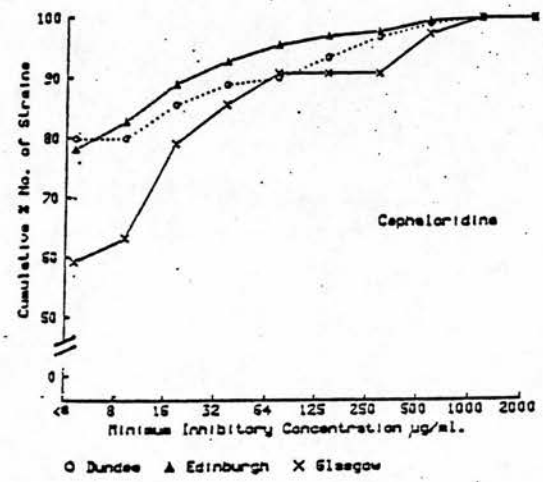
Table 12

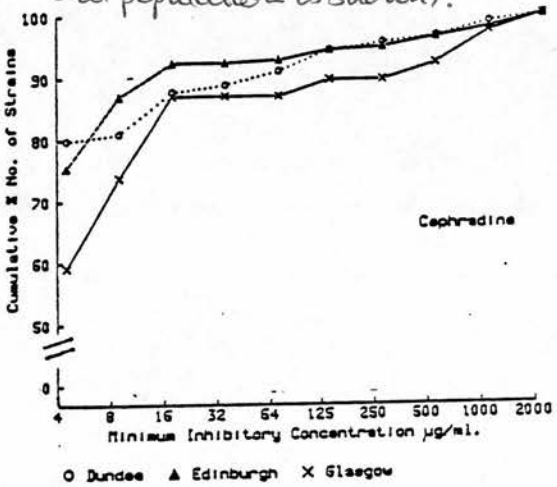
Bacterial Species Distribution in Three Centres of Cephaloridine Resistant Strains

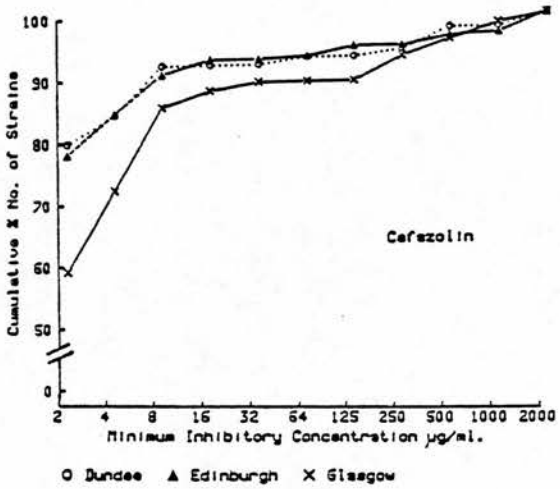
Bacterial Species	Number of Strains			
	Dundee	Edinburgh	Glasgow	Total
Acinetobacter calco.	0	1	1	2
Citrobacter freundii	3	3	1	7
Enterobacter aerogenes	0	2	0	2
E. agglomerans	1	0	0	1
E. cloacae	9	12	4	25
E. sakazakii	0	1	0	1
Escherichia coli	22	47	35	104
Hafnia alvei	0	1	0	1
Klebsiella oxytoca	7	2	3	12
K. ozaenae	1	2	0	3
K. pneumoniae	3	5	0	8
Proteus mirabilis	2	10	0	12
P. morganii	4	1	4	9
P. vulgaris	2	7	1	10
Providencia stuartii	0	0	1	1
Pseudomonas aeruginosa	3	14	5	22
Ps. fluorescens group	0	2	1	3
Ps. maltophilia	0	1	0	1
Serratia marcescens	2	1	5	8
S. odorifera	0	2	0	2
TOTAL	59	114	61	234

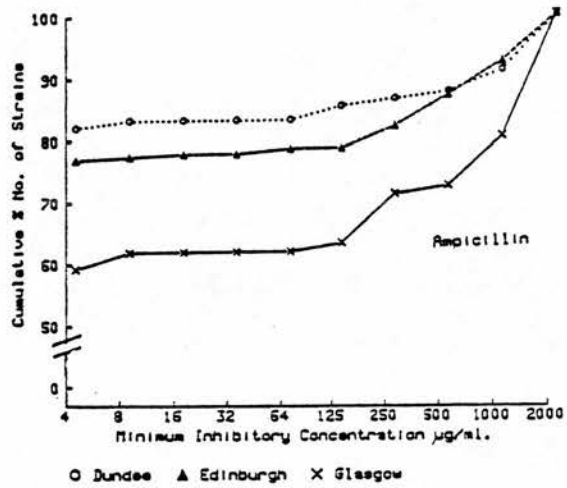
Figure 10a

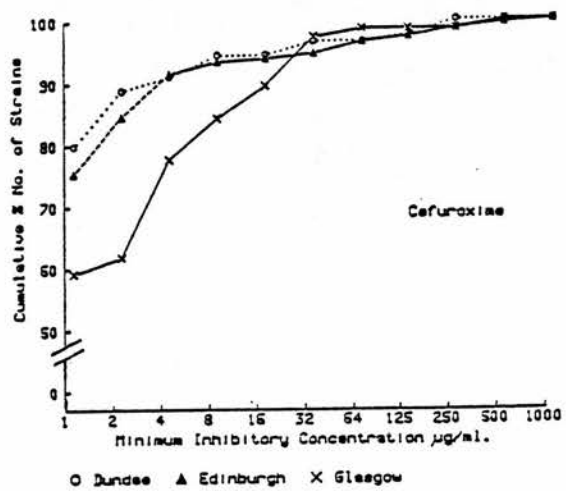
Cumulative minimum inhibitory concentrations of cephaloridine, cephradine, cefazolin, ampicillin, cefuroxime and cefamandole for cephaloridine resistant clinical strains, (except for cephaloridine where the total population is shown).











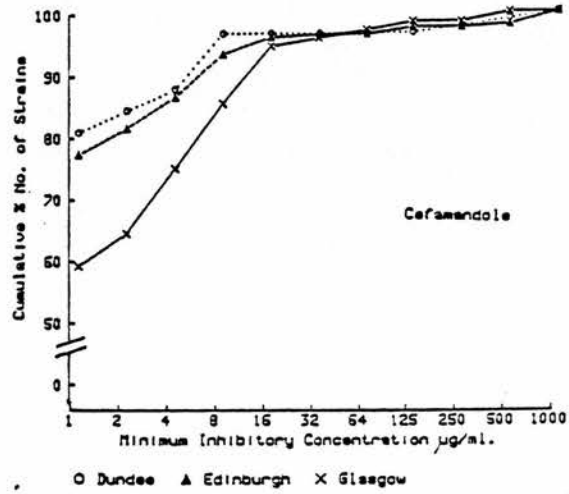
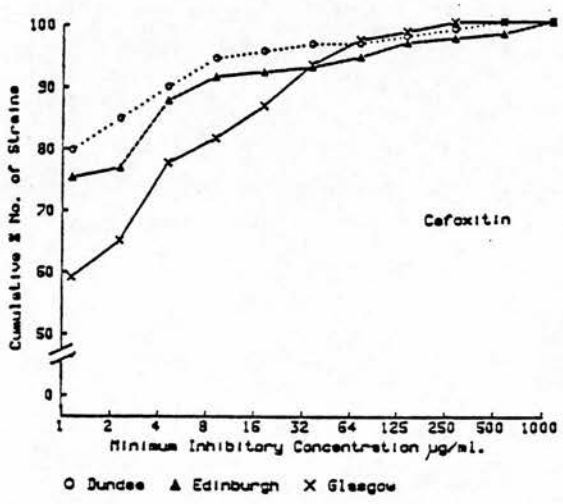
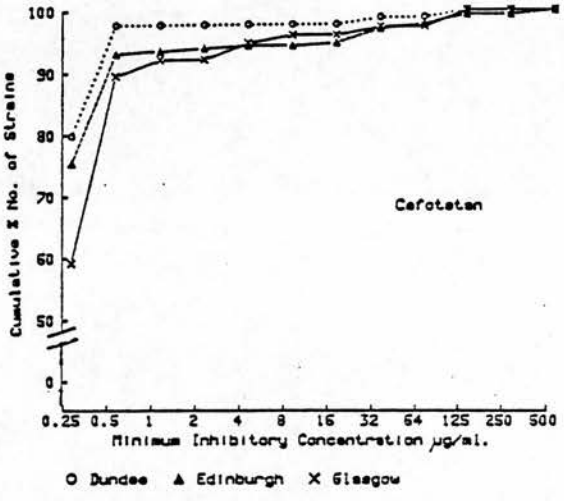
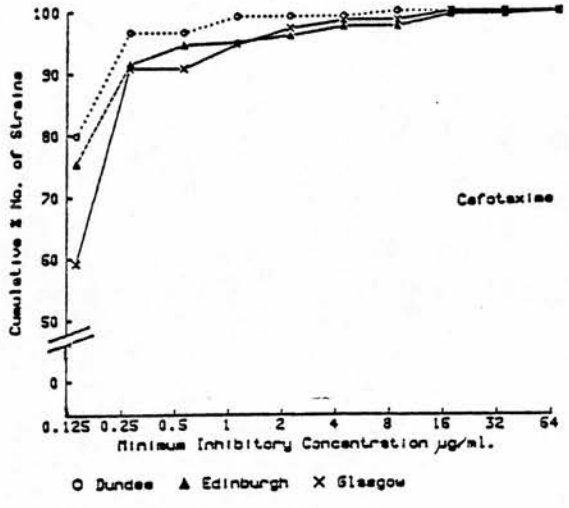


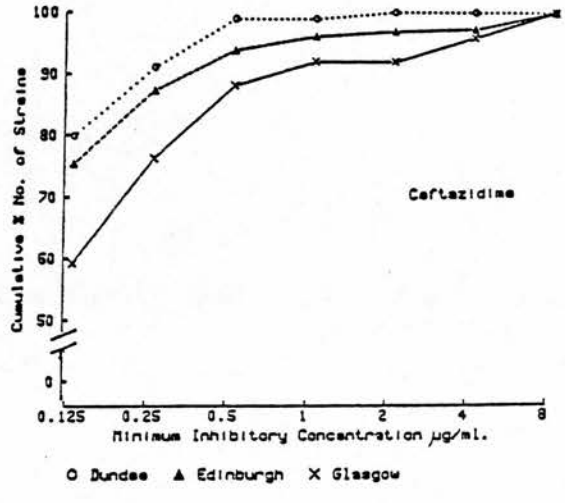
Figure 10b

Cumulative minimum inhibitory concentrations of cefoxitin, cefotetan, cefotaxime, ceftazidime and ceftriaxone for cephaloridine resistant clinical strains.









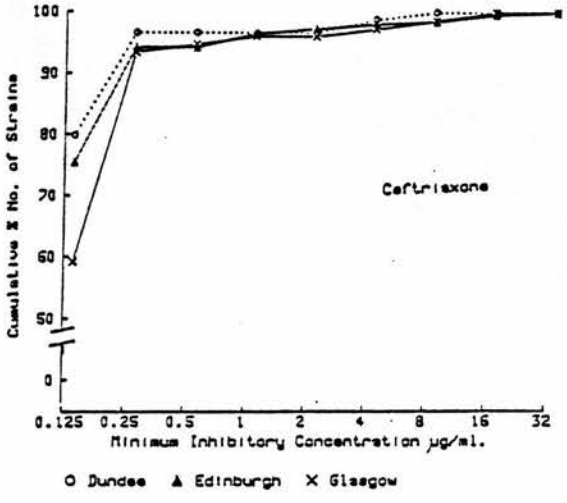


Table 13

Cumulative MIC90 Values for Clinical Strains (excluding Pseudomonas) Resistant to 8mgL^{-1} Cephaloridine

β-lactam	MIC$_{90}$ Values (mgL^{-1}) Dundee	Edinburgh	Glasgow
Cephaloridine	64	16	64
Cephradine	64	16	125
Cefazolin	8	8	32
Ampicillin	500	500	2000
Cefuroxime	2	4	16
Cefamandole	4	8	16
Cefoxitin	4	8	32
Cefotetan	0.5	0.5	0.5
Cefotaxime	0.25	0.25	0.25
Ceftazidime	0.25	0.25	0.5
Ceftriaxone	0.25	0.25	0.25

these Pseudomonas strains was shown to be 32mgL^{-1} (Figure 10c).

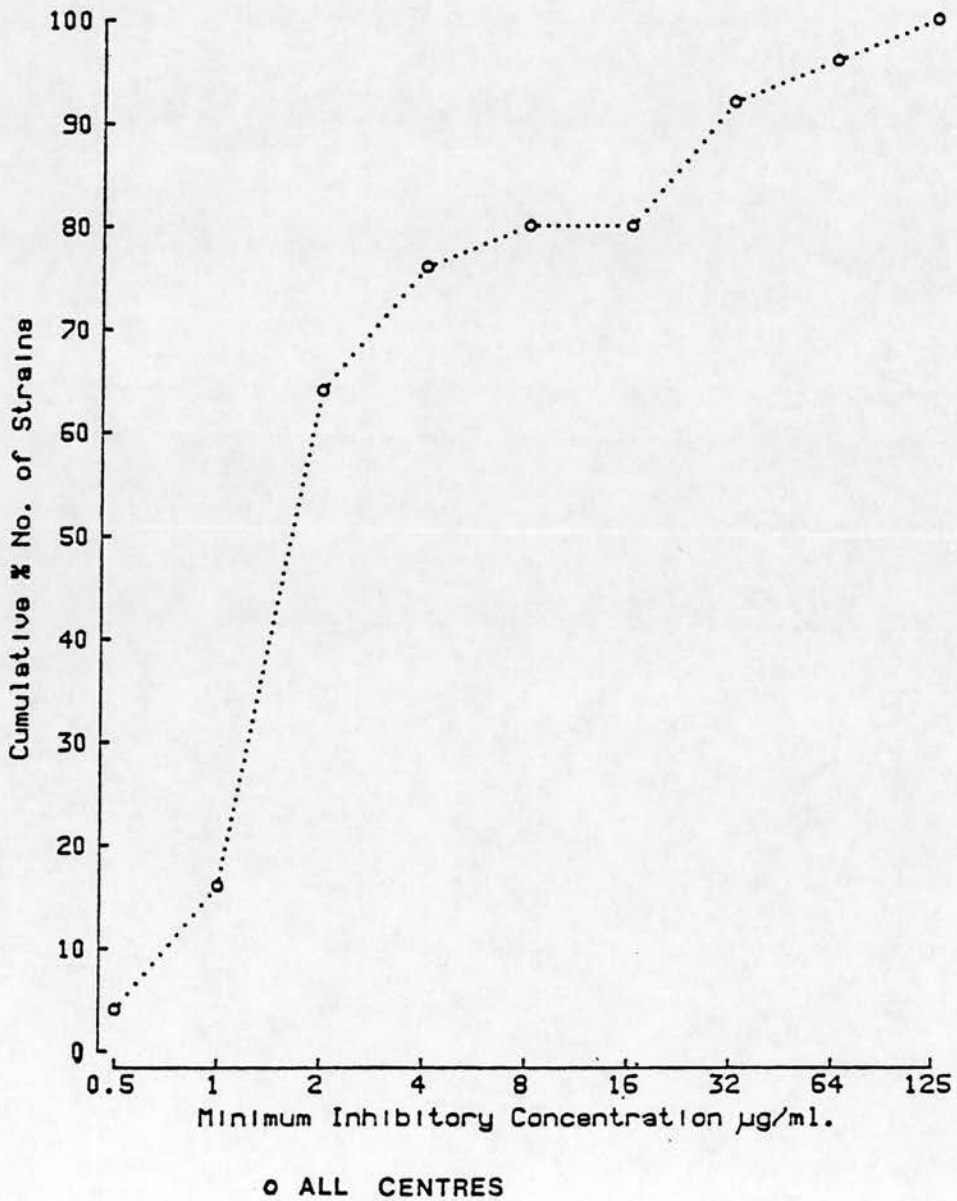
Although the population was selected on cephaloridine resistance, the MIC_{90} values of ampicillin were higher than those of cephaloridine in all three centres. Cephaloridine and cephradine had similar MIC_{90} values for each centre with strains from Glasgow and Dundee having higher MIC_{90} values than strains from Edinburgh. Cefazolin, another first generation cephalosporin, was more active with MIC_{90} values of 8mgL^{-1} in Dundee and Edinburgh and 32mgL^{-1} in Glasgow.

The second generation cephalosporins, cefuroxime, cefamandole and cefoxitin, were similarly active against strains from each centre, with the MIC_{90} values for Glasgow isolates being slightly higher than those for strains from Dundee and Edinburgh. Cefotetan, a cephamycin (like cefoxitin) is a very active compound and this is reflected in the low MIC_{90} values for strains from all three centres.

Third generation cephalosporins were also tested. These included cefotaxime, one of the earliest extended spectrum cephalosporins, ceftazidime and ceftriaxone. All were extremely active against this cephaloridine resistant population with 90% of the strains from all three centres being inhibited by 0.25mgL^{-1} of cefotaxime and ceftriaxone. Ceftazidime inhibited 90% of strains from Dundee and Edinburgh at 0.25mgL^{-1} and 90% of strains from Glasgow at 0.5mgL^{-1}.

Figure 10c

Cumulative minimum inhibitory concentrations of cefsulodin for Pseudomonas strains.



Other resistance markers associated with cephaloridine resistance were also examined during the survey (Table 14). Streptomycin resistance was associated with cephaloridine resistance in 46.2% of the 234 strains.

Also commonly associated with cephaloridine resistance were chloramphenicol (42.3% of strains), tetracycline (41.0%) and sulphamethoxazole resistances (39.7% of strains). This association changes when Pseudomonas strains, which are intrinsically resistant to many drugs, are excluded. Streptomycin resistance is still most commonly found associated with cephaloridine resistance (45.7% of strains). Next most common are tetracycline (42.8%), sulphamethoxazole (39.9%) and chloramphenicol (36.0%) resistances. The 26 Pseudomonas strains were almost uniformly resistant to nalidixic acid (96.2%), trimethoprim and chloramphenicol (92.3%), and kanamycin (88.5%) at the concentrations tested. Norfloxacin, streptomycin and sulphamethoxazole resistances were also commonly found amongst the Pseudomonas strains.

1.3 β-lactamase Enzymes Produced by the Cephaloridine Resistant Population

The β-lactamase enzymes produced by the 234 strains were identified by analytical isoelectric focusing (IEF) of small-scale crude enzyme extracts. Resistant strains which did not appear to produce a β-lactamase enzyme were induced with cefoxitin (materials and methods). Comparison with

Table 14

Other Resistance Markers Associated with Cephaloridine Resistance

Antimicrobial Agent+	Percentage Number of Strains		
	Non-Pseudomonas Species	Pseudomonas Species	All Strains
Chloramphenicol	36.0	92.3	42.3
Colistin	25.5	3.8	23.1
Gentamicin	8.6	3.8	8.1
Kanamycin	16.3	88.5	24.4
Nalidixic Acid	7.2	96.2	17.1
Norfloxacin	8.2	53.8	13.2
Rifampicin	10.1	19.2	11.1
Streptomycin	45.7	50.0	46.2
Sulphamethoxazole	39.9	38.5	39.7
Tetracycline	42.8	26.9	41.0
Trimethoprim	23.1	92.3	30.8

+ Antimicrobial sensitivities were tested at the concentrations given in the materials and methods section.

standard marker enzymes enabled preliminary identification of the β-lactamases as to whether they may be plasmid-mediated or of probable chromosomal origin. Later these affiliations were confirmed by genetic experiments (Section 1.3.4).

Certain types of chromosomal enzymes are well characterised and standard marker enzymes were used for specific identifications, otherwise typing of chromosomal enzymes was by bacterial species (Simpson et al 1980).

1.3.1 Distribution of β-lactamase enzymes

Only three enterobacterial strains did not produce a detectable β-lactamase enzyme, all were Proteus mirabilis.

One hundred and nineteen strains produced only a typically chromosomally-mediated β-lactamase and no plasmid enzyme.

One hundred and twelve strains produced an enzyme which is typically plasmid-mediated, in addition to the chromosomal β-lactamase (Table 15).

In almost all cases, the β-lactamase predicted plasmid-mediated was produced in greater quantities than the chromosomal β-lactamase in cultures which had not been induced with cefoxitin. This was determined from the intensity of the focus ed bands in IEF gels, after overlay with nitrocefin solution. This result strongly suggested that the predicted plasmid-mediated β-lactamase was the

Table 15

Distribution of β-lactamase enzymes and bacterial species in three centres*

Species	Number of Isolates																														
	No detectable β-lactamase			Chromosomal β-lactamase only			Predicted Plasmid-mediated β-lactamases																								
							TEM-1			TEM-2			OXA-1			OXA-2			OXA-3			PSE-4			Unclassified			SHV-1 +TEM-1		
	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G	D	E	G
A. calco					1				1																					
C. freundii				2	2	1	1	1																						
Ent. aerogenes					2																									
Ent. agglomerans				1																										
Ent. cloacae				6	9	1	2	2			3						1						1							
Ent. sakazakii								1																						
E. coli				7	7	11	15	38	23		1	1								1										
H. alvei														1																
K. oxytoca				6	1	2	1	1			1																			
K. ozaenae				1	1																									1
K. pneumoniae				3	2			1															1			1+				1
P. mirabilis	1	2		1	2			4			2																			
P. morganii				4		3		2																						
P. vulgaris				2	7	1																								
Prov. stuartii						1																								
Ps. aeruginosa				2	13	5	1	1																						
Ps. fluorescens gp.					2	1																								
Ps. maltophilia								1																						
S. marcescens				1	1	5														1										
S. odorifera					2																									

* D, Dundee; E, Edinburgh; G, Glasgow
+ Also entered under SHV-1+TEM-1 (strain produces 3 β-lactamase enzymes)

principal enzyme in the majority of the cephaloridine resistant strains.

1.3.2 Chromosomally-mediated β-lactamase enzymes

Thirty six (61%) of bacterial strains from Dundee resistant to cephaloridine produced one β-lactamase with an isoelectric point which was always typical of the species concerned. This compared with 52 strains isolated in Edinburgh (46%) and 31 strains isolated in Glasgow (51%).

There are two major groups of Gram negative chromosomal β-lactamase enzymes. The broad spectrum enzymes hydrolyse benzyl penicillin and carbenicillin as well as cephalosporins, and the cephalosporinase enzymes which have little or no activity against the penicillins. It was possible to establish what proportion of the chromosomal enzymes produced by the 119 strains were "typical cephalosporinases" and what proportion could be described as "broad spectrum" enzymes on the basis of published data for the bacterial species involved.

The majority of chromosomal β-lactamases produced by this group were cephalosporinases - 93 strains, 21 strains produced broad spectrum enzymes (Table 16).

The overall incidence of each type of chromosomal β-lactamase enzyme produced by strains which did not have a typically plasmid-mediated enzyme was 39.7% for cephalosporinase enzymes and 11.1% for broad spectrum enzymes.

Table 16

Incidence of β-lactamase Enzymes in Cephaloridine Resistant Gram Negative Bacteria

Description	Predicted Genetic Location*	Action	Source			Total	Overall Incidence
			Dundee	Edinburgh	Glasgow		
Chromosomal	Chromosome	Cephalosporinase	24	41	28	93	39.2
Chromosomal	Chromosome	Broad Spectrum	12	11	3	26	11.0
TEM-1	R-plasmid	Broad Spectrum	20	54	24	98	41.4
TEM-2	R-plasmid	Broad Spectrum	0	3	5	8	3.4
OXA-1	R-plasmid	Penicillinase	0	1	0	1	0.4
OXA-2	R-plasmid	Penicillinase	0	1	0	1	0.4
OXA-3	R-plasmid	Penicillinase	1	1	0	2	0.8
PSE-4	R-plasmid	Carbenicillinase	1	1	0	2	0.8
Unclassified	R-plasmid	Unknown	0	1	0	1	0.4
SHV-1	R-plasmid/ Chromosome	Broad Spectrum	0	2	0	2	0.8
None Detectable	-	-	1	2	0	3	1.3

* On the basis of IEF results

1.3.3 Typically plasmid-mediated β-lactamase enzymes

The proportion of β-lactamase enzymes which could be plasmid-mediated on the basis of IEF results is detailed in Table 16. Of the 59 Dundee strains, 22 produced β-lactamases that could be plasmid-mediated. Amongst the Edinburgh strains such enzymes were found on 61 occasions. In addition, one strain *K. pneumoniae* 175, produced an unclassified β-lactamase (Results section 2). The β-lactamase SHV-1 which may be chromosomal or plasmid-mediated was found in two *Klebsiella* strains which also produced the TEM-1 β-lactamase. Twenty nine strains from Glasgow produced the TEM-1 or TEM-2 β-lactamase enzymes.

A large proportion of cephaloridine resistant strains from Edinburgh and Glasgow (53.5% and 47.5% respectively) produced β-lactamase which can be plasmid-mediated. A lower percentage (37.3%) of strains isolated in Dundee produced such β-lactamases.

Seven well-characterised β-lactamase enzymes were identified amongst the survey strains (Tables 15 and 16). These were the TEM-1, TEM-2, OXA-1, OXA-2, OXA-3, PSE-4 and SHV-1 β-lactamases (Figure 11). The most commonly found β-lactamase was TEM-1 found in 98 of the 234 cephaloridine resistant strains - 41.9%). The incidence of TEM-1 amongst Dundee strains was 33.9%, in Edinburgh it was 47.4% and in Glasgow TEM-1 was found in 39.3% of strains.

Figure 11

Isoelectric focusing patterns of standard plasmid-mediated ß-lactamase enzymes identified amongst survey strains.

Track A - PSE-4 (pI 5.3)
Track B - TEM-1 (pI 5.4)
Track C - TEM-2 (pI 5.6)
Track D - OXA-3 (pI 7.1)
Track E - OXA-1 (pI 7.4)
Track F - SHV-1 (pI 7.6)
Track G - OXA-2 (pI 7.45,7.7)

The TEM-2 β-lactamase was produced by eight strains and was the second most commonly found β-lactamase. None of the strains isolated in Dundee produced TEM-2. Two strains produced the OXA-3 β-lactamase, whilst OXA-1 and OXA-2 were each found in single isolates. The SHV-1 β-lactamase was produced by two Klebsiella strains. The carbenicillinase, PSE-4, was identified in a K. pneumoniae isolate and an E. cloacae isolate. This β-lactamase was previously believed to be confined to P. aeruginosa due to the transfer deficient nature of the PSE-4 element (Results section 3).

1.3.4 Establishing the genetic location of β-lactamase enzymes predicted as being plasmid-mediated

Isoelectric focusing identified plasmid-like β-lactamase enzymes in 112 strains. Conjugation and mobilisation experiments were carried out to confirm the genetic location of the β-lactamases in these strains.

Conjugation experiments were performed as described in the materials and methods. Matings were carried out overnight into E. coli J62-$_2$ Rif^R pro$^-$his$^-$trp$^-$ with selection made for transfer of ampicillin resistance, the principle substrate of the β-lactamases in question. Any clinical strains resistant to rifampicin were conjugated with E. coli J62-1 Nal^R pro$^-$his$^-$trp$^-$ with appropriate selection.

Seventy eight strains transferred the marker β-lactamase by simple conjugation as demonstrated by IEF of crude enzyme

extracts of transconjugants. Mobilisation experiments were carried out (materials and methods) with the plasmids X^+, Sa or R751 with appropriate selection and then mating from the clinical strains into *E. coli* $J62\text{-}_2$ as before. In total 15 mobilisations were successful, 10 with X^+, four with Sa and one involving R751 (Results sections 3 and 4).

Thus in a total of 93 strains the β-lactamase predicted as being plasmid-mediated by IEF was confirmed as being transferable by genetic experiments. Nineteen strains did not transfer their β-lactamase enzymes to an *E. coli* recipient (Table 17).

The 19 strains may be capable of transferring these β-lactamases under different conditions but they are not freely transferable.

1.3.5 The specific enzyme activity of β-lactamase producing strains

The specific enzyme activities of cell extract preparations from the cephaloridine resistant strains were calculated as described in the materials and methods with cephaloridine as substrate. Strains producing a β-lactamase which had required induction in order to visualise the enzyme on IEF gels were assayed as uninduced and induced preparations. The uninduced value was used in any comparative studies with other strains. The values obtained were expressed per mg

Table 17

Cephaloridine Resistant Strains which did not Transfer a ß-lactamase which is Normally Plasmid-mediated

Strain Species	Number of Strains	ß-lactamase
E. coli	14	TEM-1
C. freundii	1	TEM-1
K. oxytoca	1	TEM-2
E. coli	1	TEM-2
H. alvei	1	OXA-1
S. marcescens	1	OXA-3

protein and details for each strain are presented in the appendix.

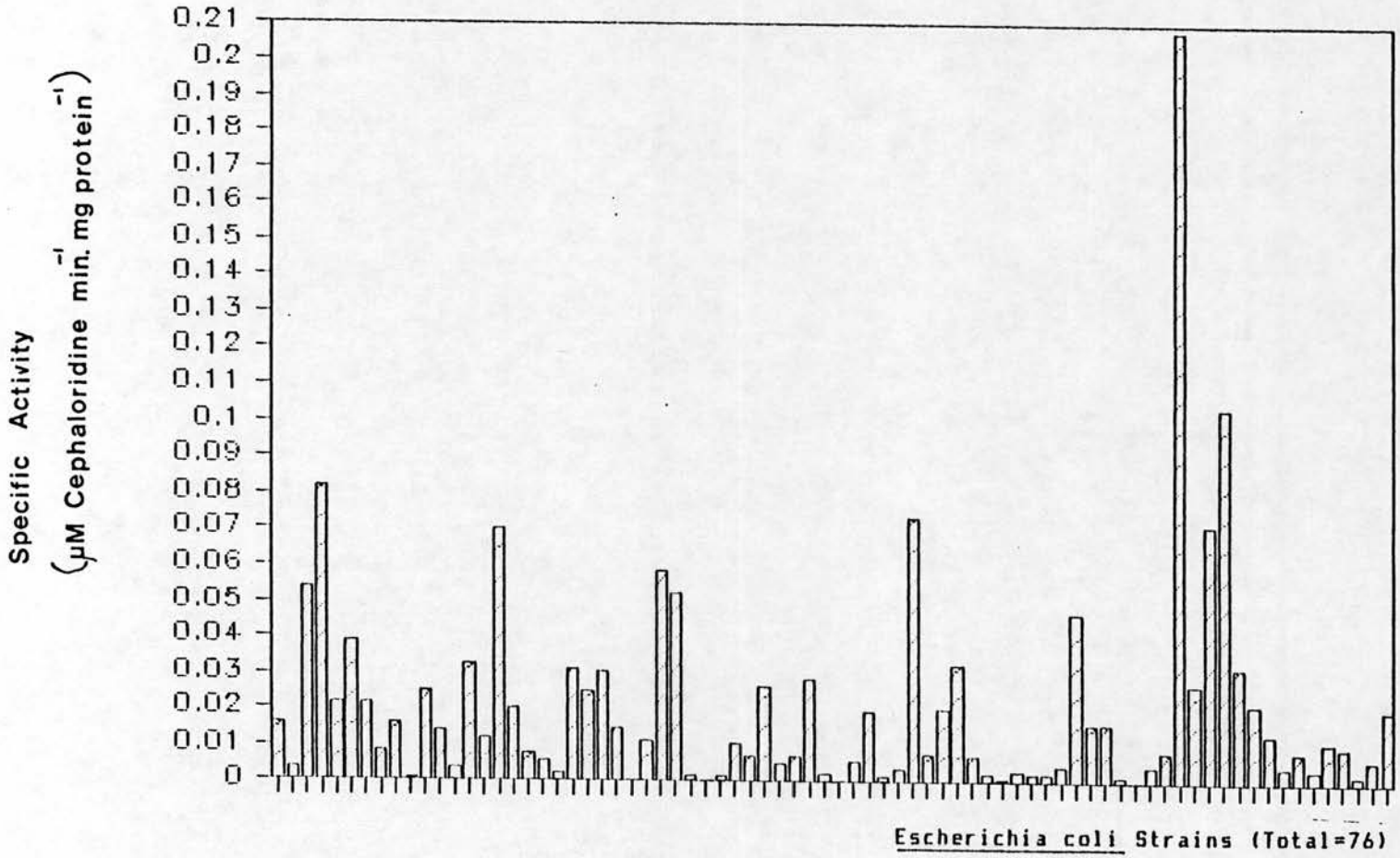
1.4 A Study of the TEM-1 β-lactamase in the Clinical E. coli Population

The largest single population of strains found in the survey were E. coli producing the TEM-1 β-lactamase (76 strains). These strains were examined initially in order to determine the variation in enzyme activity within a single species producing the same β-lactamase. The variation was considerable. Figure 12 illustrates as a histogram the specific enzyme activity of individual clinical E. coli TEM-1 strains. The majority of strains (85%) had a specific enzyme activity value of between 1 and 35nM cephaloridine hydrolysed minute^{-1} mg protein^{-1}.

It was apparent that high levels of TEM-1 β-lactamase production as measured by in vitro hydrolysis of cephaloridine, did not necessarily confer high levels of resistance to cephaloridine. For instance, strain E. coli 374 possessed the highest specific enzyme activity value of 208.4nM cephaloridine hydrolysed minute^{-1} mg protein^{-1} and yet MIC values of β-lactam antibiotics were surprisingly low with cephaloridine inhibiting the growth of this strain at 64mgL^{-1}. One of the strains with a very low specific enzyme activity (0.32nM cephaloridine hydrolysed minute^{-1} mg protein^{-1}) E. coli 135 had very high MIC values, being inhibited only by 2000mgL^{-1} cephaloridine.

Figure 12

Histogram of the specific activities of individual clinical E.coli TEM-1 producing strains.



The TEM-1 enzymes were transferred to *E. coli* J62-$_2$ Rif^R *pro*$^-$*his*$^-$*trp*$^-$ as has been previously described. The β-lactamase was transferred by simple conjugation from 51 clinical strains, Sa and X$^+$ were used to mobilise the enzyme from 11 strains. Fourteen *E. coli* strains, however, did not transfer the TEM-1 β-lactamase and therefore only 62 transconjugants were available for study.

The transfer frequency of the TEM-1 β-lactamase to *E. coli* J62-$_2$ ranged from 1.3×10^{-2} per donor cell to 5×10^{-8} per donor cell, with the majority of strains transferring TEM-1 at a frequency in the range of 10^{-4} to 10^{-6} per donor cell. Agarose gel electrophoresis of DNA extracted from transconjugants revealed the number of plasmids present in the 51 recipient *E. coli* J62-2 strains after simple conjugation (Table 18). The majority of transconjugants (30/51) contained only one plasmid.

The antimicrobial resistance patterns of the *E. coli* transconjugants containing one plasmid were examined. Table 19 shows the seven resistance profiles found amongst these 30 transconjugants. The molecular sizes of the plasmids are also given.

The most common plasmid resistance profile was that of ampicillin and cephaloridine resistance alone. Twelve plasmids of varying sizes from 56 to 120kbases had this profile. The second commonest plasmid resistance profile found was ampicillin, cephaloridine, streptomycin and

Table 18

Number of Plasmids in E. coli TEM-1 Transconjugants

Number of Plasmids	Number of Strains
1	30
2	6
3	9
4	4
5	1
6	1

Table 19

Resistance Profile and Molecular Sizes of the Single Plasmid Present in 30 E. coli Transconjugants

Resistance Profile	Number of Strains	Molecular Sizes (kbases)
Ap Cer	12	56 - 120
Ap Cer Sm Su	8	65 - 90
Ap Cer Tc	6	64 - 115
Ap Cer Ka Sm	1	110
Ap Cer Sp Sm Tp Su	1	76
Ap Cer Tc Sm Tp Su	1	69
Ap Cer Tc Tp Su Gm Cm	1	120

Abbreviations

Ap, Ampicillin; Cer, Cephaloridine; Gm, Gentamicin; Ka, Kanamycin; Sm, Streptomycin; Sp, Spectinomycin; Su, Sulphamethoxazole; Tc, Tetracycline; Tp, Trimethoprim.

sulphamethaxole which characterised 8 plasmids. Four of these plasmids were of the same size (80kbases), two from Glasgow, one from Edinburgh and one from Dundee. A further two plasmids found in transconjugants of Edinburgh strains were 83kbases in size and may be similar or identical to the four 80kbase plasmids. Similarly, three plasmids of the six which had the ampicillin, cephaloridine and tetracycline resistance profile were 115kbases, all were from Edinburgh's City Hospital.

It appears that certain plamids may be more successful than others and cross-infection of different E. coli strains by certain plasmids has occurred in some cases.

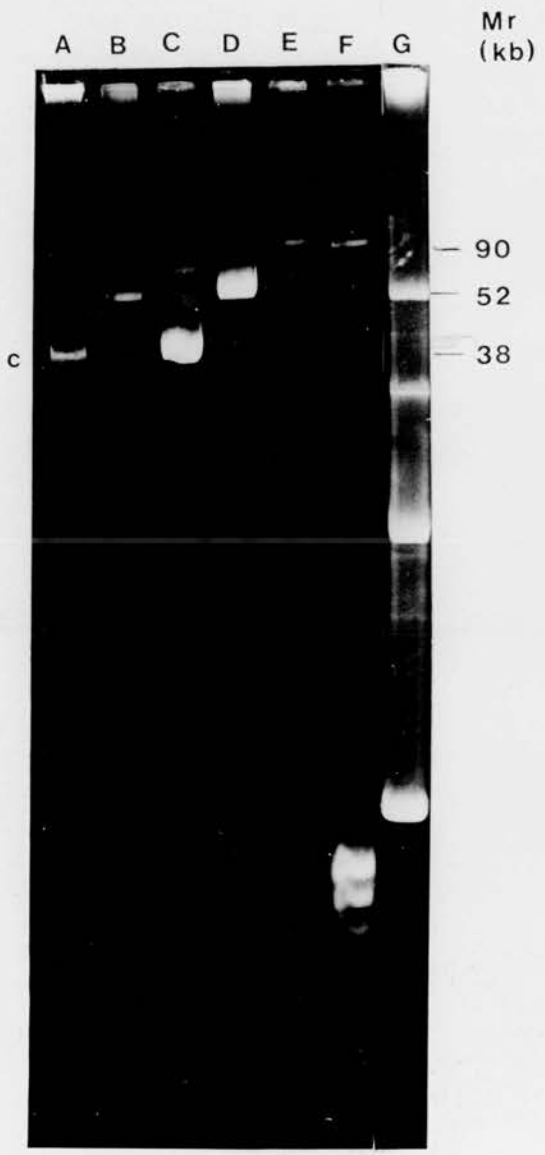
Transconjugants of the 11 strains which transferred TEM-1 β-lactamase only after mobilisation were also examined for plasmid content. Seven transconjugants contained a plasmid which was larger than the mobilising plasmid introduced into the clinical strain (Figure 13, Track E). This increase in size probably resulted from the acquisition by the mobilising plasmid of one or more copies of TnA which encodes TEM-1 and is 3kb in size. One of the seven strains also had six small plasmids of less than 10kbases in size (Figure 13, Track F). The four remaining transconjugants from successful mobilisation experiments contained the mobilising plasmid which had not apparently increased in size, however the strains had smaller plasmids of varying

Figure 13

Agarose gel electrophoresis of DNA extracted from representative E.coli J62-$_2$ transconjugants following mobilisation of the TEM-1 genes by the X^+ factor.
Method of Takahashi and Nagano (1984).

Track A - R1
Track B - RP4
Track C - R6K
Track D - X^+
Track E - E.coli J62-$_2$ 144(X^+)
Track F - E.coli J62-$_2$ 135(X^+)
Track G - E.coli J62-$_2$ 458(X^+)

c = chromosomal



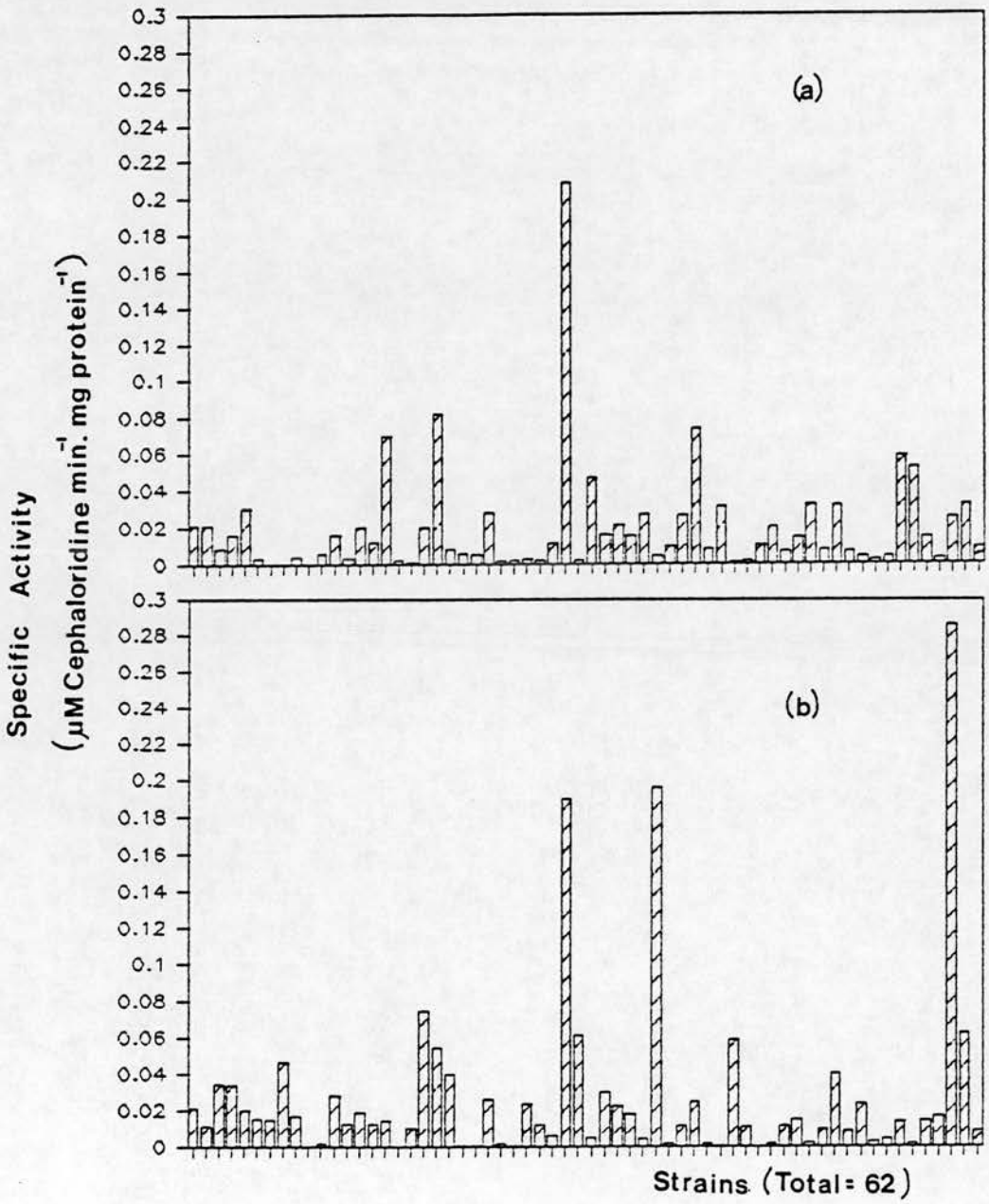
sizes (Figure 13, Track G), one or more of which was thought to encode the TEM-1 ß-lactamase.

The 62 transconjugants which produced TEM-1 all had an MIC of ampicillin of 1000mgL^{-1} or above, in contrast to the varied sensitivity of the clinical *E. coli* strains (Appendix). All were sensitive to 32mgL^{-1} cephaloridine, and the majority (40/62) were sensitive to 8mgL^{-1} of cephaloridine. The specific enzyme activity of the TEM-1 ß-lactamase in the *E. coli* J62-$_2$ transconjugants did not mimic that of the corresponding clinical isolates. Figure 14 compares the histogram of the specific enzyme activities of the 62 *E. coli* clinical isolates which produced TEM-1 with that of their *E. coli* J62-$_2$ transconjugants. The ß-lactamase activity of the clinical isolates did not appear to correlate with that of the transconjugants. The majority of strains (82%) had a specific enzyme activity value of between 0.58 and 35nmoles cephaloridine hydrolysed minute^{-1} mg protein^{-1}, as had been found with the clinical isolates. It appears that there was still diversity in the levels of TEM-1 ß-lactamase production even in the *E. coli* K12 strain J62-$_2$, variation of almost 800 fold in activity (compared with variation of around 2000 fold in the clinical strains). Seventeen of the 62 transconjugants had MICs of cephaloridine of 16 or 32mgL^{-1}. The average specific enzyme activity of these strains was around 2.5 times that of the cephaloridine sensitive transconjugants. Nine of these 17 strains were also tetracycline resistant.

Figure 14

Histograms comparing the specific enzyme activities of TEM-1 producing E.coli clinical isolates compared with that of their E.coli J62$_2$ transconjugants.

(Clinical strains are shown above and transconjugants below, both in the same order).



CHARACTERIZATION OF A NOVEL PLASMID-MEDIATED β-LACTAMASE ENZYME

2. Introduction

Various plasmid-mediated β-lactamases have been identified in *Klebsiella* spp. These include TEM-1, TEM-2, SHV-1, OXA-1 and OXA-3 β-lactamase enzymes (Matthew 1979). The SHV-1 β-lactamase in most commonly found in *Klebsiella* spp. This β-lactamase is known to be transposon-mediated and it is now distributed widely in many bacterial genera (Nugent and Hedges 1979, Matthew 1979).

During the survey of β-lactamases produced by cephaloridine resistant Gram negative bacteria (Results section 1), a *K. pneumoniae* strain was isolated which produced three β-lactamase enzymes. One of these was a novel plasmid-mediated β-lactamase with a unique combination of biochemical properties.

2.1 Results

K. pneumoniae 175 was isolated from a sputum specimen taken from a patient in Edinburgh Royal Infirmary. The strain was highly resistant to cephaloridine, ampicillin and other β-lactams as well as nalidixic acid, gentamicin, streptomycin, trimethoprim, sulphamethoxazole, kanamycin, chloramphenicol and tetracycline.

2.2 Conjugation Experiments

Resistance to ß-lactam antibiotics was transferred to *E. coli* J62-2 (3.5×10^{-6} per donor cell after an overnight mating) when selection was made for cephaloridine and rifampicin resistant transconjugants. These transconjugants acquired resistance to ampicillin, carbenicillin, sulphamethoxazole, and streptomycin as well as cephaloridine. Streptomycin and sulphamethoxazole resistance could be transferred together to *E. coli* J62-2 independently of the ß-lactam resistance by selecting for transfer of resistance to either streptomycin or sulphamethoxazole (frequencies 9×10^{-7} and 2×10^{-6} per donor cell after overnight incubation). Both resistances transferred together to *E. coli* J62-2 at a frequency of 3×10^{-6} per donor cell after overnight incubation.

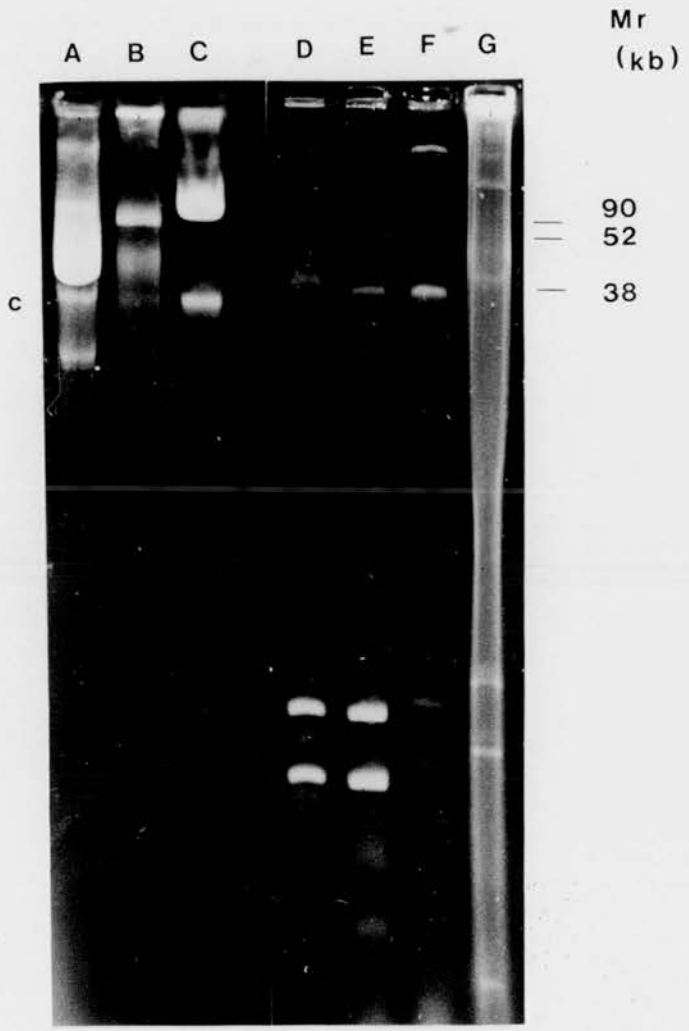
2.3 Analysis of the Plasmid Profile of K. pneumoniae 175 and Transconjugants

K. pneumoniae 175 and the three transconjugants selected on cephaloridine, streptomycin and sulphamethoxazole were examined for plasmid profile (Figure 15). The clinical strain 175 contained two large plasmids of approximately 100 and 133kbases and three other plasmids of less than 10kbases (Figure 15, Track G). The mucoid character of *K. pneumoniae* 175 did not allow the cells to lyse completely and plasmids, although visible, were not as clear as those seen in the transconjugants.

Figure 15

Agarose gel electrophoresis of DNA extracted from K.pneumoniae 175 and E.coli $J62_2$ transconjugants selected on streptomycin (Sm), sulphamethoxazole (Su) or cephaloridine (CER).
Method of Birnboim and Doly (1979).

Track A - R6K
Track B - RP4
Track C - R1
Track D - E.coli J62-2 (Su)
Track E - E.coli J62-2 (Sm)
Track F - E.coli J62-2 (CER)
Track G - K.pneumoniae 175
c = chromosomal



The first transconjugant, selected on cephaloridine, contained a large plasmid of 135kbases (designated pUK 702) which appeared as two bands (Figure 15, Track F) probably open and covalently closed circular DNA. This transconjugant also had three smaller plasmids. The transconjugants selected on streptomycin and sulphamethoxazole did not carry pUK 702 but had the three smaller plasmids seen previously in the cephaloridine transconjugant (Figure 15, Tracks D and E). These results suggest that one or more of the small plasmids encoded sulphamethoxazole and streptomycin resistance and they show that pUK 702 carried the β-lactam resistance.

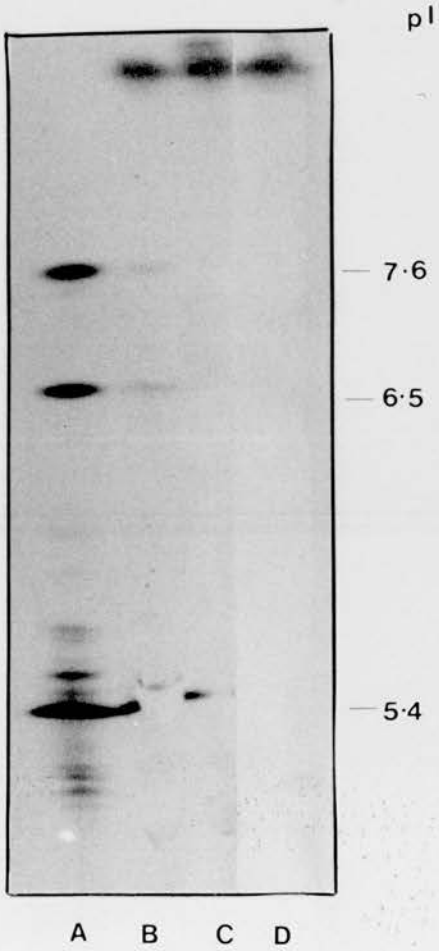
2.4 Isoelectric Focusing

Sonicated extracts from *K. pneumoniae* 175, and the cephaloridine, streptomycin and sulphamethoxazole *E. coli* transconjugants were examined by analytical IEF. *K. pneumoniae* 175 produced three β-lactamase bands with isoelectric points (pI) corresponding to 5.4, 6.5 and 7.6 (Figure 16, Track A). The *E. coli* transconjugant containing pUK 702, which confers cephaloridine, ampicillin and carbenicillin resistance, produced these three β-lactamase bands and also the *E. coli* chromosomal enzyme of pI 8.9 (Figure 16, Track B). Conjugation experiments, selecting for transfer of cephaloridine resistance were carried out for a period of 1 hour, 2 hours, 3 hours and 5 hours. All transconjugants produced all three β-lactamases. No

Figure 16

Isoelectric focusing pattern of K.pneumoniae 175 and its E.coli $J62_2$ transconjugants (selected on Streptomycin (Sm), sulphamethoxazole (Su) or cephaloridine (CER)).

Track A - K.pneumoniae 175
Track B - E.coli J62-2 (CER)
Track C - E.coli J62-2 (Sm)
Track D - E.coli J62-2 (Su)



transfer of cephaloridine resistance took place with conjugation experiments of less than one hour.

The streptomycin and sulphamethoxazole transconjugants were sensitive to β-lactams and when examined by IEF they did not produce a β-lactamase apart from the E. coli chromosomal enzyme (Figure 16, Tracks C and D). These results strongly suggest that pUK 702 alone carries the genetic information for all three β-lactamase enzymes.

As can be seen from Figure 17, the β-lactamase of pI 5.4 corresponded to the TEM-1 β-lactamase, while the enzyme of pI 7.6 corresponded to SHV-1. However, the β-lactamase of pI 6.5 did not correspond to any of the known β-lactamase enzymes.

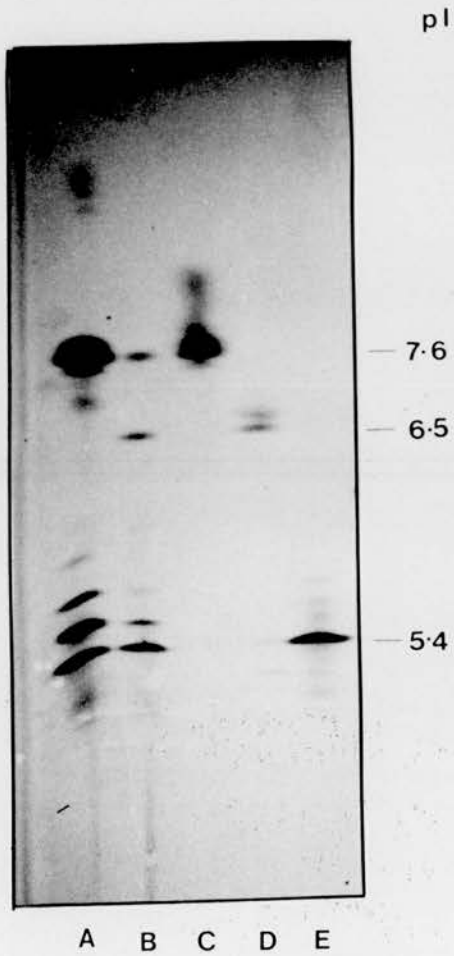
2.5 Purification of the Novel β-lactamase Enzyme

The novel β-lactamase was purified from the original clinical isolate K. pneumoniae 175. As three β-lactamases were present in this strain, preparative IEF was used as the purification technique. Sixty millilitres of crude extract was prepared from five litres of sonicated cells. This was electrofocused with pH 3.5 - 10.0 ampholines and all the fractions which contained the novel β-lactamase (as shown by analytical IEF) were eluted from the ultradex. The pooled fractions were electrofocused in a gradient made from a mixture of pH 3.5 - 10.0 and pH 5.0 - 7.0 ampholines (50% v/v). At this point, fractions containing only the new

Figure 17

Analytical isoelectric focusing of three β-lactamase enzymes purified from K.pneumoniae 175.

Track A - SHV-1, TEM-1 (standards) Track B - K.pneumoniae 175
Track C - Purified SHV-1 from K.pneumoniae 175
Track D - Purified novel enzyme from K.pneumoniae 175
Track E - Purified TEM-1 from K.pneumoniae 175



enzyme were identified by analytical IEF and retained (Figure 17).

2.6 Molecular Weight Determination

The novel ß-lactamase, when purified from SHV-1 and TEM-1 enzymes was run through a calibrated Sephadex G75 column (material and methods). The enzymic peak, as determined by enzymic activity and protein concentration corresponded to a molecular weight of 19,000 (Figure 18).

2.7 Immunology

In order to compare the novel ß-lactamase with SHV-1, TEM-1 and N-29 (which has the pI closest to that of the novel enzyme) antisera was raised to each of the enzymes (materials and methods), and cross reactions were observed by Ouchterlony gel diffusion tests and immuno-IEF.

Antisera was raised against TEM-1, SHV-1 and the novel ß-lactamase, however no detectable antisera was produced to the N-29 ß-lactamase. The novel enzyme appeared to contain two antigens (2 bands on gel diffusion) and TEM-1 contains at least three antigens, while SHV-1 appears to have only one antigen (Figure 19).

The novel ß-lactamase antiserum cross-precipitates with one antigen of TEM-1, while TEM-1 antiserum cross-precipitates

Figure 18

Molecular weight determination of the novel β-lactamase as measured by gel filtration on Sephadex G-75.

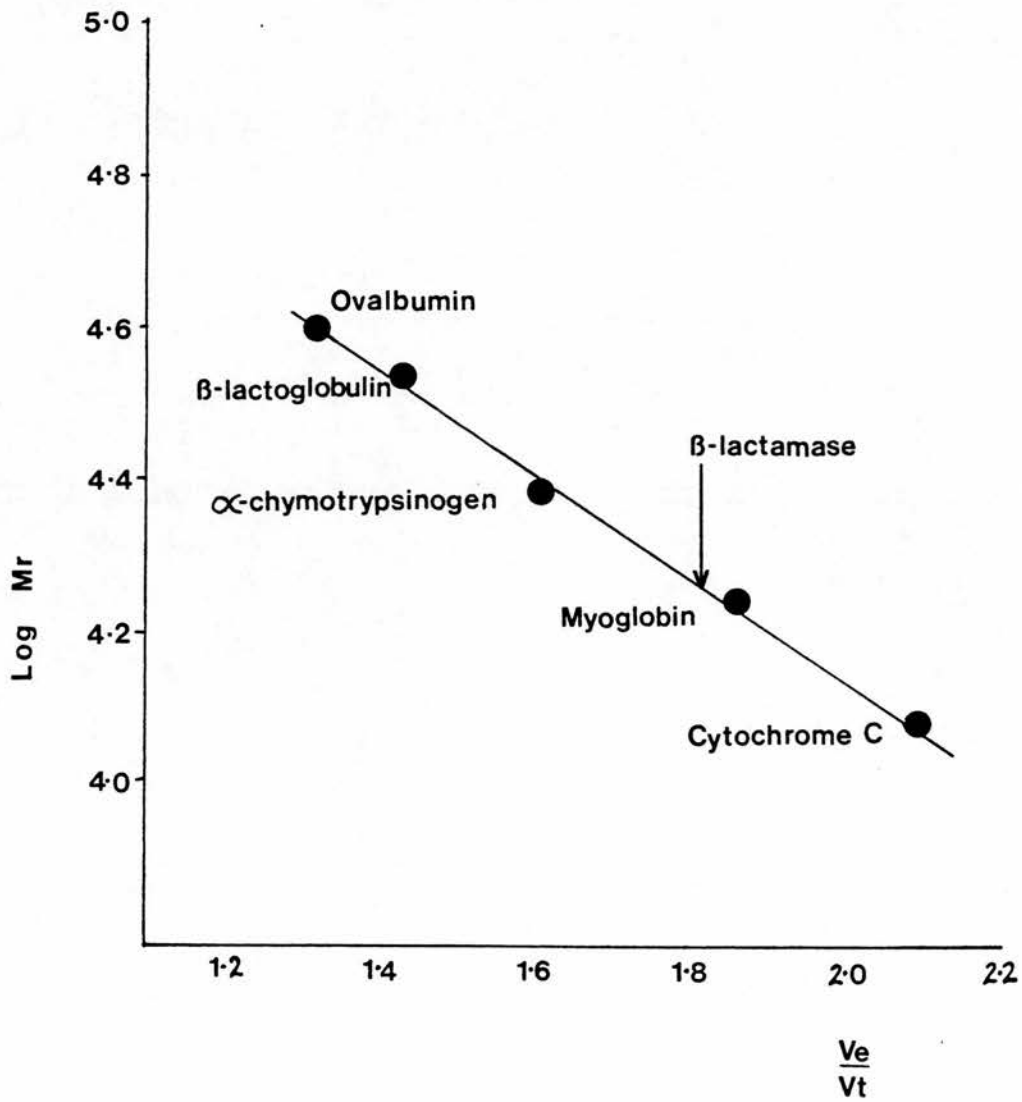
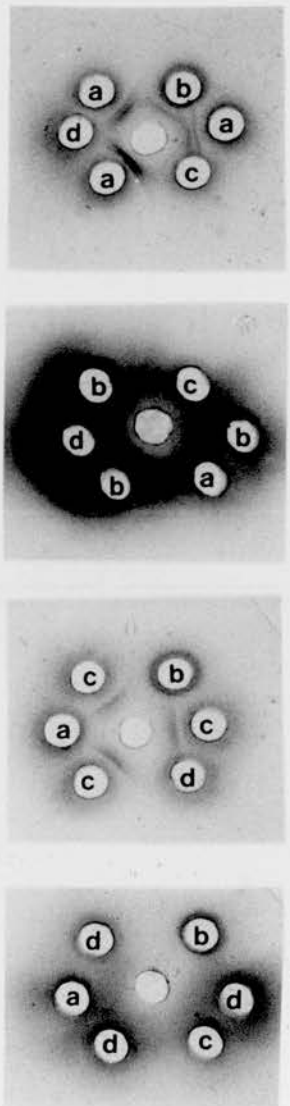


Figure 19

Ouchterlony gel diffusion tests of β-lactamase antisera and antigens.

1. Novel β-lactamase antigen (centre), antisera to novel β-lactamase (a), TEM-1 (b), SHV-1 (c) and N29 (d) (surrounding wells).
2. TEM-1 β-lactamase antigen (centre), antisera to novel β-lactamase (a), TEM-1 (b), SHV-1 (c) and N29 (d) (surrounding wells).
3. SHV-1 β-lactamase antigen (centre), antisera to novel β-lactamase (a), TEM-1 (b), SHV-1 (c) and N29 (d) (surrounding wells).
4. N29 β-lactamase antigen (centre), antisera to novel β-lactamase (a), TEM-1 (b), SHV-1 (c) and N29 (d) (surrounding wells).



with one of the novel β-lactamase antigens. SHV-1 is monospecific (Figure 19).

The immuno-IEF revealed the reactions of each β-lactamase to their respective antisera. No cross reactions were observed between TEM-1, SHV-1 and the novel β-lactamase (Figure 20).

Immuno-IEF is a specific test for antisera to a β-lactamase, as inhibition arcs are revealed when the gel is stained for β-lactamase activity with nitrocefin. The Auchterlony gel diffusion will pick up all antigen/antibody precipitation reactions. The cross precipitation observed between TEM-1 and the novel β-lactamase appears to result from an antigen common to both preparations, the results of the immuno-IEF strongly suggest that this antigen is not a β-lactamase. The enzymes were purified by preparative IEF. This separation by isoelectric point may have resulted in an antigen such as a cell wall or cell membrane component of intermediate pI being present in both β-lactamase preparations and resulting in a cross reaction.

2.8 The Substrate Profile and Michaelis Menten Kinetics of the Novel β-lactamase

The novel β-lactamase hydrolysed several β-lactams when examined by the spectrophotometric assay system (materials and methods). As can be seen in Table 20, benzyl penicillin. ampicillin, carbenicillin, cephaloridine,

Figure 20

Immuno isoelectric focusing of K.pneumoniae 175 β-lactamase enzymes.

Antisera: Track A - Control sera, Track B - Novel β-lactamase, Track C - TEM-1 , Track D - SHV-1 , Track E - Control sera, Track F - N29

Plates were incubated for 48 hours at 37 C after the addition of the antisera. The novel enzyme did not retain any β-lactamase activity after this incubation, however, no cross reactions were seen between TEM-1 enzyme and the novel β-lactamase antiserum.

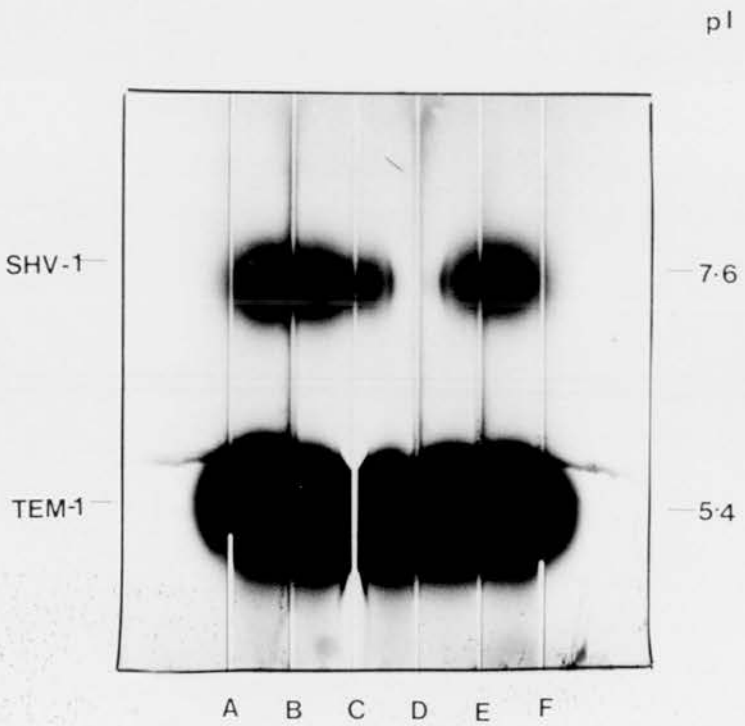


Table 20

Enzyme kinetics of the novel β-lactamase isolated from K. pneumoniae 175[a]

Substrate	Km (μM)	Vmax[b]	Relative Vmax[c]	Efficiency of hydrolysis[d] ($x10^{-5}$)	Relative efficiency[d]
Benzyl penicillin	1110	0.0446	100	4.02	100
Ampicillin	1602	0.0625	140	3.90	97
Carbenicillin	NM[e]	0.0058[f]	13	NM	NM
Cephaloridine	Does not obey first order kinetics (see text)				
Nitrocefin	2080	0.044	99	2.12	53
Cephradine	NM	0.00024[f]	0.5	NM	NM
Cefuroxime	No hydrolysis				
Cloxacillin	No hydrolysis				
Methicillin	No hydrolysis				
Cefamandole	No hydrolysis				

a Spectrophotometric assay method

b Vmax, μM substrate hydrolysed $minute^{-1}$ ml $enzyme^{-1}$

c Expressed as a percentage of the value for benzyl penicillin

d $\frac{\text{Vmax per ml enzyme}}{\text{Km}}$

e NM, not measurable

f Vmax values for these substrates were obtained from the initial reaction rates at maximal substrate concentration.

cephradine and nitrocefin were all hydrolysed by this β-lactamase. There was no detectable hydrolysis of cefuroxime, cloxacillin, methicillin or cefamandole by spectrophotometric or macroiodometric assay techniques.

The Michaelis-Menten kinetics of the β-lactamase for the hydrolysable substrates were examined by plotting substrate concentration (S) against reaction velocity (V). The hydrolysis of benzyl penicillin, ampicillin and nitrocefin all obeyed first order kinetics and produced a hyperbolic curve when S was plotted against V. The hydrolysis of cephaloridine however, did not obey first order kinetics and a sigmoid curve was obtained from a plot of S against V (Figure 21). It was impossible therefore to obtain Km and Vmax (maximum velocity) values for this enzyme with cephaloridine as substrate. In fact, from Figure 21, it would appear that the Vmax for this substrate was never reached. It was not possible to measure the Km values of cephradine and carbenicillin as these substrates were hydrolysed very slowly by the novel β-lactamase.

The Vmax and Km values for benzyl penicillin, ampicillin and nitrocefin were obtained from Lineweaver-Burk plots of $\frac{1}{S}$ against $\frac{1}{V}$ (Figure 22). Table 20 lists the Vmax and Km values obtained with each substrate. From the relative Vmax values, the novel β-lactamase appeared to be primarily a penicillinase enzyme. It had a higher Vmax value for ampicillin than obtained for benzyl penicillin. Nitrocefin

Figure 21

Reaction velocity (v) of the novel β-lactamase, as a function of cephaloridine concentration [S].

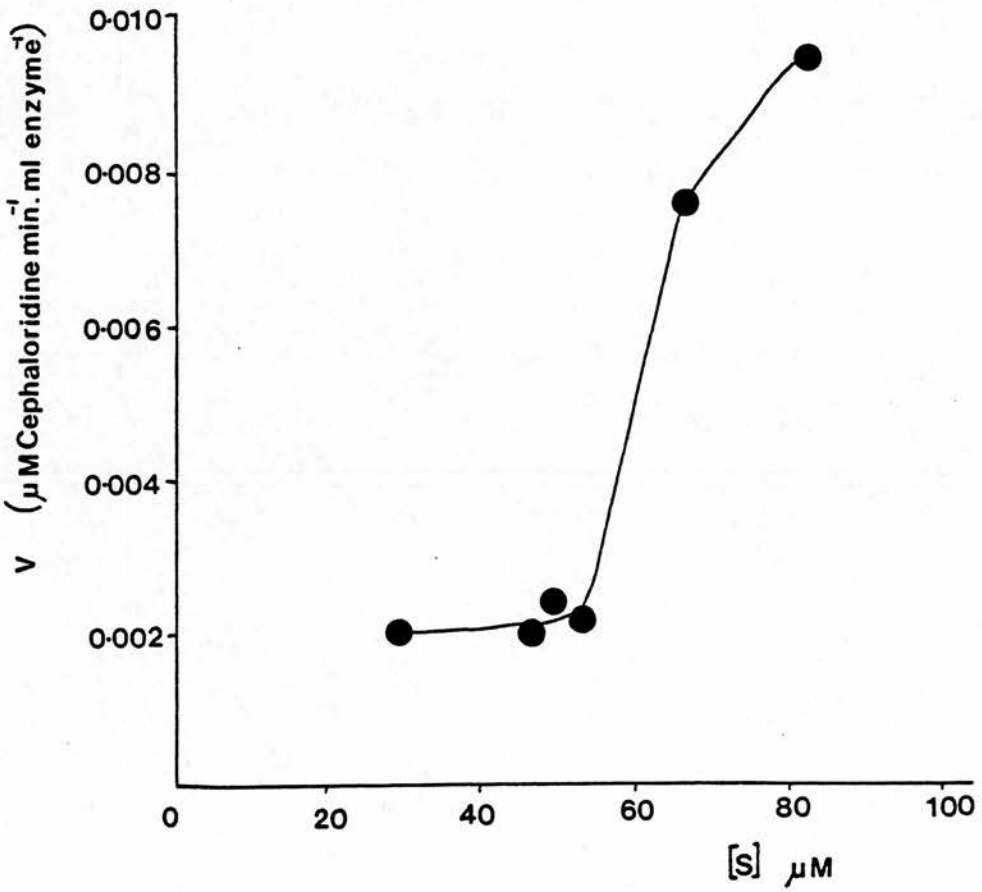
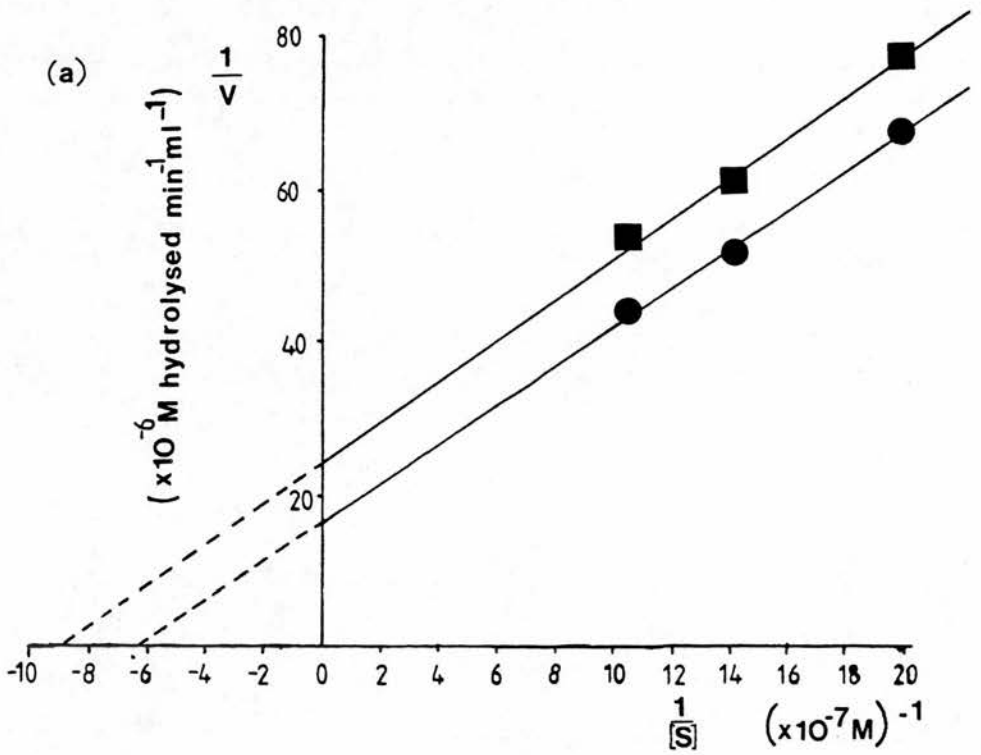
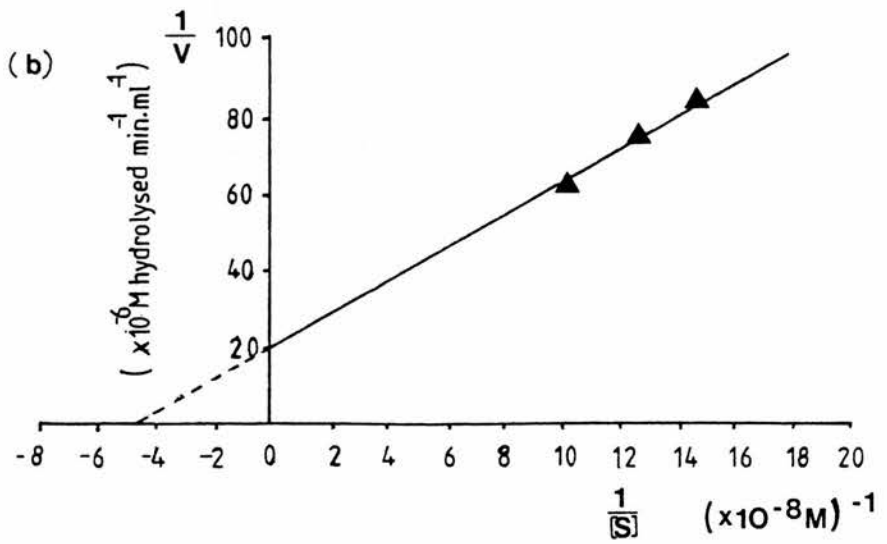


Figure 22

Lineweaver-Burk plots of the novel β-lactamase hydrolysing (a) ampicillin (●) and benzyl penicillin (■), and (b) nitrocefin (▲).

The reciprocal of the substrate concentration $\frac{1}{[S]}$ is plotted against the reciprocal of the β-lactamase activity $\frac{1}{V}$.





was hydrolysed at almost the same rate as benzyl penicillin and at a faster rate than carbenicillin or cephradine.

Similar Km values were obtained with benzyl penicillin, ampicillin and nitrocefin, with the β-lactamase having the lowest affinity for the latter. The relative efficiency of hydrolysis of ampicillin was lower due to the enzyme having a higher Km (lower affinity) for this substrate. The same was true of nitrocefin when the relative efficiency of hydrolysis of this substrate was compared with that of benzyl penicillin.

The relative substrate profile of the novel β-lactamase is similar to that published for the SHV and TEM-type enzymes. The fact that the novel β-lactamase does not hydrolyse cephaloridine by first order kinetics, however, distinguishes the new enzyme from the TEM and SHV enzymes.

2.9 β-lactamase Inhibition Profile

The concentration of inhibitor required to inhibit by 50% the hydrolysis of a substrate by a β-lactamase (ID_{50}) was determined by spectrophotometric assay. The novel β-lactamase was sensitive to inhibition by cloxacillin, clavulanic acid and parachloromercuribenzoic acid (pCMB) when nitrocefin was used as a substrate (Table 21). When benzyl penicillin was the substrate, the β-lactamase was resistant to inhibition by pCMB. This differential response

to pCMB as an inhibitor is also found with the SHV-1 β-lactamase (Matthew et al 1979) and has also been demonstrated with the TEM-1 β-lactamase.

Hydrolysis of nitrocefin by the novel β-lactamase was inhibited by addition of benzyl penicillin, carbenicillin and to a much lesser extent by cefuroxime (Table 21).

Table 21

Inhibition profile of the novel β-lactamase

(Nitrocefin 0.1mM as substrate)

Potential Inhibitor	ID_{50} Value (M)
Clavulanic Acid	8×10^{-8}
Cloxacillin	9×10^{-5}
pCMB	2×10^{-7}
Carbenicillin	8×10^{-5}
Cefuroxime	1×10^{-2}
Benzyl penicillin	9×10^{-6}

These ID_{50} results did not distinguish the novel β-lactamase from the TEM and SHV-type enzymes. A newer technique, the relative substrate affinity index (RSAI) (materials and methods) can distinguish between TEM-1 and SHV-1 on the basis of a differential response to inhibition by the

cephalosporins. As this technique is semi-automated a large number of β-lactams can be examined.

The RSAI value is the ratio of β-lactam concentration giving 50% inhibition of nitrocefin cleavage to the nitrocefin concentration used in the assay. Table 22 shows the average RSAI values obtained with cefotetan, cefoxitin and cefsulodin in assays with TEM-1, SHV-1 and the novel β-lactamase.

Table 22

Average RSAI values

β-lactamase	Cefotetan	Cefoxitin	Cefsulodin
TEM-1	27	165	> 200
SHV-1	> 200	>200	> 200
Novel β-lactamase	1.7	135	7

From these studies, TEM-1 can be clearly distinguished from SHV-1 by comparing the RSAI values for cefotetan. The novel β-lactamase can be differentiated from both SHV-1 and TEM-1 by comparing the results obtained with cefotetan and cefsulodin for each β-lactamase.

Biochemical characterisation has shown that the β-lactamase enzyme purified from K. pneumoniae 175 is a novel β-lactamase. The presence of three β-lactamases of similar molecular weight in the bacterial strain made a classical purification impractical.

The enzyme had a substrate profile very similar to that of the SHV and TEM-type β-lactamases. The novel β-lactamase could be distinguished from the TEM and SHV β-lactamases in its failure to obey first order kinetics with cephaloridine as a substrate. The inhibition profile of the novel β-lactamase with conventional inhibitors is the same as that found for SHV and TEM-type β-lactamases (sensitivity to cloxacillin, clavulanate and pCMB). The RSAI results with cefotetan and cefsulodin distinguished the novel β-lactamase enzyme from SHV-1 and TEM-1. TLE-1 β-lactamase has the closest molecular weight (19,800) to that of the novel β-lactamase (19,000). The novel β-lactamase has a different isoelectric point from those enzymes with a similar substrate profile. Antisera raised to TEM-1 and SHV-1 do not appear to cross-react specifically with the novel β-lactamase.

From the information gathered, it appeared that the novel enzyme was similar to the TEM-type β-lactamases, and it was given the designation TEM-like enzyme (TLE-2).

THE PSE-4 β-LACTAMASE AND ITS MIGRATION INTO THE ENTEROBACTERIACEAE

3. Introduction

The four pseudomonas specific enzymes (PSE) were originally believed to be mediated by genetic determinants confined to *P. aeruginosa* (Hedges and Matthew 1979). Recent reports have shown that there has been a spread of two of these β-lactamases, PSE-1 and PSE-2, into the *Enterobacteriaceae* (Medeiros *et al* 1982, Livermore *et al* 1984). No similar migration had been observed with the PSE-4 β-lactamase, even though it is the most common plasmid-mediated β-lactamase found in *P. aeruginosa* in the United Kingdom (Williams *et al* 1984).

The PSE-4 genetic determinant is not transferable by conjugation amongst *P. aeruginosa* strains (Livermore *et al* 1985), although Sinclair and Holloway (1982) have demonstrated that the PSE-4 gene can reside in a chromosomally located transposon (Tn2521) in this species.

During the β-lactamase survey of cephaloridine resistant strains, two non-pseudomonal clinical isolates were found which produced the PSE-4 β-lactamase enzyme. The characteristics of the genetic determinant and biochemical expression of the enzyme in different bacterial hosts was examined.

3.1 Results

Two strains, *K. pneumoniae* 241 and *E. cloacae* A113 were isolated in Edinburgh and Dundee respectively, and were found to produce the PSE-4 β-lactamase during the β-lactamase survey. *K. pneumoniae* 241 was multiply resistant to β-lactam antibiotics and other antimicrobial drugs, *E. cloacae* A113 was resistant to carbenicillin, ampicillin and cephaloridine (Table 23).

3.2 Transfer of the PSE-4 Determinant from Clinical Bacteria to Standard Strains

The PSE-4 determinants were transferred from the clinical strains to *E. coli* J62-2 and *P. aeruginosa* PA08 selecting for carbenicillin resistance at 1000mgL^{-1} (Figure 23).

As is shown in Figure 23, the PSE-4 determinant from *K. pneumoniae* 241 (designated *dal1*) transferred by direct conjugation to *E. coli* J62-2 and *P. aeruginosa* PA08. The transfer frequency to strain PA08 was two orders of magnitude less than the frequency of transfer to strain J62-2. The determinant *dal1* transferred freely from *E. coli* J62-2 to *E. coli* J53, however, further conjugation experiments with *P. aeruginosa* PA08 *dal1* transconjugant failed to transfer *dal1* to either *E. coli* or *P. aeruginosa* recipients.

In the case of the *E. cloacae* A113 PSE-4 gene, designated *dal2*, free transfer to *E. coli* J62-2 and *P. aeruginosa* PA08

Table 23

Resistance Profiles of K. pneumoniae 241, E. cloacae A113 and their transconjugants

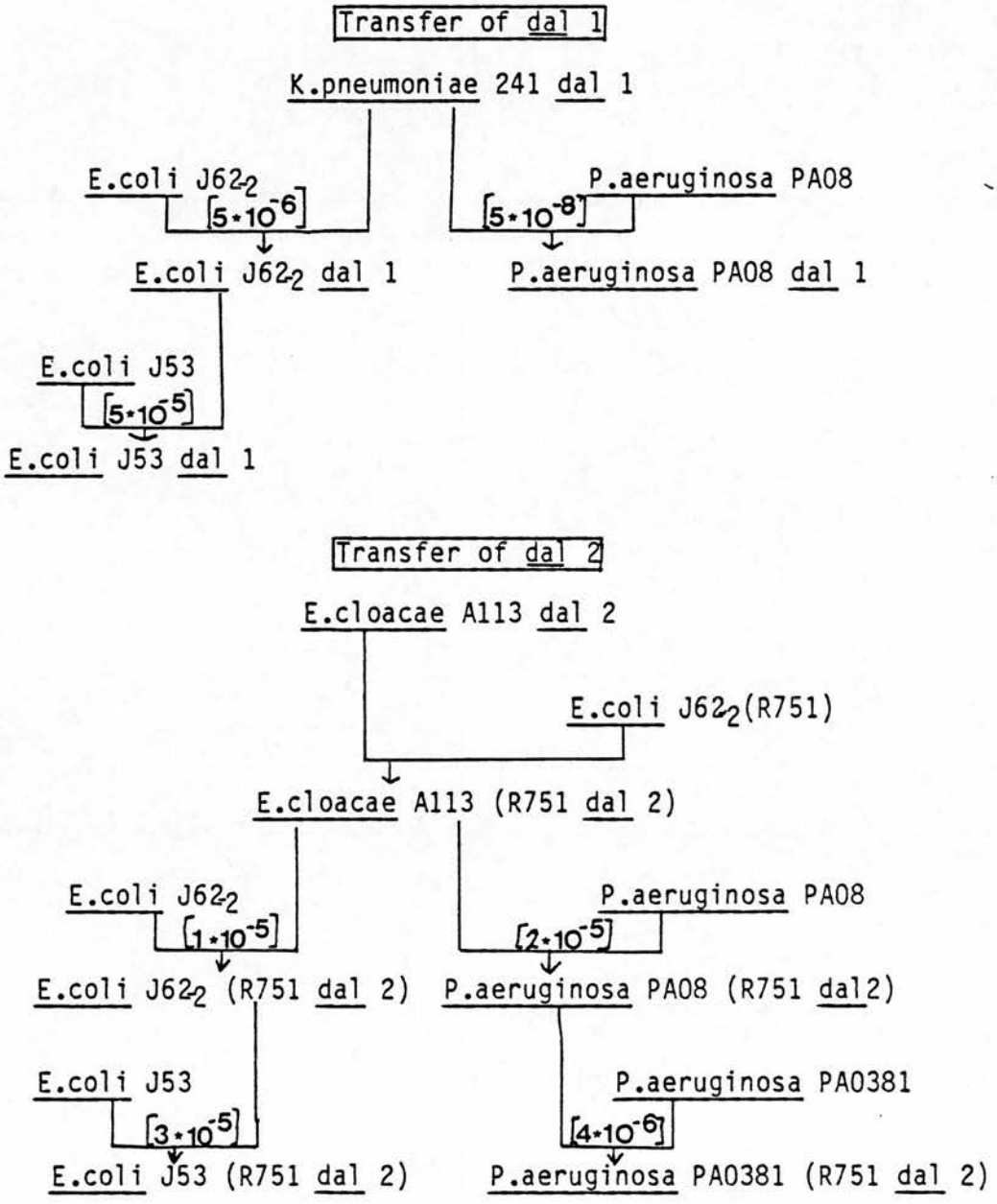
Strain	MIC (mgL^{-1}) CARB	AMP	CER	Resistance Pattern[a]
K. pneumoniae 241 *dal1*	>1000	>1000	64	SpSmSuKmTcCmGmTp
E. coli J62-$_2$ *dal1*	>1000	1000	32	SpSmSuKmTcCmGmTp
P. aeruginosa PA08 *dal1*	>1000	>1000	>1000	SpSmSuKm
E. cloacae A113 *dal2*	32	32	64	
E. coli J62-$_2$ (R751 *dal2*)	>1000	1000	16	TpSp (SuSm)[b]
P. aeruginosa PA08 (R751 *dal2*)	>1000	>1000	>1000	TpSpSuSmKm
E. coli J62-$_2$	<1	<1	<1	
P. aeruginosa PA08	64	125	>1000	SpSxSmKm

a All antimicrobials were tested at the concentrations given in the text, except trimethoprim (Tp) was tested at $1000mgL^{-1}$

b Growth on agar plates containing sulphamethoxazole (Su) and streptomycin (Sm) was poor when compared with a positive control strain *E. coli* J53-$_1$ (Sa).

Figure 23

Transfer of PSE-4 determinants from clinical bacteria to laboratory standard strains.(frequency of transfer is given in square brackets)



by direct conjugation was not possible and the dal2 determinant was mobilised by the introduction of the trimethoprim resistant incP-1 plasmid R751. The R751 recombinant plasmid transferred to E. coli J62-2 and P. aeruginosa PA08 after overnight conjugation at similar frequencies (Figure 23). Both the E. coli J62-2 and P. aeruginosa PA08 transconjugants containing R751 (dal1) could transfer resistance to carbenicillin to E. coli J53 and P. aeruginosa PA0381 recipients respectively (Figure 23).

The E. coli J62-2 dal1 transconjugants of K. pneumoniae 241 had acquired resistance to carbenicillin, ampicillin and cephaloridine as well as other antimicrobials (Table 23). The PA08 transconjugant encoding dal1 was also multiply resistant to carbenicillin, ampicillin, cephaloridine, spectinomycin, streptomycin, sulphamethoxazole and kanamycin. It was however sensitive to tetracycline, chloramphenicol, gentamicin and trimethoprim, in contrast to the E. coli J62-2 transconjugant (Table 23).

The R751 recombinant plasmid which encoded dal2 conferred resistance in E. coli J62-2 to carbenicillin, cephaloridine, ampicillin, spectinomycin, sulphamethoxazole and streptomycin as well as trimethoprim (Table 23).

3.3 Plasmid Profiles of the PSE-4 Producing Strains

K. pneumoniae 241 was found to contain two large plasmids of approximately 127kb and 290kb in size (Figure 24, Track D). The smaller plasmid was the only plasmid visualised in the *E. coli* J62-2 and J53 transconjugants (Figure 24, Tracks B and C), this plasmid (pUK700) must therefore carry the gene for PSE-4 production. The host *E. coli* J62-2 strain was not sensitive to phage PR4 and therefore pUK700 was not *incP*, N or W.

Plasmid pUK700 was determined as being 132kb in size by restriction endonuclease digest. No plasmid DNA could be visualised in the *P. aeruginosa* PA08 transconjugant encoding *dal1* (Figure 24, Track A). This may be due to difficulty in extracting intact large plasmids from Pseudomonas by traditional DNA preparation techniques. However, the fact that the PSE-4 determinant could not be transferred from the PA08 transconjugant and that several pUK700 resistance markers were not seen in this transconjugant strongly suggested that the *dal1* gene had integrated into the *P. aeruginosa* PA08 chromosome.

The *E. cloacae* A113 transconjugants contained the recombinant R751 plasmid which had increased in size by approximately 13kb (Figure 25, Tracks B, C and D). This additional piece of DNA encoding the *dal2* determinant most probably originated from the *E. cloacae* A113 chromosome, as no plasmid DNA was visualised in this strain (Figure 25,

Figure 24

Agarose gel electrophoresis of DNA extracted from the *dal* 1 containing strains.
Method of Kado and Lui (1981).

Track A - *P.aeruginosa* PA08 *dal*1 Track B - *E.coli* J53 *dal*1
Track C - *E.coli* J62-2 *dal* 1 Track D - *K.pneumoniae* 241 *dal* 1
Track E - R6K Track F - R1010
Track G - R1
c=chromosomal DNA

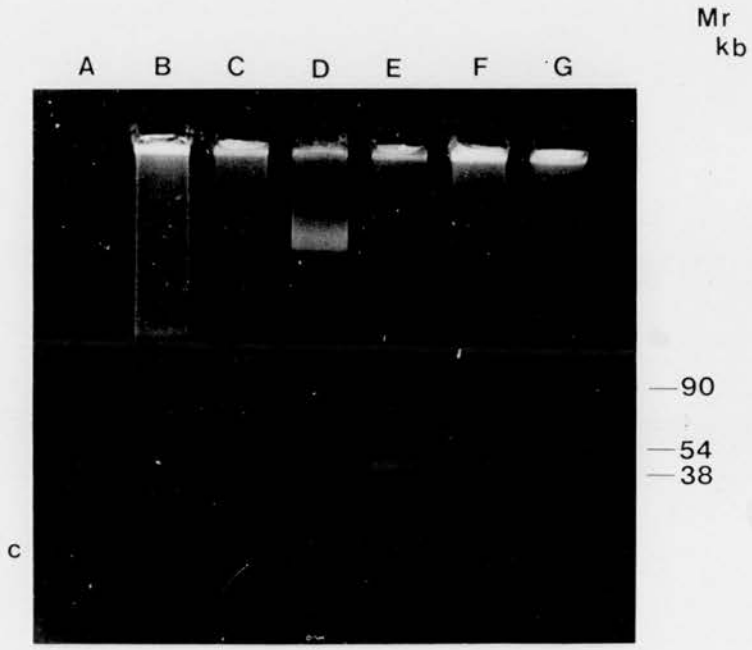
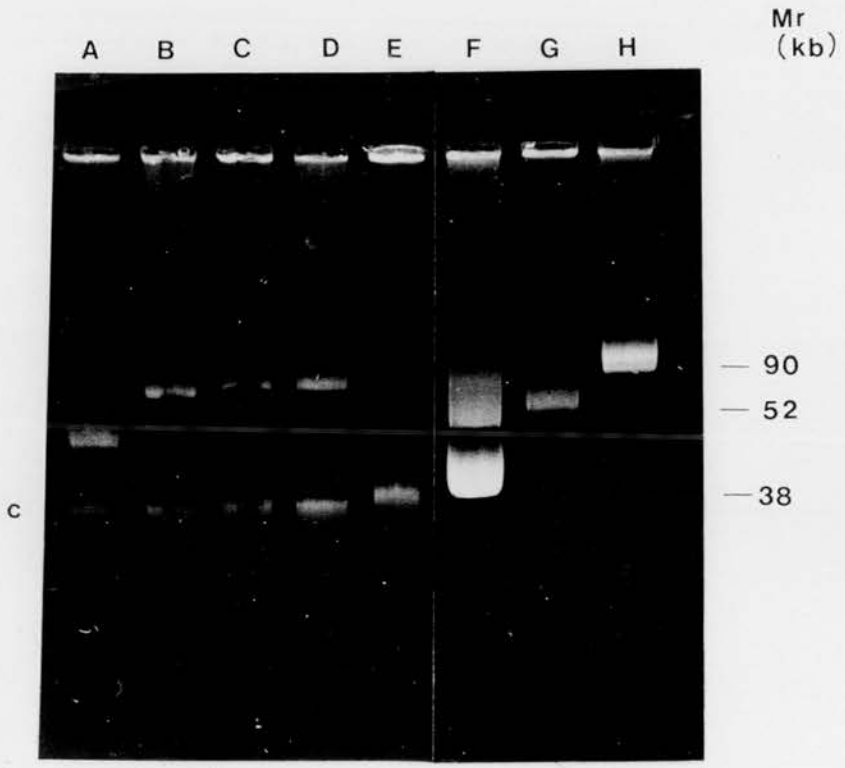


Figure 25

Agarose gel electrophoresis of DNA extracted from the *dal* 2 containing strains.
Method of Birnboim and Doly (1979).

Track A - R751
Track B - *P.aeruginosa* PA08 *dal* 2
Track C - *E.coli* J53 *dal* 2
Track D - *E.coli* J62-2 *dal* 2
Track E - *E. cloacae* A113 *dal* 2
Track F - R6K
Track G - RP4
Track H - R1
c=chromosomal DNA



Track E). The original R751 plasmid has monomeric and dimeric forms (Figure 25, Track A).

3.4 Mobilisation of the dal 1 Determinant from P. aeruginosa PA08 and Restriction Enzyme Analysis

Initially the incP-1 plasmid was used as the mobilising plasmid in the case of the dal2 determinant. During this work R751 was found to promote a significant degree of carbenicillin resistance (MIC = 16mgL^{-1}) in the host E. coli J62-$_2$ strain. This phenomenon was examined further (Chapter 4).

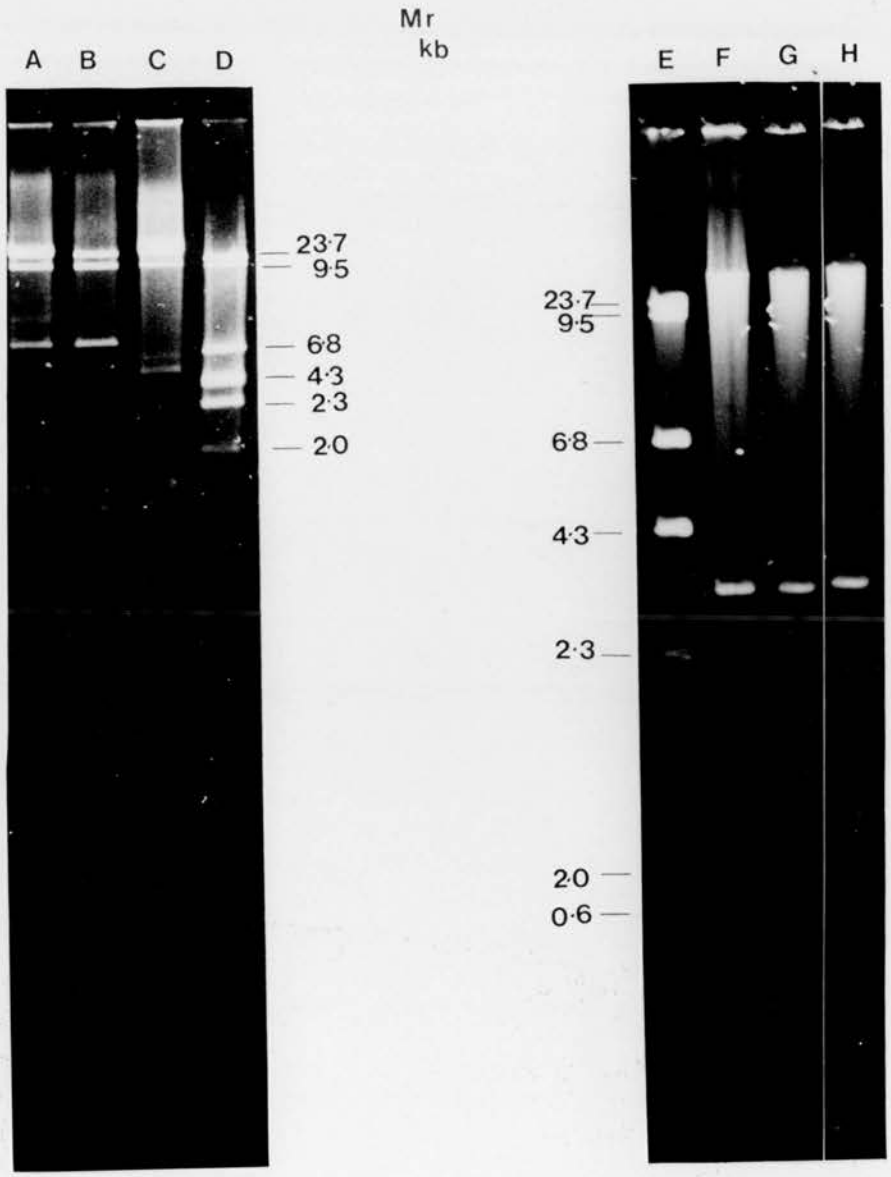
In order to continue the study of the dal1 and dal2 genes, the kanamycin and tetracycline resistant incP-1 plasmid R18-18 (derived from R18, materials and methods) was used in place of R751. This plasmid showed no predisposition towards the induction of carbenicillin resistance.

R18-18 was introduced into P. aeruginosa PA08 dal1 selecting for tetracycline resistance. Conjugation experiments were then carried out with E. coli J62-$_2$ selecting for carbenicillin resistance, which transferred to E. coli J62-$_2$ at a frequency of 7×10^{-6} per donor cell after overnight conjugation and from there to E. coli J53 at 2×10^{-5} per donor cell after overnight incubation. The recombinant R18-18 plasmid increased in size, and restriction enzyme analysis was carried out (Figure 26).

Figure 26

Agarose gel electrophoresis of restricted DNA extracted from R18-18:*dal* 1 strains.

Track D,E - λ (HindIII) Track C - R18-18 (PstI)
Track A - *E.coli* J53 R18-18:*dal* 1 (PstI)
Track B - *E.coli* J62$_2$R18-18:*dal* 1 (PstI)
Tracks F G and H, as for B,C and A except digested with HindIII.



A PstI digest of R18-18 gave four fragments of 25, 20.5, 6.0 and 1.9kb (Figure 26, Track C). Two recombinant plasmid clones gave six fragments after digest with PstI (25, 20.5, 8.4, 1.9, 1.8 and 1.7kb). The recombinant plasmids had increased in size by 6.0kb and the insertion had two additional PstI sites (Figure 26, Tracks A, B). No additional HindIII sites were found (Figure 26 , Tracks F, G and H).

The 6.0kb dal1 determinant conferred carbenicillin, ampicillin, spectinomycin, streptomycin and sulphamethoxazole resistance.

3.5 Isoelectric Focusing

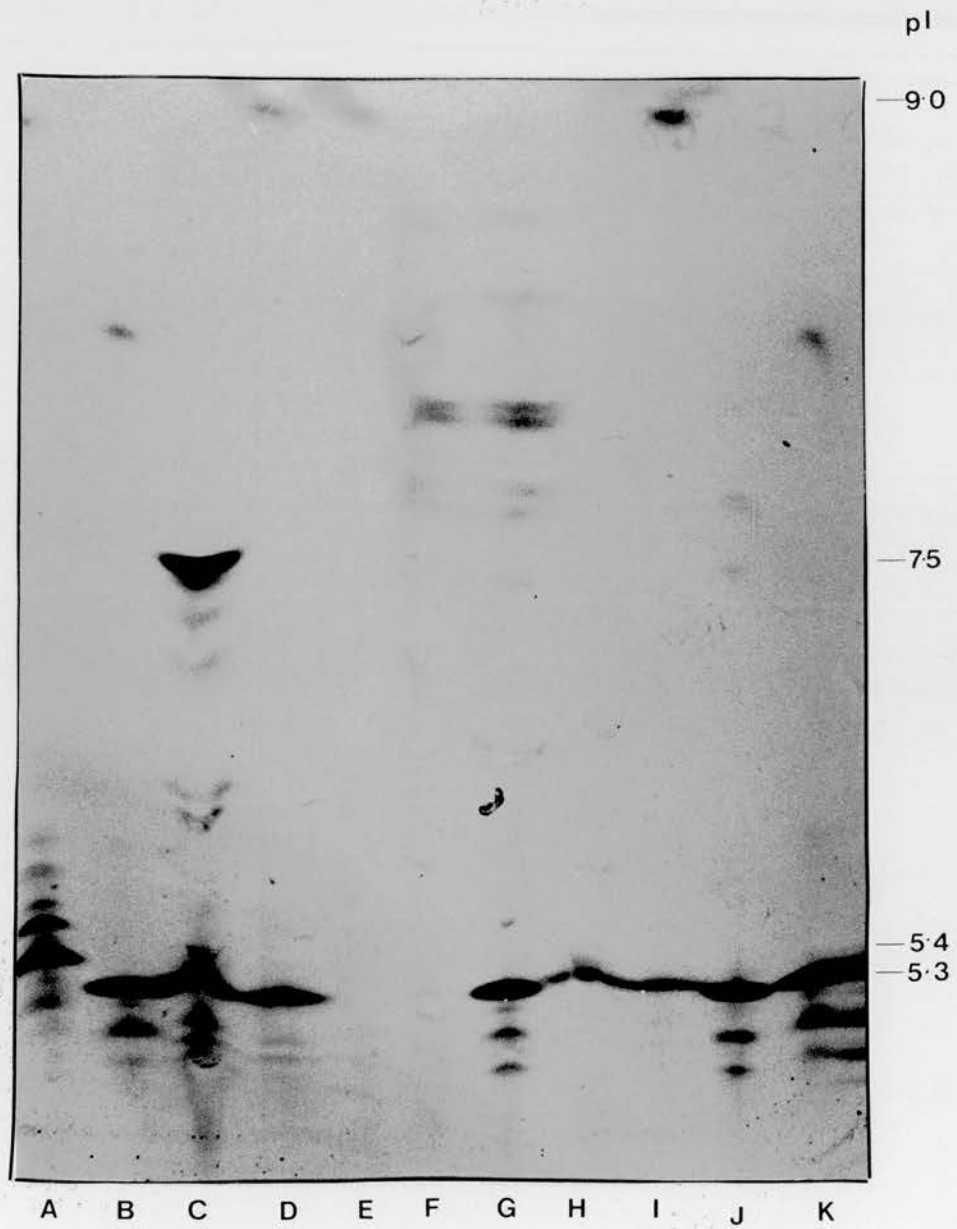
During the preliminary ß-lactamase survey K. pneumoniae 241 and E. cloacae A113 were found to produce a ß-lactamase, which when focused on a pH 3.5 - 10.0 IEF gradient appeared identical to the prototype PSE-4 of the P. aeruginosa Dalgleish strain (Figure 27). The ß-lactamases from both strains focused as a main band with a pI of 5.3 with two satellite bands at pIs 5.0 and 4.8. Species specific ß-lactamases were observed in both strains, the K. pneumoniae ß-lactamase at pI 7.5 and the E. cloacae ß-lactamase at pI 9.0.

When the IEF was repeated with a mixture of narrow range (pH 4-6) and broad range (pH 3.6-10) ampholines (50% v/v), finer determination of the isoelectric points was possible (Figure

Figure 27

Comparative isoelectric focusing (pH 3.5-10.0) of β-lactamase enzymes extracted from dal 1 and dal 2 containing strains.

Track A - TEM-1
Track B - PSE-4
Track C - K.pneumoniae 241 dal 1
Track D - E.coli J62-2 dal 1
Track E - E.coli J62-2
Track F - P.aeruginosa PA08
Track G - P.aeruginosa PA08 dal 1
Track H - E.cloacae A113 dal 2
Track I - E.coli J62-2 dal 2
Track J - P.aeruginosa PA08 dal 2
Track K - PSE-4



28). The pI of the β-lactamases from the two clinical strains was marginally higher (0.05 pH units) than the Dalgleish PSE-4 enzyme.

When the PA08 transconjugants of both K. pneumoniae 241 and E. cloacae A113 were examined by expanded gradient IEF (Figure 28, Tracks F and K), the pI of the PSE-4 β-lactamase produced was identical to that of the prototype "Dalgleish" enzyme. However, the E. coli transconjugants showed a similar discrepancy in pI to that found with the original clinical isolates (Figure 28, Tracks D, E, I and J). Mobilisations of dal1 from the PA08 transconjugant with the R18-18 resulted in transconjugant E. coli J62-$_2$ which produced the prototype PSE-4 β-lactamase on IEF (Figure 29, Tracks A and B).

3.6 Substrate Profiles of the PSE-4 β-lactamase Enzymes in the Original Clinical Strains and Their Respective Transconjugants

The PSE enzymes can be distinguished by their rapid hydrolysis of carbenicillin and slower hydrolysis of cephaloridine. The rates of hydrolysis of penicillin G, carbenicillin, ampicillin and cephaloridine were measured by spectrophotometric assay for both the clinical strains and their transconjugants (Table 24).

In both cases the specific enzyme activities and the relative rates of hydrolysis of the enzymes from the

Figure 28

Comparative isoelectric focusing (expanded pH 4.0 - 6.0 range) of β-lactamase enzymes extracted from *dal* 1 and *dal* 2 containing strains.

Track A - TEM-1
Track B - PSE-4
Track C - *K.pneumoniae* 241 *dal* 1
Track D - *E.coli* $J62_2$ *dal* 1
Track E - *E.coli* J53 *dal* 1
Track F - *P.aeruginosa* PA08 *dal* 1
Track G - PSE-4
Track H - *E.cloacae* A113 *dal* 2
Track I - *E.coli* $J62_2$ *dal* 2
Track J - *E.coli* J53 *dal* 2
Track K - *P.aeruginosa* PA08 *dal* 2

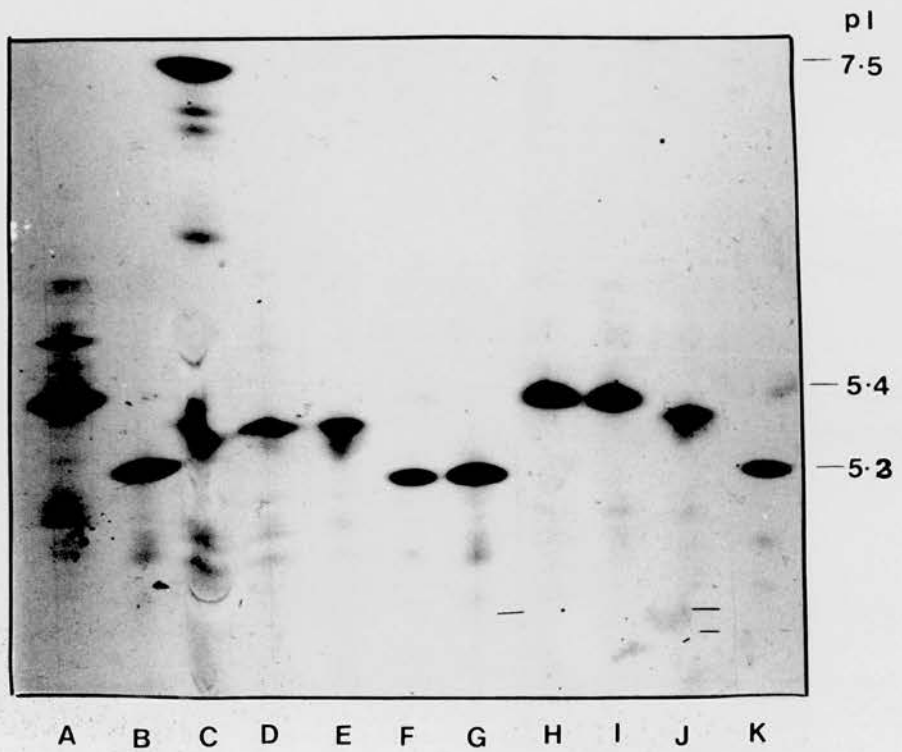


Figure 29
Comparative isoelectric focusing (expanded pH 4.0 - 6.0 range) of β-lactamase enzymes extracted from E.coli J62$_2$and E.coli J53 strains encoding dal 1 : R18-18.

Track A - PSE-4
Track B - E.coli J62$_2$dal 1 : R18-18
Track C - E.coli J53 dal 1 : R18-18

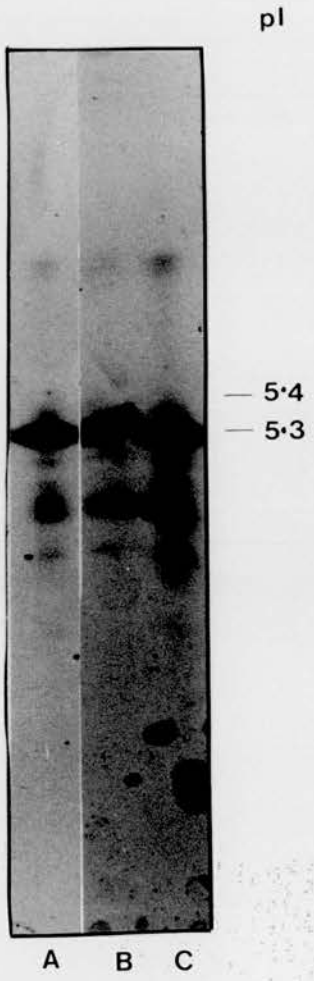


Table 24

Rates of Hydrolysis of PSE-4 Producing Strains

Enzyme Source	Benzyl Penicillin		Carbenicillin		Ampicillin		Cephaloridine	
	SA[a]	RR[b]	SA	RR	SA	RR	SA	RR
P. aeruginosa "Dalgleish"	493.0	100	485.0	98	477.5	97	20.4	4
K. pneumoniae 241 *dal1*	49.6	100	5.3	10	40.6	82	16.1	32
E. coli J62-$_2$ *dal1*	34.0	100	5.0	15	6.4	19	20.2	59
P. aeruginosa PA08 *dal1*	700.0	100	652.0	93	586.7	84	42.2	6
E. cloacae dal2	26.4	100	6.1	23	9.8	37	7.3	28
E. coli (R751 *dal2*)	40.0	100	6.0	16	6.1	20	21.0	60
P. aeruginosa PA08 (R751 *dal2*)	690.0	100	697.0	98	524.0	80	73.0	10
E. coli J62-$_2$	1.0	-	1.0	-	1.0	-	1.0	-
P. aeruginosa PA08	1.0	-	1.0	-	1.0	-	10.0	-

[a]SA = Specific activity is expressed as n Moles of substrate hydrolysed per minute per mg protein.

[b]RR = Relative rate - activity relative to that of benzyl penicillin which is set at 100%.

P. aeruginosa PA08 transconjugants closely resembled that of the P. aeruginosa "Dalgleish" strain. The β-lactamase enzymes from the clinical strains and their E. coli transconjugants resembled each other and they differed from the Dalgleish enzyme in their slower hydrolysis of carbenicillin and ampicillin. The values for the K. pneumoniae 241 β-lactamase extract are inflated because of the presence of the active chromosomal enzyme.

3.7 The Enzyme Kinetics of the β-lactamase Enzymes from the K. pneumoniae dal1 Transconjugants

The β-lactamase enzymes from the E. coli J62-$_2$ (pUK700 dal1), P. aeruginosa PA08 dal1 and E. coli J62-$_2$ (R18-18 dal1) transconjugant strains were purified from the chromosomal β-lactamase by preparative isoelectric focusing.

The Km and Vmax values of these three β-lactamases for carbenicillin and cephaloridine were determined as previously described by spectrophotometric assay (Table 25).

The Km values for cephaloridine were similar for all three β-lactamases with the E. coli (R18-18 dal1) β-lactamase having the highest affinity. The β-lactamase from the E. coli dal1 transconjugant had the highest affinity for carbenicillin, with the other transconjugants having similar affinity to each other.

Table 25

Enzyme Kinetics of the dal1 β-lactamase from Three transconjugants

	β-lactamase					
	E. coli J62-2 *dal1*		*P. aeruginosa dal1*		*E. coli* J62-2 (R18-18 *dal1*)	
Substrate	Km[a]	Vmax[b]	Km	Vmax	Km	Vmax
Cephaloridine	357	2.8	179	0.22	79	0.04
Carbenicillin	446	0.49	2380	11.1	2780	0.91

a μM

b μM substrate hydrolysed $minute^{-1}$ ml $enzyme^{-1}$

The Vmax values varied considerably for the β-lactamases produced by the transconjugants. The *E. coli dal1* transconjugant had the highest Vmax value for cephaloridine, which was greater than the specific acitivity value implying that in the previous assay the Vmax had not been reached. The *P. aeruginosa* PA08 *dal1* transconjugant had the highest Vmax value for carbenicillin with both *E. coli* β-lactamase enzymes giving similar values. Again the Vmax values were much higher than the specific activity values obtained previously.

3.8 Molecular Weight Determinations

The molecular weights of the PSE-4 β-lactamases purified from *E. coli* J62-2 (pUK700 *dal1*), *P. aeruginosa* PA08 *dal1*, *E. coli* J62-2 (R18-18 *dal1*) and *P. aeruginosa* "Dalgleish" were determined by gel filtration (Table 26).

Table 26

Molecular Weights of PSE-4 β-lactamase Enzymes

Strain	β-lactamase Molecular Wt.
E. coli J62-2 (pUK700 *dal1*)	31,000
P. aeruginosa PA08 *dal1*	31,000
E. coli J62-2 (R18-18 *dal1*)	31,500
P. aeruginosa "Dalgleish"	31,800

The molecular weights of these β-lactamase enzymes did not differ significantly either from each other or from the prototype Dalgleish PSE-4 β-lactamase.

The dal1 and dal2 determinants express much higher levels of PSE-4 production in P. aeruginosa PA08 than in the Enterobacteriaceae. The differences observed in pI and the Vmax values of PSE-4 in the different strains suggests that the biochemical and biophysical properties of the PSE-4 enzyme are host dependent.

THE INC P-1 PLASMID R751

4. Introduction

The incP-1 plasmids such as R751 are invaluable genetic tools because of their exceptionally broad host range (Ward and Grinsted 1982). The plasmid R751 (Jobanputra and Datta 1974) has been used for incompatibility testing (Datta 1974) as well as for genetic analysis and manipulation (Hedges and Jacob 1975, Meyer and Shapiro 1980, Ward and Grinsted 1982).

The majority of the incP-1 plasmids are resistant to ampicillin and can therefore not be used in the analysis of β-lactam resistance genes. R751 however, confers resistance to trimethoprim only, which is encoded by Tn402 (Shapiro and Sporn 1977) and this plasmid is therefore used in the manipulation of β-lactam resistance determinants (Hedges and Jacob 1975).

As described in the previous chapter, R751 was used as a broad host range plasmid to mobilise the PSE-4 determinant dal2 from E. cloacae to E. coli $J62\text{-}_2$ and P. aeruginosa PA08. During this work, R751 itself was found to have several previously undescribed properties which have been examined in the following chapter.

4.1 Results

The incP-1 plasmid R751 was found to confer a significant degree of carbenicillin and ampicillin resistance on its host strain E. coli $J62\text{-}_2$ during routine sensitivity testing. In order to confirm that this was not an isolated occurrence with the plasmid from our own laboratory culture collection, a culture of E. coli $J62\text{-}_1$ (R751) was obtained from the National Plasmid Collection at the Central Public Health Laboratories, Colindale. The MICs of carbenicillin (CARB), ampicillin (AMP), cefuroxime (CXM), cephaloridine (CER) and trimethoprim (Tp) were determined for the laboratory strain E. coli $J62\text{-}_2$ (R751) and the Colindale strain E. coli $J62\text{-}_1$ (R751) (Table 27). Strains were inoculated on to DSTA plates containing doubling dilutions of antimicrobial drugs, the inoculum used was 10^6 CFU ml^{-1}.

Table 27

Antimicrobial Sensitivity of R751 Strains

Strain	MIC (mgL^{-1})				
	CARB	AMP	CXM	CER	Tp
E. coli $J62\text{-}_2$ (R751)	16	16	4	8	>1000
E. coli $J62\text{-}_1$ (R751)	16	16	4	8	>1000
E. coli J62-2	0.5	0.5	0.5	1	2
E. coli $J62\text{-}_1$	0.5	0.5	0.5	1	2

The two strains did not appear to differ and the *E. coli* $J62\text{-}1$ (R751) strain from the National Plasmid Collection was also found to be resistant to low levels of carbenicillin and ampicillin. The *E. coli* $J62\text{-}1$ (R751) strain from Colindale was used in further transfer experiments.

4.2 Transfer Studies

Trimethoprim resistance encoded by *E. coli* $J62\text{-}1$ (R751) transferred to *E. coli* $J62\text{-}2$ at a frequency of 7.2×10^{-3} per donor cell after an overnight mating, selecting for trimethoprim ($10 mgL^{-1}$) and rifampicin ($20 mgL^{-1}$) resistant transconjugants on suitably supplemented minimal medium. When selection was made for ampicillin ($8 mgL^{-1}$) and rifampicin resistant transconjugants, no transfer of R751 was observed. Similarly, no transconjugants were isolated when selection was made on minimal plates containing trimethoprim, ampicillin and rifampicin at the same concentrations.

In order to study the properties of the ß-lactam resistance of R751, this plasmid was transferred to *E. coli* J62 which had no chromosomal resistance mutations. This conjugation was carried out from *E. coli* $J62\text{-}1$ via *E. coli* J53 to allow selection to be made for the transfer of the R751 plasmid.

The transfers of R751 to *E. coli* J53, *E. coli* J62 and the other bacterial strains are detailed in Table 28. All

donors were E. coli J62-1 (R751) except, as described above, for the transfer into E. coli J62. All matings lasted 18 hours and selection was made on Davis and Mingioli agar plates containing the supplements listed and trimethoprim at the concentration given in Table 28.

Table 28

Transfer of the R751 Plasmids to Various Bacterial Host Strains

Recipient strains	Selection	Frequency of transfer per viable donor cell
E. coli J53	Tp_{10} Pro Met	4.4×10^{-3}
E. coli J62	Tp_{10} His Pro Trp	6.6×10^{-4}
E. coli PB1150 recA	Tp_{10} His	6.7×10^{-4}
E. coli 114	Tp_{10}	9.8×10^{-7}
P. aeruginosa PA08	Tp_{1000} Ilv Met	3.7×10^{-5}

These transconjugants were used in further studies of the R751 plasmid.

4.3 Mutation Frequencies of Strains Containing the R751 Plasmid to Carbenicillin Resistance

The mutation frequencies to carbenicillin resistance ($10 mgL^{-1}$) for E. coli J62 (R751), E. coli PB1150 recA (R751) and E. coli 114 (R751) and also carbenicillin resistance ($125 mgL^{-1}$) for P. aeruginosa PA08 (R751) were compared with

the mutation frequencies for the isogenic strains lacking the plasmid. Overnight nutrient broth cultures (4.5mls) were washed in Davis and Mingioli minimal medium and then resuspended in (4.5mls) minimal medium. These suspensions were plated on to DSTA plates containing carbenicillin. The culture was diluted in tenfold steps (in minimal medium) to 10^{-6} and each dilution was plated on the same medium. The mutation ~~rates~~ frequencies per viable cell for each strain are given in Table 29.

The plasmid R751 confers a low level of resistance to carbenicillin and ampicillin in E. coli J62 and in E. coli PB1150 recA and also increases the mutation frequency of resistance to carbenicillin in E. coli J62 and E. coli PB1150 recA (Table 29). Elevated mutation frequencies to ampicillin resistance ($10mgL^{-1}$) were also found, 3.7×10^{-4} for E. coli J62 (R751) and 4.0×10^{-6} for E. coli PB1150 recA.

The expression of this resistance to ampicillin and carbenicillin and its mechanism were examined further.

Colonies of E. coli J62 (R751) taken from both a carbenicillin plate and an ampicillin plate ($10mgL^{-1}$) were purified and the MICs of both carbenicillin and ampicillin for these strains were determined.

Both the strains isolated from the carbenicillin ($10mgL^{-1}$) plate E. coli (R751C) and the strain isolated from the ampicillin ($10mgL^{-1}$) plate E. coli J62 (R751A) had the same

Table 29

Mutation Rates of R751 Containing Strains to Carbenicillin Resistance

	Mutation Frequency per Viable Cell		Fold Increase $\left(\frac{\text{R751 strain}}{\text{isogenic strain}}\right)$
	Strain containing R751	Isogenic strain	
E. coli J62	1.6×10^{-4}	2.5×10^{-8}	6.4×10^{3}
E. coli PB1150 *recA*	1.27×10^{-6}	9.3×10^{-9}	1.4×10^{2}
E. coli 114	1.1×10^{-8}	3.4×10^{-8}	0.32
P. aeruginosa PA08	2.0×10^{-9}	1.0×10^{-9}	2

MICs of carbenicillin and ampicillin, (125mgL^{-1} compared with 16mgL^{-1} for strains which had not been pre-exposed to these β-lactams).

In order to determine whether these changes had arisen from physical alterations of the R751 plasmids in these strains, restriction enzyme analysis was carried out.

4.4 Restriction Enzyme Analysis of the R751 Plasmid

The R751 plasmid isolated from *E. coli* J62 was compared by restriction endonuclease digest to the plasmids isolated from both the high level ampicillin resistant strain, *E. coli* J62 (R751A) and the high level carbenicillin resistant strain, *E. coli* J62 (R751C). The plasmids were isolated from the three strains and digested with either *Hind*III or *Eco*R1 restriction endonucleases (Figure 30). No differences were seen in the restriction patterns of these three plasmids. It appears, therefore, that there are almost certainly no changes in the DNA of plasmids R751A or R751C when compared with R751, and that these plasmids are the same.

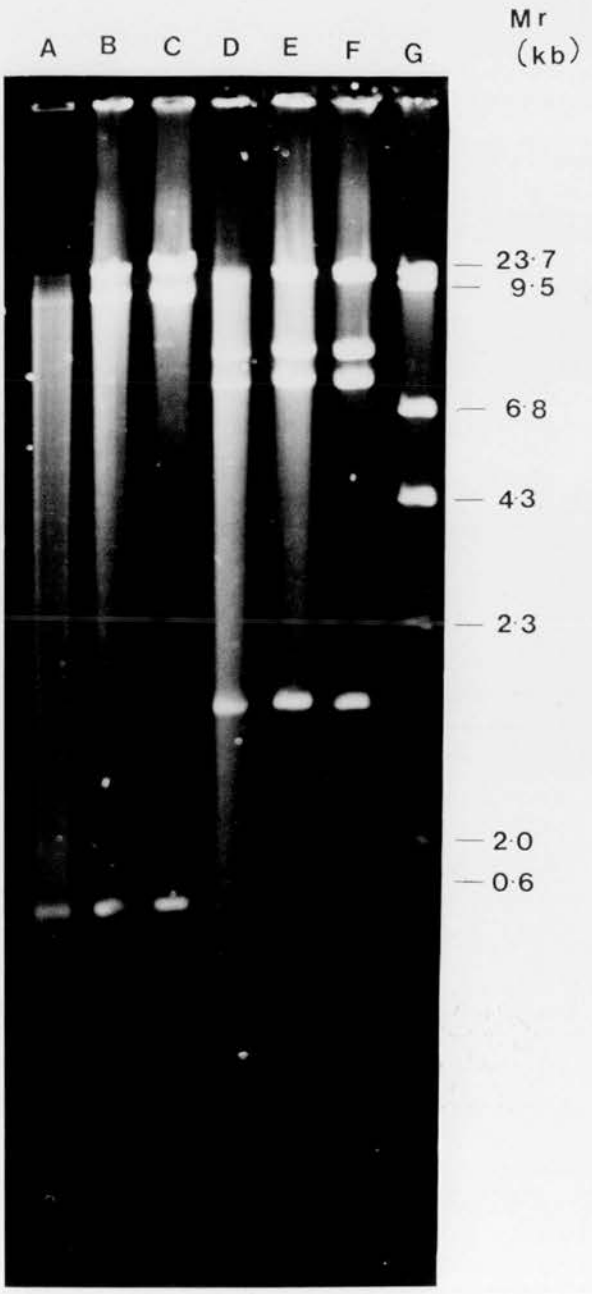
4.5 Mutation Frequencies of R751 Containing Strains to a Variety of Antimicrobial Agents

It had been found that R751 in *E. coli* J62 and *E. coli* PB1150 increased the mutation frequency to carbenicillin resistance. It was decided to see if R751 increased the

Figure 30

Agarose gel electrophoresis of restricted DNA extracted from R751 containing strains.

Track A - E.coli J62 (R751A) Track B - E.coli J62 (R751C)
Track C - E.coli J62 (R751) Track D - E.coli J62 (R751A)
Track E - E.coli J62 (R751C) Track F - E.coli (R751)
Tracks A,B and C were digested with EcoRI; Tracks D,E and F were digested with HindIII.
Track G - λ (HindIII)



mutation rates of resistance to other β-lactam and non-β-lactam antimicrobial drugs in *E. coli* J62 and *E. coli* 114.

Mutation rates were determined as before, plating tenfold dilutions of washed overnight cultures of the strains. The antimicrobial drugs tested were cephaloridine (CER), streptomycin (Sm), chloramphenicol (Cm), nalidixic acid (Na) and tetracycline (Tc). All were tested at $10mgL^{-1}$ in DSTA plates and comparison was made with the results previously obtained for carbenicillin (4.3). The results obtained are detailed in Table 30.

As expected, low mutation frequencies were observed with *E. coli* J62 and *E. coli* 114 which did not contain R751. The *E. coli* J62 (R751) strain had increased mutation frequencies to cephaloridine, streptomycin and tetracycline as well as carbenicillin. However the *E. coli* J62 (R751) strain which had mutated to high level ampicillin and carbenicillin resistance had low mutation frequencies to cephaloridine, streptomycin and tetracycline resistance and increased mutation frequencies to chloramphenicol and nalidixic acid resistance. The *E. coli* 114 (R751) strain had low mutation frequencies for all the antimicrobial drugs tested.

Colonies of *E. coli* J62 which had mutated to antimicrobial drug resistance were taken from the DSTA plates containing the individual drugs and tested for cross-resistance to the other antimicrobial agents. None of the mutant colonies had cross-resistance to the remaining antimicrobial drugs

Table 30

Mutation Frequencies of R751 Containing Strains to a Variety of Antimicrobial Agents

Strains	Mutation Frequencies					
	CARB	CER	Sm	Cm	Na	Tc
E. coli J62	2.5×10^{-8}	1.4×10^{-9}	3.6×10^{-9}	$< 10^{-11}$	1.1×10^{-9}	1.7×10^{-10}
E. coli J62 (R751)	1.6×10^{-4}	7.4×10^{-3}	6.9×10^{-3}	9.3×10^{-10}	5.9×10^{-9}	7.7×10^{-3}
E. coli J62 (R751A)	-	1.2×10^{-8}	7.2×10^{-9}	1.7×10^{-4}	8.7×10^{-3}	$< 10^{-11}$
E. coli 114	3.2×10^{-8}	3.2×10^{-9}	6.7×10^{-9}	$< 10^{-11}$	$< 10^{-11}$	$< 10^{-11}$
E. coli 114 (R751)	1.1×10^{-8}	1.2×10^{-9}	4.6×10^{-8}	$< 10^{-11}$	8.9×10^{-10}	$< 10^{-11}$

although some mutant colonies from carbenicillin plates grew on cephaloridine plates and vice versa.

In contrast, with strains containing the R751 plasmid, mutations which had resulted in resistance to a particular antimicrobial drug also produced cross-resistance to several other antimicrobial agents. This was especially true of E. coli J62 (R751) mutant colonies isolated from DSTA plates containing individual antimicrobial drugs (Table 31).

Table 31

Cross Resistance of E. coli J62 (R751) Mutant Strains

Antimicrobial drug from which mutant colonies were isolated	Cross resistance to other antimicrobial drugs[a]					
	CARB	CER	Sm	Na	Tc	Cm
Carbenicillin	+	-	+/-	+	-	+
Cephaloridine	+	+	+	-	+	-
Streptomycin	+	+	+	+	+	-
Nalidixic Acid	+	-	-	+	-	+
Tetracycline	+	+	+	+	+	+
Chloramphenicol	+	+	+	+	+	+

a +, growth; -, no growth; +/-, poor growth

It appeared from these results that the presence of the R751 plasmid in E. coli J62 may encourage a mutation or mutations which produced broad spectrum antimicrobial drug resistance.

4.6 Response of E. coli J62 (R751) to Increasing Doses of Ampicillin

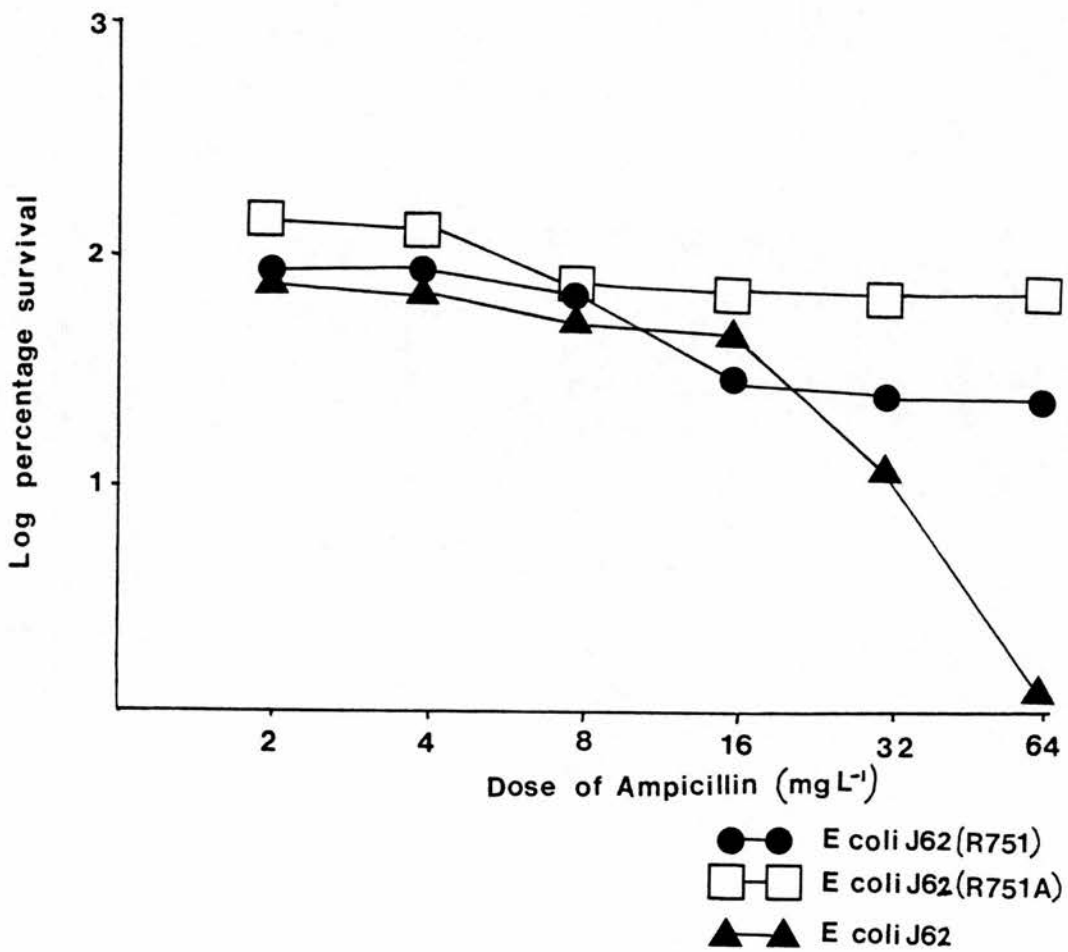
Two E. coli J62 strains containing R751 were studied further; E. coli J62 (R751) which had not been pre-exposed to ampicillin or carbenicillin (MIC = 16mgL^{-1}), and E. coli J62 (R751A) which had been pre-exposed to ampicillin and had an MIC of 125mgL^{-1}. In order to assess whether these two strains had different responses to ampicillin, they were challenged by increasing doses of ampicillin and their response was compared to that of the sensitive strain E. coli J62.

Overnight broth cultures were diluted in isosensitest broth to give a viable count of 9.1 x 10^6 bacteria ml^{-1} (E. coli J62); 3.2 x 10^7 bacterial ml^{-1} (E. coli J62 (R751)) and 8.8 x 10^6 bacterial ml^{-1} (E. coli J62 (R751A)). Doubling dilutions of ampicillin (64 - 2mgL^{-1}) were added to 5ml amounts of the cultures which were incubated statically overnight and the viable counts determined (Figure 31).

The results show that E. coli J62 exhibited the expected bactericidal response to increasing doses of ampicillin. Both E. coli J62 (R751) and E. coli J62 (R751A) were less susceptible to the action of ampicillin and were

Figure 31

The growth of plasmid-containing strains (E.coli J62 (R751) and E.coli J62 (R751A)) in increasing concentrations of ampicillin compared to the growth of the isogenic strain (E.coli J62).



bacteriostatic in their response. Although the two strains containing R751 differed markedly in their MICs of ampicillin no great difference was seen in their dose response to ampicillin.

It appeared that E. coli J62 (R751) and E. coli J62 (R751A) had a resistance mechanism(s) to ampicillin that enabled them to overcome the bacteriocidal action of this drug. Therefore, growth of E. coli J62 (R751) was examined in the presence of a fixed concentration of ampicillin.

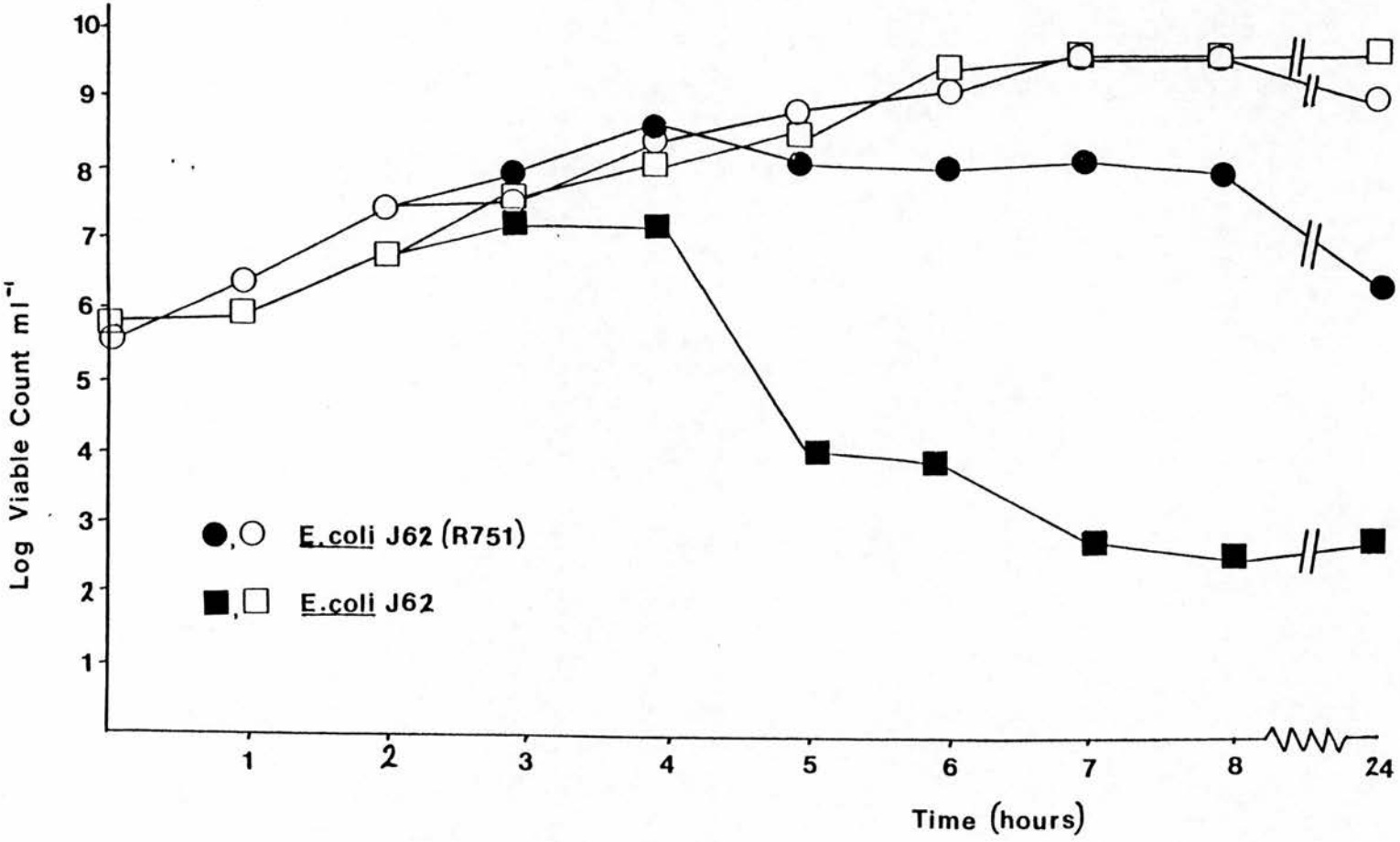
4.7 Growth of E. coli J62 (R751) in the Presence of a Constant Concentration of Ampicillin

The effect of ampicillin ($5mgL^{-1}$) on the growth of a logarithmic (log) phase culture of E. coli J62 (R751) was examined. This was compared with the effect of the same concentration of ampicillin on a log phase culture of E. coli J62.

Static overnight isosensitest broth cultures of the two strains were diluted 1 in 10^{-4} in prewarmed broth to get cultures into log phase, and the viable counts were determined every hour. Each culture was divided in half at two hours and ampicillin ($5mgL^{-1}$) added to one half only (Figure 32).

Ampicillin had a bactericidal effect on a log phase culture of E. coli J62 and the viable count dropped after the addition of ampicillin $5mgL^{-1}$ at two hours. The effect of

Figure 32 The growth curve of E.coli J62 (R751) in the presence and absence of 5mgL^{-1} of ampicillin, compared with the isogenic strain E.coli J62
Shaded symbols are cultures to which 5mgL^{-1} ampicillin was added at time=2hours.



ampicillin on the viable count of the E. coli J62 (R751) strain was markedly different from that of E. coli J62. Ampicillin had no immediate effect on the growing E. coli J62 (R751) culture. The culture however did enter stationary phase at around 10^8 organisms/ml. This was one order of magnitude lower than seen with the control culture.

These results, in effect, confirmed those of the dose response curve, indicating that R751 had allowed the E. coli J62 strain to overcome the bactericidal effect of ampicillin.

4.8 Possible Resistance Mechanisms to Ampicillin and Carbenicillin Mediated by the R751 Plasmid

Outer membrane proteins and penicillin binding proteins

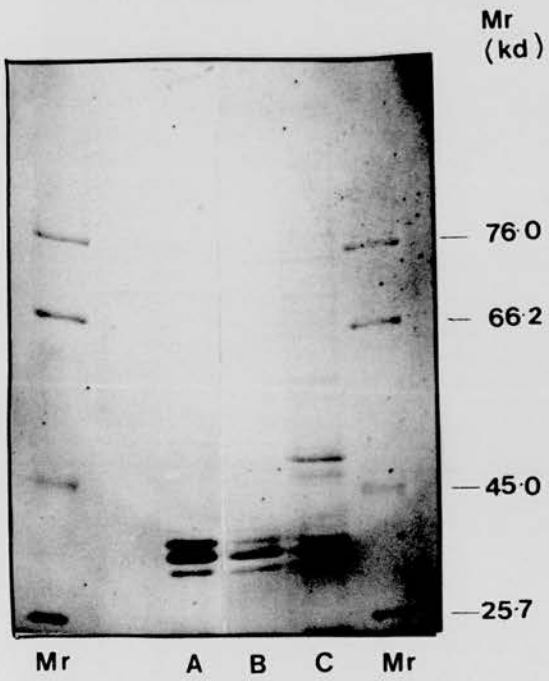
The outer membrane proteins (OMPs) and penicillin binding proteins (PBPs) of E. coli J62 (R751) and E. coli J62 (R751A) were examined in order to determine whether R751 was causing a mutation in these types of protein which resulted in a resistant phenotype.

No major changes were seen in the outer membrane proteins of E. coli J62 (R751) when compared with E. coli J62 (Figure 33, Lanes A and B). The major outer membrane proteins, OmpA, OmpC and OmpF were present in both strains. An outer membrane preparation of E. coli J62 (R751A) was also examined (Figure 33, Lane C). The three major OMPs were

Figure 33

SDS-PAGE of outer membrane proteins extracted from R751 containing strains.

Track A - E.coli J62 Track B - E.coli J62 (R751)
Track C - E.coli J62 (R751A)



present in this strain, however, some minor outer membrane proteins appeared to be over-produced when compared to the *E. coli* J62 profile.

Densitometry traces of the SDS-PAGE gel were made to compare the relative amounts of each major outer membrane protein in the three strains (Figure 34). OmpC and OmpA appeared to be present in the same relative amounts, however the porin OmpF was produced in smaller proportions in both strains which contained R751 when compared with *E. coli* J62 without the plasmid. A mutation, resulting in the reduction of the amount of porin protein OmpF (the larger porin channel in *E. coli*) may explain the broad spectrum drug resistance of *E. coli* J62 (R751) strains (Section 4.5). Reduction in the permeability of the outer membrane can affect the sensitivity of bacteria to a variety of antimicrobial compounds.

The PBPs of both R751 containing strains were compared with those produced by *E. coli* J62 which did not contain the plasmid. Competition assays with non-radioactive ampicillin were carried out in order to obtain S_{50} values for each of the PBPs (materials and methods). Low S_{50} values indicate that the competing ampicillin had high affinity for the PBP.

Figure 35 shows the fluorographs obtained for strains *E. coli* J62, *E. coli* J62 (R751) and *E. coli* J62 (R751A). The most obvious difference between the strains was that *E. coli* J62 (R751^{A}) appeared to have a very low affinity

Figure 34

Densitometry traces of the outer membrane protein profiles of E.coli J62 strains containing the plasmids R751A (a) and R751 (b), compared to the isogenic strain E.coli J62 (c)

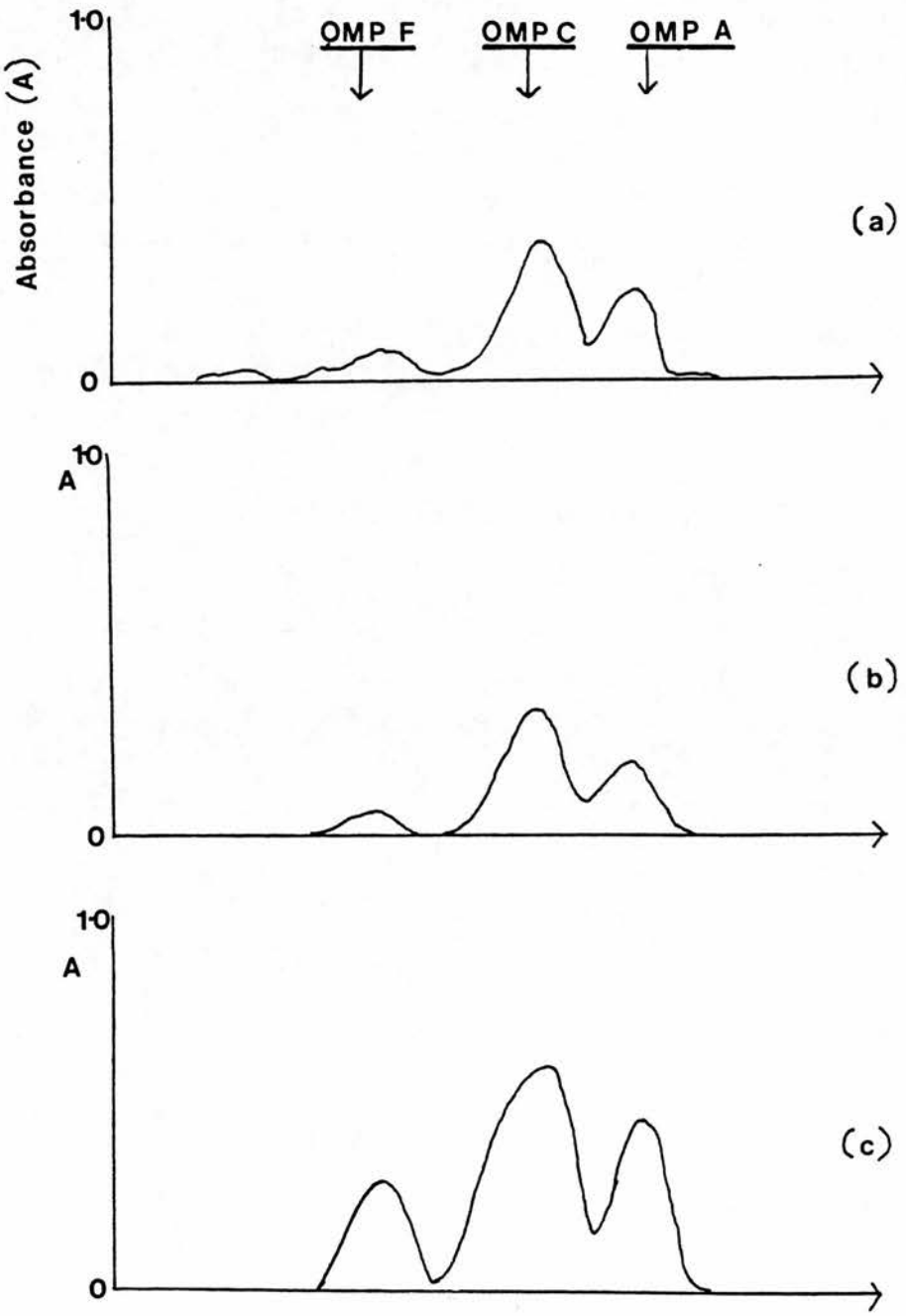
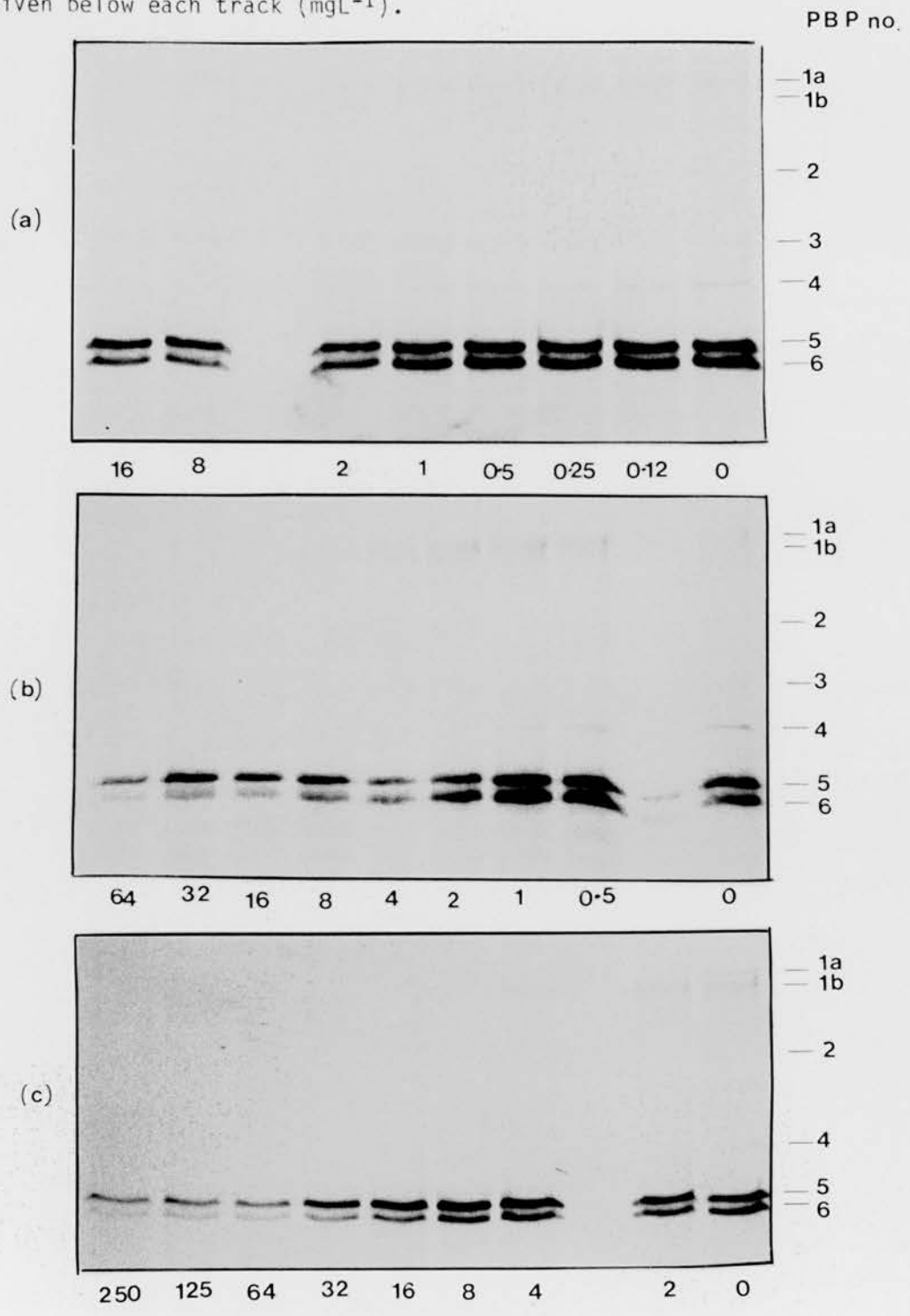


Figure 35

Fluorographs of penicillin binding proteins extracted from (a) E.coli J62 (b) E.coli J62 (R751) and (c) E.coli J62 (R751A). The concentrations of ampicillin used in the competion assays are given below each track (mgL^{-1}).



PBP3. This PBP is the principal lethal target for ampicillin in *E. coli* and its loss would explain the high MIC of ampicillin for *E. coli* J62 (R751A) of 125mgL^{-1}. The ampicillin S_{50} values for each of the strains are given in Table 32.

Table 32

Binding of Ampicillin to Target Proteins

Strain	Ampicillin MIC (mgL-1)	Ampicillin S50 value for PBPs[a]						
		1a	1b	2	3	4	5	6
E. coli J62	0.5	0.5	4	0.5	0.5	2	>16	>16
E. coli J62 (R751)	16	0.5	4	4	1.0	2	64	8
E. coli J62 (R751A)	125	2	4	2	-	2	64	16

a See materials and methods (mgL^{-1})

The *E. coli* J62 (R751) strain appears to have a similar PBP profile to that of *E. coli* J62, although PBP2 may have less affinity for ampicillin. It is probable that changes in the affinity of the PBPs do not account for the ampicillin resistance of *E. coli* J62 (R751) in the same way as they do for *E. coli* J62 (R751A).

4.9 Isoelectric Focusing of Cell-Free Extracts from R751 Containing Strains

The results obtained from OMP and PBP studies might not account wholly for the ampicillin resistance of the R751-containing strains. Therefore, the resistant strains were examined for the presence of β-lactamase enzymes. IEF of cell free extracts from *E. coli* J62 (R751A) and *E. coli* J62 (R751C) did reveal a β-lactamase in addition to the chromosomal enzyme. The β-lactamase pI corresponded to that of SHV-1 on IEF (Figure 36a, Lanes E and F). No β-lactamase enzyme was seen in extracts from *E. coli* J62 (R751) which had not been pre-exposed to ampicillin. Similarly mutant *E. coli* J62 (R751) colonies from the cephaloridine and nalidixic acid plates (section 4.5) did not produce SHV-1 (Figure 36a, Lanes A, B and C). The *E. coli* 114 (R751) strain taken from an ampicillin plate ($10 mgL^{-1}$) produced very weak SHV-1 activity on IEF.

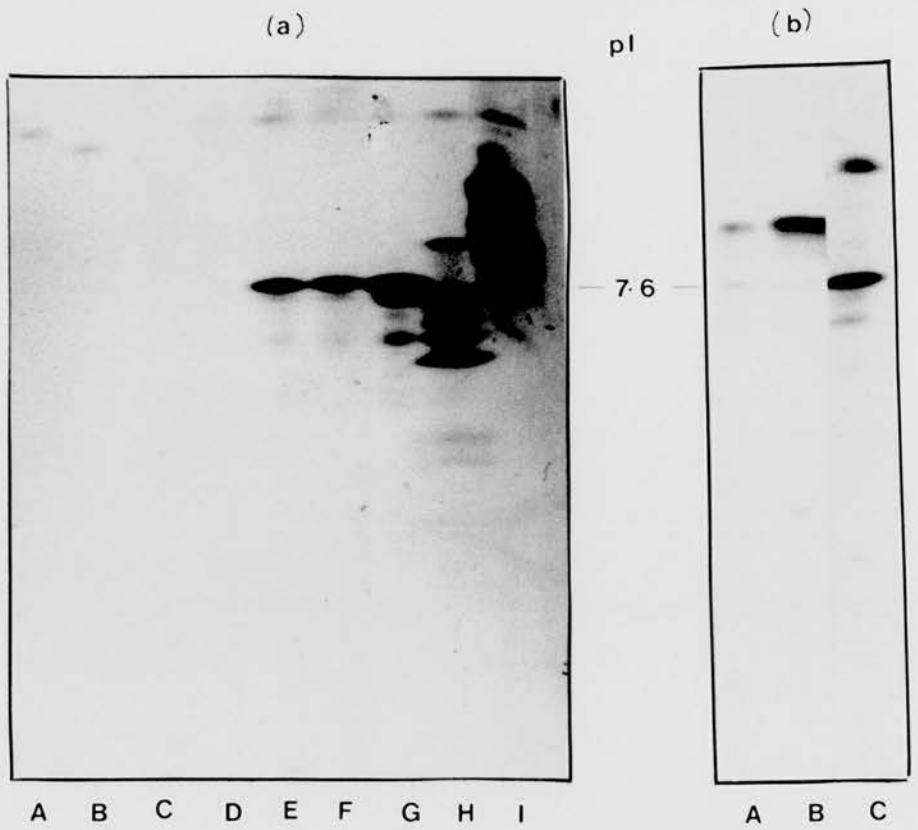
The cultures of *P. aeruginosa* PA08 (R751) and *E. coli* PB1150 *recA* (R751) did not produce this β-lactamase even after mutation to higher levels of ampicillin resistance (section 4.5). However, the culture of *P. aeruginosa* PA08 (R751) did produce large amounts of chromosomal β-lactamase in the region of the SHV-1 β-lactamase (pI 7.0 to 8.0) which made identification of SHV-1 difficult in this strain (Figure 36b, Lanes A, B and C). The SHV-1 β-lactamase has been identified in both chromosomal and plasmid locations.

Figure 36

Isoelectric focusing of cell-free extracts from R751-containing strains.

(a) Track A - *E.coli* J62(R751) Track B - *E.coli* J62(R751CER)
Track C - *E.coli* J62(R751Na) Track D - *E.coli* J62
Track E - *E.coli* J62(R751A) Track F - *E.coli* J62(R751C)
Track G - SHV-1 Track H - OXA-1 Track I - OXA-2

(b) Track A - *P.aeruginosa* PA08 Track B - *P.aeruginosa* PA08(R751A)
Track C - SHV-1



Therefore it was decided to cure the SHV-1 producing strain (*E. coli* J62 (R751A)) in order to determine the location of the β-lactamase gene.

4.10 Curing the E. coli J62 (R751) SHV-1 Producing Strains

To determine the genetic location of the SHV-1 β-lactamase gene a culture of *E. coli* J62 (R751A) which produced SHV-1 was cured of R751 and tested for the presence of the SHV-1 β-lactamase. Nutrient broth cultures (4.5mls) of *E. coli* J62 (R751A) were grown statically to log phase and 100µl of phage PRR1 was added (10^9 pfu ml^{-1}). Phage PRR1 only lyses those cells which contain R751. After overnight incubation, the culture was enriched for cells which had spontaneously lost the R751 plasmid. The overnight culture was diluted 10^{-6} in minimal media and plated on to nutrient agar, individual colonies were then tested for the loss of trimethoprim resistance. Nine trimethoprim sensitive clones were isolated (138 colonies were tested), purified and the loss of R751 from these bacteria confirmed by agarose gel electrophoresis (Figure 37). All nine clones were found to be resistant to both carbenicillin and ampicillin ($10mgL^{-1}$) but sensitive to tetracycline, chloramphenicol, gentamicin, nalidixic acid and streptomycin as well as trimethoprim (all tested at $10mgL^{-1}$).

When examined by IEF at least three of the nine clones produced SHV-1, albeit at a very low level (Figure 38). When sensitivity of all nine to ampicillin or carbenicillin

Figure 37

Agarose gel elecrophoresis of DNA extracted from ampicillin resistant clones of E.coli J62 cured of the R751 plasmid. Method of Takahashi and Nagano (1984).

Tracks A - I - E.coli J62 clones 1 to 9.
Track J - E.coli J62 (R751)
c=chromosomal DNA.

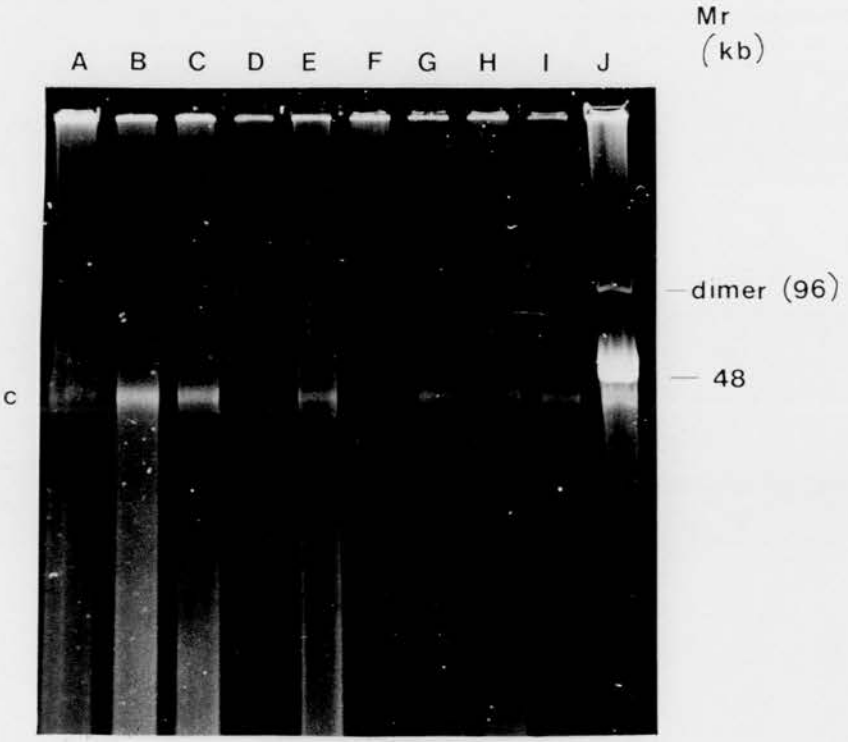
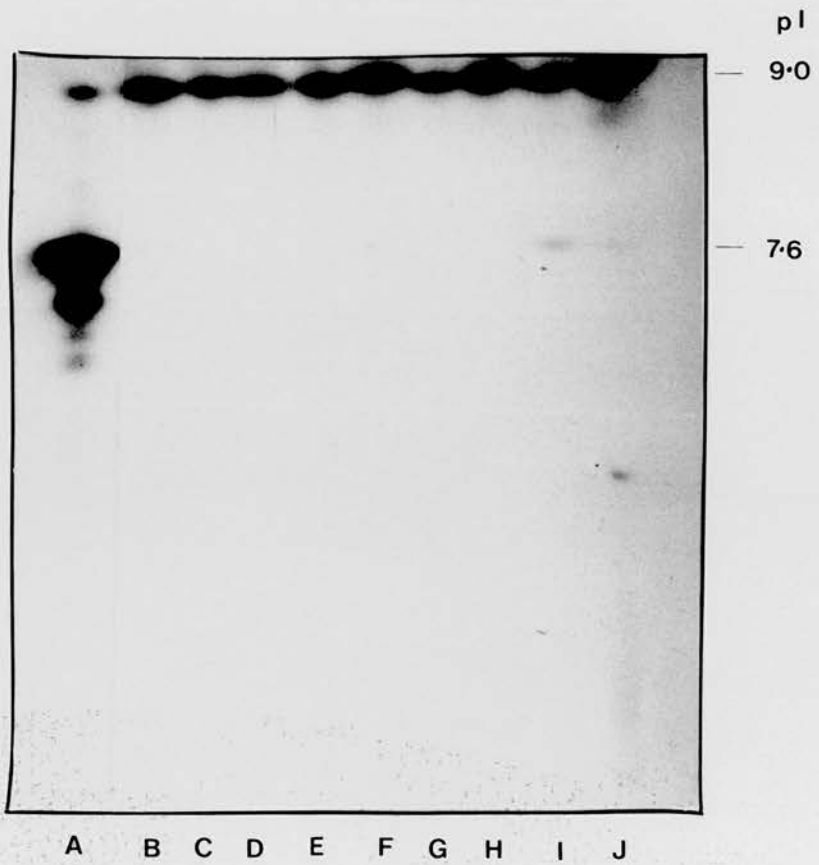


Figure 38

Isoelectric focusing of cell-free extracts from ampicillin resistant E.coli J62 cured of the R751 plasmid.

Track A - SHV-1
Tracks B to J, nine E.coli J62 ampicillin resistant clones cured of R751.



was tested in the presence of clavulanic acid (16mgL^{-1}), all were sensitive. As clavulanic acid is an inhibitor of the SHV-1 β-lactamase this would strongly suggest that SHV-1 is the resistance mechanism to ampicillin and carbenicillin in the nine cured strains and that the SHV-1 β-lactamase is situated on the chromosome of E. coli J62. The levels of production of SHV-1 were extremely low and it would be necessary to concentrate the enzyme extracts in order to visualise SHV-1 in the six cured E. coli J62 strains which did not appear to produce the β-lactamase.

It is possible that the SHV-1 gene may have transposed from the R751 plasmid to the chromosome of the cured strains. The high incidence of the β-lactamase amongst the cured strains (at least a third and probably as high as nine out of nine, in view of the clavulanic acid results) would be too high a frequency for such a transposition event.

An E. coli J62 strain which had been mutated to ampicillin resistance was also examined by IEF, and no additional β-lactamase activity was found. It appears therefore that the presence of R751 is required for the expression of the SHV-1 β-lactamase in the E. coli J62 chromosome.

The R751 plasmid appears to have the effect of mutating E. coli J62 strains to antimicrobial drug resistance. The same effect was also seen with carbenicillin when the plasmid was present in E. coli PB1150 recA. The recA strain was included to confirm that no generalised recombination

between R751 and the E. coli J62 chromosome was responsible for the increased mutation frequencies observed. The mechanism by which the R751 plasmid causes mutation remains undiscovered. It appears, however, that the presence of the R751 plasmid is necessary to maintain such a mutation or mutations conferring broad spectrum resistance to a variety of antimicrobial drugs. Cured E. coli J62 strains were found to be sensitive to all antimicrobial drugs tested except carbenicillin and ampicillin (section 4.10).

The mutation mechanism present on the R751 plasmid has resulted in the identification of a previously unexpressed SHV-1 β-lactamase enzyme, probably located on the chromosome of E. coli J62. When selection was made on ampicillin or carbenicillin containing plates, R751 appeared to promote a mutation which allowed the expression of this otherwise silent β-lactamase. It is possible that this enzyme is also produced by a non-K12 strain E. coli 114, in the presence of the R751 plasmid, however this result remains to be confirmed.

β-LACTAM RESISTANCE NOT ASSOCIATED WITH THE PRODUCTION OF A β-LACTAMASE ENZYME

5. Introduction

β-lactamase enzymes play a major role in bacterial resistance to β-lactam antibiotics (Richmond and Sykes 1973, Medeiros 1984). There are, however, increasing numbers of reports of enterobacterial strains which owe their β-lactam resistance to a mechanism other than the production of a β-lactamase. Changes in the PBPs and OMPs can both result in β-lactam resistance (reviewed by Spratt 1983, Nikaido 1985).

Transferable β-lactam resistance is mainly attributable to plasmid-mediated β-lactamase enzymes (reviewed by Medeiros 1984). There have been no reports of transferable genes encoding altered PBPs, although there have been reports of reductions in the porin proteins being associated with the presence of a plasmid (Iyer et al 1978, Rossouw and Rowbury 1984).

During the survey of cephaloridine resistant strains, a group of six strains was identified which could transfer resistance to β-lactams which was not β-lactamase mediated.

5.1 Results

Six enterobacterial strains were isolated from patients in Edinburgh's Royal Infirmary. The strains were resistant to low levels of ampicillin (AMP), cephaloridine (CER) and

cephradine (CED) in addition, some of the strains were resistant to tetracycline (Tc) and spectinomycin (Sp) (Table 33). The strains comprised one E. coli isolate, and five P. vulgaris isolates.

Table 33

Sensitivity of Clinical Isolates

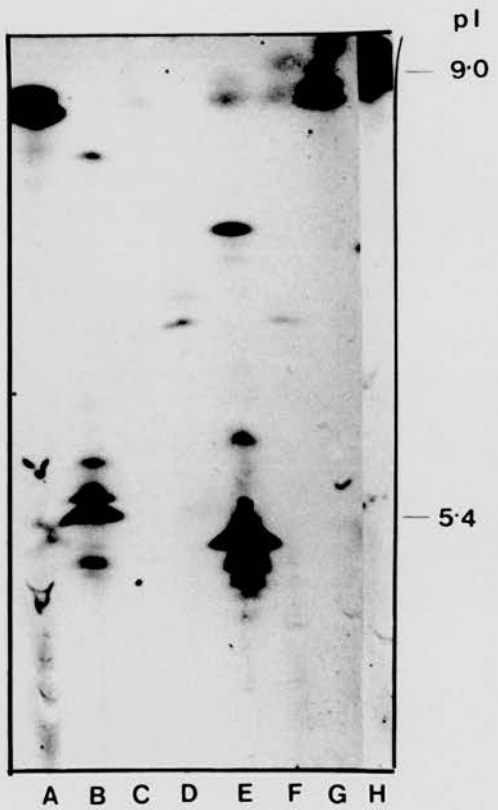
Strain number	Species	MICs CER	CED	AMP	Other Resistances
A182	E. coli	8	16	4	Tc
A261	P. vulgaris	32	32	16	TcSp
A263	P. vulgaris	16	64	16	Tc
A264	P. vulgaris	32	8	8	TcSp
A265	P. vulgaris	16	2	16	
A266	P. vulgaris	8	16	16	Sp

Cell free extracts were examined by analytical IEF and all six strains produced a ß-lactamase enzyme (Figure 39). Conjugation experiments were carried out to ascertain whether these ß-lactamase enzymes transferred to other bacterial strains.

Figure 39

Isoelectric focusing of β-lactamase enzymes from clinical isolates.

Track A - P.vulgaris A261
Track B - TEM-1
Track C - P.vulgaris A263
Track D - P.vulgaris A264
Track E - PSE-4
Track F - P.vulgaris A265
Track G - P.vulgaris A266
Track H - E.coli A182



5.2 Transfer of the β-lactam Resistance Genes

Conjugation experiments were carried out with *E. coli* J62-2 and *P. aeruginosa* PA08 as potential recipients. Overnight conjugation mixtures were plated on to Davis and Mingioli minimal agar plates containing appropriate supplements and antimicrobial drugs.

Initially, strain A182 was mated overnight with *E. coli* $J62-2$ and selection was made for transfer of either cephradine resistance ($8mgL^{-1}$), ampicillin resistance ($2mgL^{-1}$) or cephaloridine resistance ($4mgL^{-1}$). No transfer was observed when selection was made on cephaloridine containing plates, however transfer was observed when selection was made on cephradine (2.7×10^{-5} per donor cell) or ampicillin (8.1×10^{-6} per donor cell) containing plates.

The remaining five *P. vulgaris* strains were conjugated overnight with *E. coli* $J62-2$ selecting for transfer of ampicillin resistance and all transferred their resistance at the frequencies given in Table 34. Transfer of the β-lactam resistance to *P. aeruginosa* PA08 was attempted for all six strains. An overnight conjugation mixture was plated on to minimal media supplemented with amino acids, streptomycin ($100mgL^{-1}$) and carbenicillin ($125mgL^{-1}$). No transconjugants were isolated from any of the six conjugations.

Table 34

Transfer of Ampicillin Resistance to E. coli $J62-_2$

Strain	Transfer Frequency (per viable donor cell)
A182	8.1×10^{-6}
A261	1.3 x 10-6
A263	4.9×10^{-7}
A264	4.8 x 10-7
A265	9.3×10^{-7}
A266	1.0 x 10-6

It appeared therefore that the β-lactam resistance transferred from the clinical strains to E. coli $J62-_2$ but was not freely transferable to P. aeruginosa PA08 and the resistance determinant has a narrow host range. The E. coli $J62-_2$ transconjugants were further conjugated overnight with E. coli J53, with selection again made for transfer of ampicillin resistance. This experiment was to determine whether the frequency of transfer of the β-lactam determinants was higher or lower between strains of E. coli K12. The frequencies of transfer to E. coli J53 are given in Table 35.

Table 35

Transfer of Ampicillin Resistance from E. coli $J62\text{-}_2$ to E. coli J53

Original Clinical Strain	Transfer Frequency to E. coli J53 (per viable donor cell)
A182	2.8×10^{-7}
A261	3.5×10^{-7}
A263	3.8×10^{-7}
A264	3.0×10^{-7}
A265	3.2×10^{-7}
A266	4.4×10^{-7}

The transfer frequencies between E. coli K12 strains were all slightly lower than those observed from the clinical isolates to E. coli J62-2. Although the transfer frequency of the β-lactam resistance was low, no colonies grew on the control plates and the transfer frequency was approximately two logs higher than the mutation frequency of E. coli $J62\text{-}_2$ to ampicillin resistance (1.0×10^{-9} per viable cell).

The sensitivity of the E. coli $J62\text{-}_2$ transconjugants to a variety of antimicrobial agents was then tested, as shown in Table 36.

Table 36

Sensitivity of E. coli $J62-_2$ Transconjugants

Original Clinical Strains	MICs CER	CED	AMP	Other Resistances
A182	4	8	32	Tc
A261	4	32	32	Sp
A263	4	64	32	Tc
A264	4	8	16	
A265	4	16	16	
A266	4	32	16	Tc
E. coli $J62-_2$	4	8	2	

All the transconjugants were sensitive to cephaloridine but showed similar sensitivity to cephradine and ampicillin as seen in the clinical strains. However, the transconjugant of A182 is more resistant to ampicillin than the original clinical strain.

5.3 Isoelectric Focusing and DNA Analysis of Transconjugants

Isoelectric focusing of cell free extracts from the E. coli $J62-_2$ transconjugants was carried out to determine whether any of the clinical isolates had transferred a β-lactamase enzyme to E. coli $J62-_2$ which could account for the β-lactam resistance. The only β-lactamase visualised in the

transconjugants was the *E. coli* J62-$_2$ chromosomal enzyme. In addition, spectrophotometric assays were carried out with the crude β-lactamase extracts. Cephaloridine, cephradine and ampicillin were used as substrates in the assays, but no hydrolysis was observed.

The DNA of the six *E. coli* J62-$_2$ transconjugants was also examined for the presence of plasmids by the alkaline denaturation method (Figure 40). Only one transconjugant (that of A182) contained a plasmid which was designated pUK703 and was 97kbases in size. The plasmid pUK703 was seen in the clinical isolate and also in the *E. coli* J53 transconjugant. No plasmid DNA could be visualised in the five *P. vulgaris* *E. coli* J62-2 or J53 transconjugants.

It appears that in the case of *E. coli* A182, the β-lactam and tetracycline resistance is associated with plasmid pUK703, however, the genetic carrier of resistance in the *P. vulgaris* transconjugants is less clear.

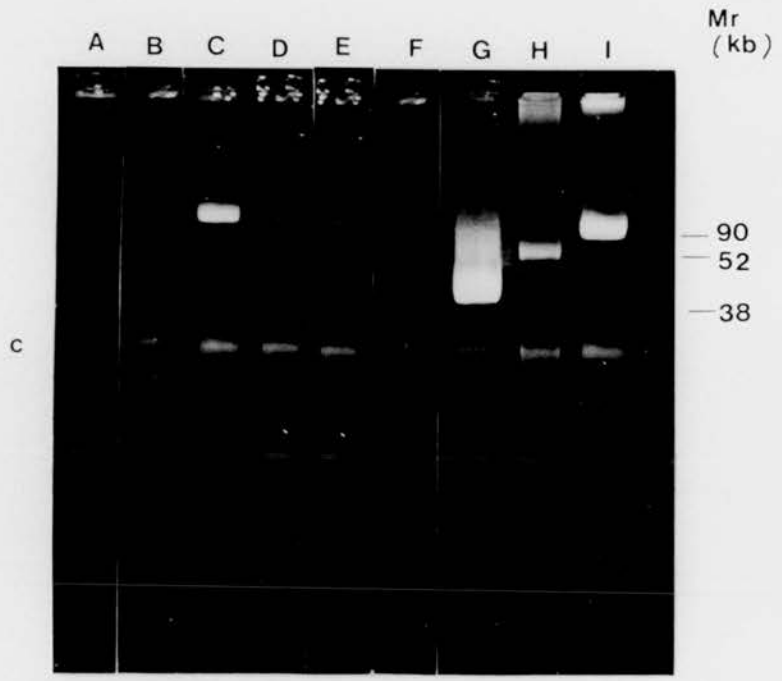
It does appear that some exchange of genetic material was taking place as the frequencies of transfer were higher than the strain mutation frequencies.

In the absence of an obvious β-lactamase to explain the resistance, the outer membrane proteins and penicillin binding proteins were examined.

Figure 40

Agarose gel electrophoresis of DNA extracted from the β-lactam resistant transconjugants.
Method of Takahashi and Nagano (1984).

Track A - E.coli $J62_2$(A261)
Track B - E.coli $J62_2$(A263)
Track C - E.coli $J62_2$(A182)
Track D - E.coli $J62_2$(A264)
Track E - E.coli $J62_2$(A265)
Track F - E.coli $J62_2$(A266)
Track G - R6K
Track H - RP4
Track I - R1
c=chromosomal DNA



5.4 Examination of the Outer Membrane Proteins of the Transconjugants

As the β-lactam resistance mechanism of the transconjugants could not be attributed to a β-lactamase, the OMPs of the six strains were examined by SDS-PAGE (Figure 41). The outer membrane profile of three of the transconjugants (A182, A264 and A265) looked very similar to that of *E. coli* J62-2. The remaining three transconjugants (A266, A263 and A261) all showed changes in their major OMPs. Densitometry traces of the proteins produced by these three strains are shown in Figure 42 and compared with that of *E. coli* $J62\text{-}_2$.

The *E. coli* $J62\text{-}_2$ transconjugant of A266 did not appear to produce OmpF which functions as a porin protein, alternatively the protein may only be present in very small amounts. Similarly, the transconjugant of A263 under-produced OmpF which is evident both from the SDS-PAGE gel and the densitometry trace (Figures 41 and 42). OmpF was also greatly diminished in the transconjugant of A261, however, this strain differed from the transconjugants of A266 and A263 in that it produced a larger amount of OmpA which does not function as a porin protein (Figures 41 and 42).

In three of the six transconjugants therefore, the β-lactam resistance may be attributable to changes in the OmpF porin protein which provides a channel for the entry of β-lactam antibiotics. However, the resistance mechanism of the

Figure 41

SDS-PAGE of outer membrane proteins extracted from transconjugants of the β-lactam resistant, β-lactamase negative strains.

Track A - E.coli $J62_2$
Track B - E.coli $J62_2$(A182)
Track C - E.coli $J62_2$(A264)
Track D - E.coli $J62_2$(A265)
Track E - E.coli $J62_2$(A266)
Track F - E.coli $J62_2$(A263)
Track G - E.coli $J62_2$(A261)

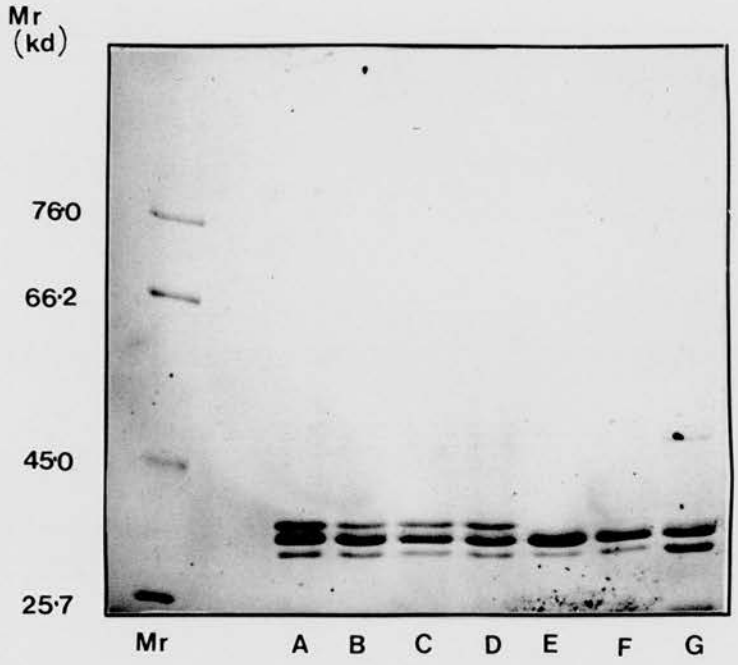
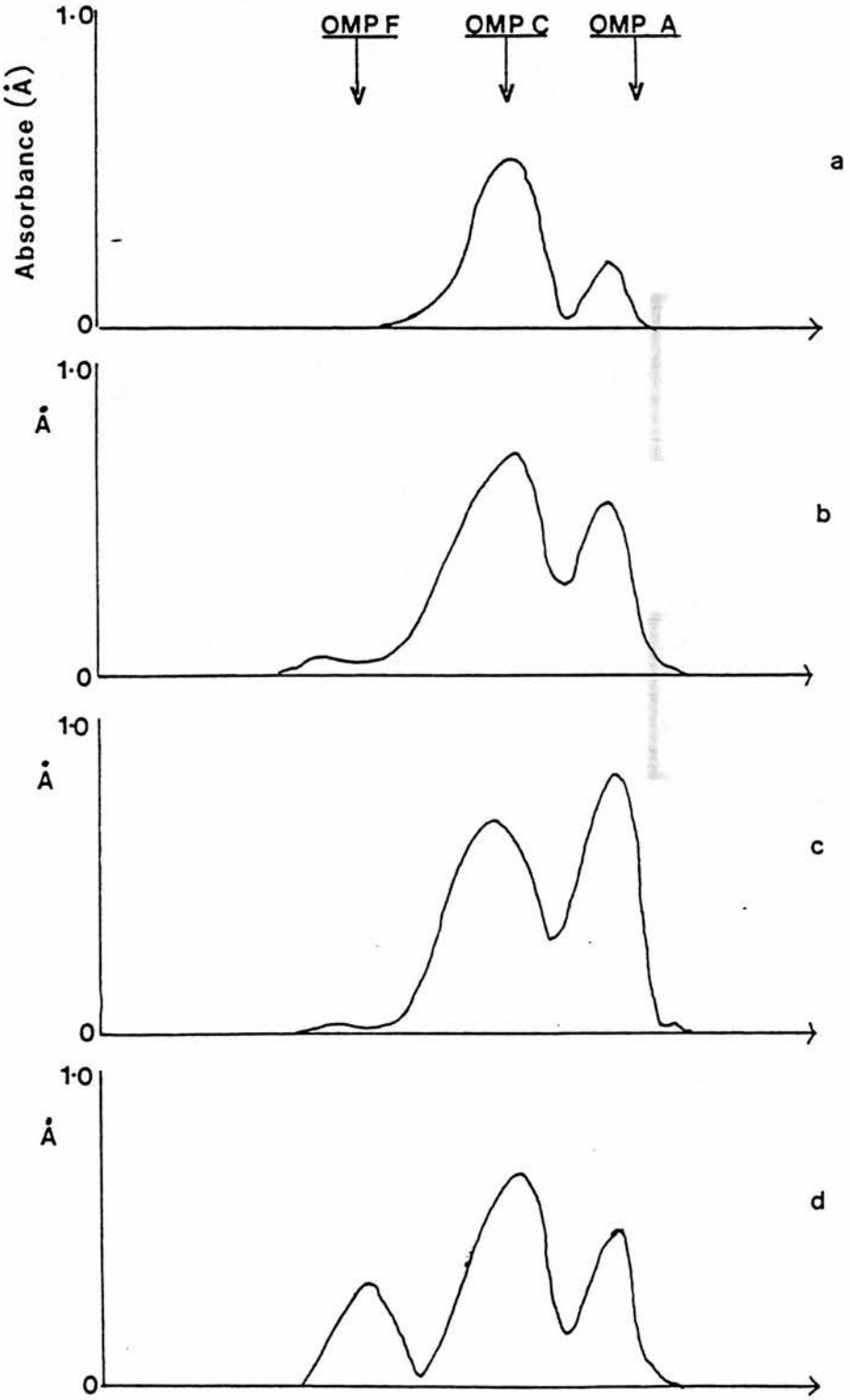


Figure 42

Densitometry traces of the outer membrane profiles of the transconjugants of strains A266, A263 and A261 compared with that of the isogenic strain E.coli J62-2.

(a) E.coli J62-2(A266) (b) E.coli J62-2(A263)
(c) E.coli J62-2(A261) (d) E.coli J62-2



remaining three transconjugants was still undetermined and therefore the PBPs of two of the strains A182 and A265 were examined.

5.5 Examination of the Penicillin Binding Proteins of Two of the Transconjugants

The transconjugants of strains A182 and A265 were examined for possible changes in their PBPs, again comparison was made with the profile of *E. coli* $J62\text{-}_2$.

Competition assays were carried out with isolated PBPs from each of the three strains. Unlabelled ampicillin was used as the competing β-lactam in doubling dilutions around the MIC of the strain (materials and methods). The concentration of ampicillin (mgl^{-1}) which reduced the binding of the radiolabelled benzyl penicillin by 50% (S_{50}) was determined by eye and the results are given in Table 37.

Table 37

Inhibition of Binding of (^{14}C) Benzyl Penicillin by Ampicillin

Strain	MIC of Ampicillin (mgL^{-1})	S_{50} (mgL^{-1}) of PBP						
		1a	1b	2	3	4	5	6
E. coli $J62\text{-}_2$	2	0.25	4	0.5	1	2	>16	8
E. coli J62-2 A182	32	0.5	4	2	2	4	>64	32
E. coli J62-2 A265	16	0.5	1	1	1	2	>64	8

No significant difference in the S_{50} values of ampicillin for any of the PBPs was observed in either transconjugant.

The MICs of ampicillin for both of the transconjugants, however, are higher than the S_{50} values for the essential PBPs. This indicates that there is another resistance mechanism playing a role in these strains. The resistance of three of the transconjugants to tetracycline and of one to spectinomycin (Table 36) suggests that the resistance mechanism may be less specific than changes in the β-lactam target sites and may have more to do with alterations in the permeability of the strains. Alterations in OMPs were found in three transconjugants and it is possible that other changes such as those in the lipopolysaccharide may account for the β-lactam resistance of the remaining three transconjugants

AMPICILLIN RESISTANCE MECHANISMS IN HAEMOPHILUS INFLUENZAE

6. Introduction

A population of 105 clinical isolates of *Haemophilus influenzae* were collected in Edinburgh in 1983/4 from sputum specimens provided by patients at the City Hospital, Edinburgh.

H. influenzae are intrinsically resistant to intermediate levels of first generation cephalosporins (Garrod *et al* 1981), therefore clinical strains were screened for resistance to ampicillin.

Ampicillin resistance in *H. influenzae* was first reported in 1974 (Thomas *et al* 1974) and since then the incidence of resistant isolates has increased worldwide (Schwartz 1978, Scheifele 1979, Lerman *et al* 1980). The most common resistance mechanism to ampicillin in *H. influenzae* is the production of the TEM-1 β-lactamase enzyme (Medeiros 1984). More recently, there have been reports that a new plasmid-mediated β-lactamase, designated ROB-1, can also mediate ampicillin resistance in this species (Rubin *et al* 1981, Medeiros 1984). Ampicillin resistant *H. influenzae* have also been isolated in which no detectable β-lactamase activity can be found (Parr and Bryan 1984, Mendelman *et al* 1984). In these studies, a limited number of strains were examined and changes in the PBPs appear to have been the causative resistance mechanism.

Changes in outer membrane proteins (OMPs) of Gram negative bacteria can also lead to β-lactam resistance as a consequence of reduced permeability (Nikaido 1985). However, studies of OMPs in H. influenzae are complicated because there may be a correlation between OMP profile and biotype (Barenkamp et al 1981, 1982, van Alphen et al 1983). Permeability changes in one H. influenzae strain have been found associated with the loss of a 27,000 dalton protein from the outer membrane (Mendelman et al 1984).

This study examined the incidence and principal mechanism of ampicillin resistance amongst 105 non-sero-typable H. influenzae strains.

6.1 Results

Fifteen (14.3%) of the 105 H. influenzae strains were resistant to ampicillin at $1mgL^{-1}$. The ampicillin resistant strains were non-serotypable. However, representatives of six of the seven H. influenzae biotypes were found (Table 38). The nitrocefin spot test (Materials and Methods) indicated that nine of the 15 strains were β-lactamase producers.

6.2 Minimum Inhibitory Concentration (MIC) Determination

The MICs (Table 39) of ampicillin for the β-lactamase positive strains were generally higher ($8-500mgL^{-1}$) than those for the β-lactamase negative strains ($2-16mgL^{-1}$).

Table 38

Biotype and β-lactamase activity of ampicillin resistant H. influenzae

Strain No.	Nitrocefin spot test	β-lactamase	β-lactamase activity*	Biotype
52	+	TEM-1	4.8	I
74	+	TEM-1	21.0	I
572	+	TEM-1	5.4	I
584	+	TEM-1	12.0	III
585	+	TEM-1	7.4	IV
588	+	TEM-1	1.2	II
639	+	TEM-1	14.2	II
714	+	TEM-1	12.6	V
733	+	TEM-1	8.0	V
118	-	NONE	0.1	VI
156	-	NONE	0.1	II
243	-	NONE	0.1	I
564	-	NONE	0.1	II
621	-	NONE	0.1	V
684	-	NONE	0.1	II
1184E	-	NONE	0.1	III

* = nmoles cephaloridine hydrolysed $minute^{-1}$ mg $protein^{-1}$

Table 39

β-lactam sensitivities of ampicillin resistant H. influenzae strains*

Strain	AMP	AMP + CLAV	PIP	CED	CFX	CXM	CTX
52	16	0.125	4	16	8	0.5	0.01
74	8	0.125	4	16	4	0.5	0.01
572	125	0.125	32	8	4	0.5	0.01
584	500	0.125	16	250	8	2	0.01
585	250	0.125	64	250	4	0.5	0.01
588	16	0.125	0.2	64	4	1	0.01
639	500	0.125	32	8	2	0.5	0.01
714	64	2	64	16	32	4	0.01
733	125	0.125	2	16	4	0.5	0.01
118	8	4	0.05	125	4	4	0.06
156	2	1	0.05	16	2	0.5	0.01
243	4	2	0.5	64	4	1	0.01
564	16	4	0.2	16	8	4	0.01
621	16	2	0.05	16	4	1	0.01
684	4	2	0.05	64	8	2	0.01
1184E	0.125	0.125	0.03	16	2	0.5	0.01

AMP = Ampicillin, PIP = Piperacillin, CED = Cephradine,
CFX = Cefoxitin, CXM = Cefuroxime, CTX = Cefotaxime,
CLAV = Clavulanic Acid.

* All concentrations, mgL^{-1}

However, in the presence of a fixed concentration of clavulanic acid at 8mgL^{-1}, an inhibitor of the TEM-1 β-lactamase, the MICs of ampicillin for eight of the β-lactamase positive strains were reduced to 0.125mgL^{-1}. The MIC of ampicillin of the remaining β-lactamase positive strain 714 was reduced from 64 to 2mgL^{-1}. The MICs of ampicillin of all the β-lactamase negative strains were only marginally reduced in the presence of clavulanic acid, giving values greater than for the β-lactamase positive strain (i.e. 1 to 4mgL^{-1}).

The MICs of the ureidopenicillin, piperacillin, were generally considerably higher for the β-lactamase positive strains (4 to 125mgL^{-1}) than the β-lactamase negative strains (0.05 to 0.2mgL^{-1}).

β-lactamase production did not appear to affect resistance to the test cephalosporins. All of the strains gave MICs of 0.01 to 0.06mgL^{-1} for cefotaxime, 0.5 to 4mgL^{-1} for cefuroxime and, all were resistant to cephradine (MICs$\geq$ 8mgL^{-1}). Strain 714 had an MIC of cefoxitin of 32mgL^{-1}, the remaining strains gave MICs of 2 to 8mgL^{-1}.

6.3 β-lactamase Production and Identification

Cell free extracts of the β-lactamase positive strains gave β-lactamase specific activities ranging from 1.2 to 21nmoles cephaloridine hydrolysed minute^{-1} mg protein^{-1} (Table 38). Isoelectric focusing (IEF) showed that in all

nine strains the β-lactamase specific activity was directly attributable to the TEM-1 β-lactamase (Figure 43). The β-lactamase specific activities of the six β-lactamase negative strains were less than 0.1nmoles cephaloridine hydrolysed minute^{-1} mg protein^{-1} and no β-lactamase enzymes could be detected by isoelectric focusing.

It was concluded from the β-lactamase specific activities and MICs in the presence of clavulanic acid that β-lactamase production was the principal mechanism of ampicillin resistance in eight of the nine β-lactamase positive strains. The β-lactamase positive strain 714 appeared to have an additional resistance mechanism(s) due to the residual ampicillin resistance in the presence of clavulanic acid. Accordingly, the penicillin binding proteins (PBPs) and outer membrane proteins (OMPs) of 714 and the six β-lactamase negative strains were examined.

6.4 Penicillin Binding Proteins

The PBPs of the six β-lactamase negative strains and the β-lactamase positive strain 714 were compared to those of the ampicillin sensitive control strain 1184E. Strain 1184E produced eight major PBPs with apparent molecular sizes of 87, 77, 69, 58, 48, 43, 39 and 37 k daltons (Figure 44).

Four of the ampicillin resistant isolates gave similar PBP profiles to the sensitive control strain 1184E. The

Figure 43

Isoelectric focusing of crude cell extracts of β-lactamase positive H. influenzae.

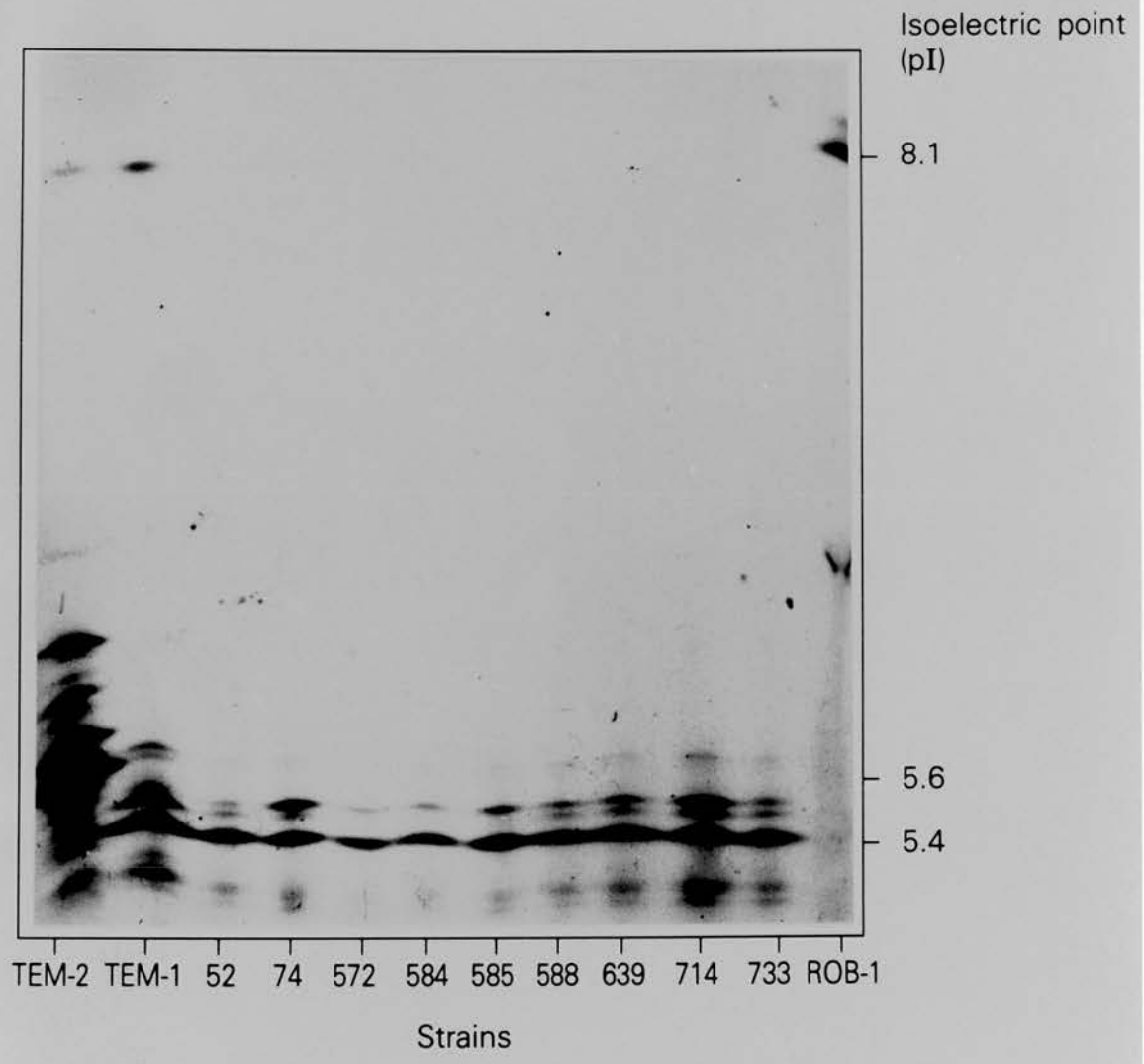
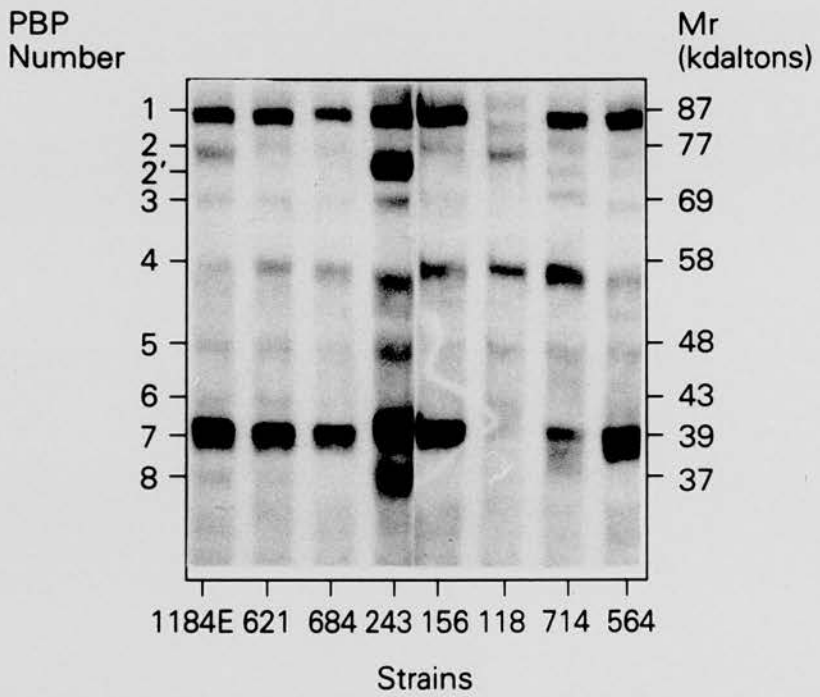


Figure 44
Fluorograph of penicillin binding proteins (PBPs) of ampicillin resistant H. influenzae.



remaining three strains (118, 243 and 714) exhibited marked changes in their PBP profiles (Figures 44 and 45).

H. influenzae 118 produced three PBPs (91, 86 and 80 k daltons) which were in the region of the expected PBPs 1 and 2 but they exhibited different mobilities. In addition, there was less binding of radiolabel to PBPs 3 and 7. Strain 243 produced a PBP (75 k daltons) which differed markedly in mobility from PBP2 seen in H. influenzae 1184E. The β-lactamase positive strain 714 also produced an unexpected PBP profile, having a total of three PBPs in the region of PBPs 2 and 3 but exhibiting different mobilities (83, 76 and 71 k daltons) from either PBP2 or PBP3 visualised in strain 1184E.

6.5 Outer Membrane Proteins

Five of the seven biotypes produced characteristic patterns of OMPs when pairs of ampicillin sensitive strains were examined (Figure 46). Strains representing the two remaining biotypes, IV and V, gave dissimilar OMP profiles. All strains produced a limited number of major OMPs with molecular sizes between 30 and 50 k daltons (Figure 46).

The OMP profiles of the seven ampicillin resistant strains were compared to that of their ampicillin sensitive biotypic counter parts. Two of the ampicillin resistant β-lactamase negative strains (621 and 243) and the β-lactamase positive strain (714) gave very similar OMP profiles to their

Figure 45

Densitometry traces of PBP fluorograms of H. influenzae strains 118, 243 and 714.

Comparison is made with the PBP profile of the ampicillin sensitive strain 1184E. Peak 6 corresponds to PBP 7 and masks the presence of PBP 6.

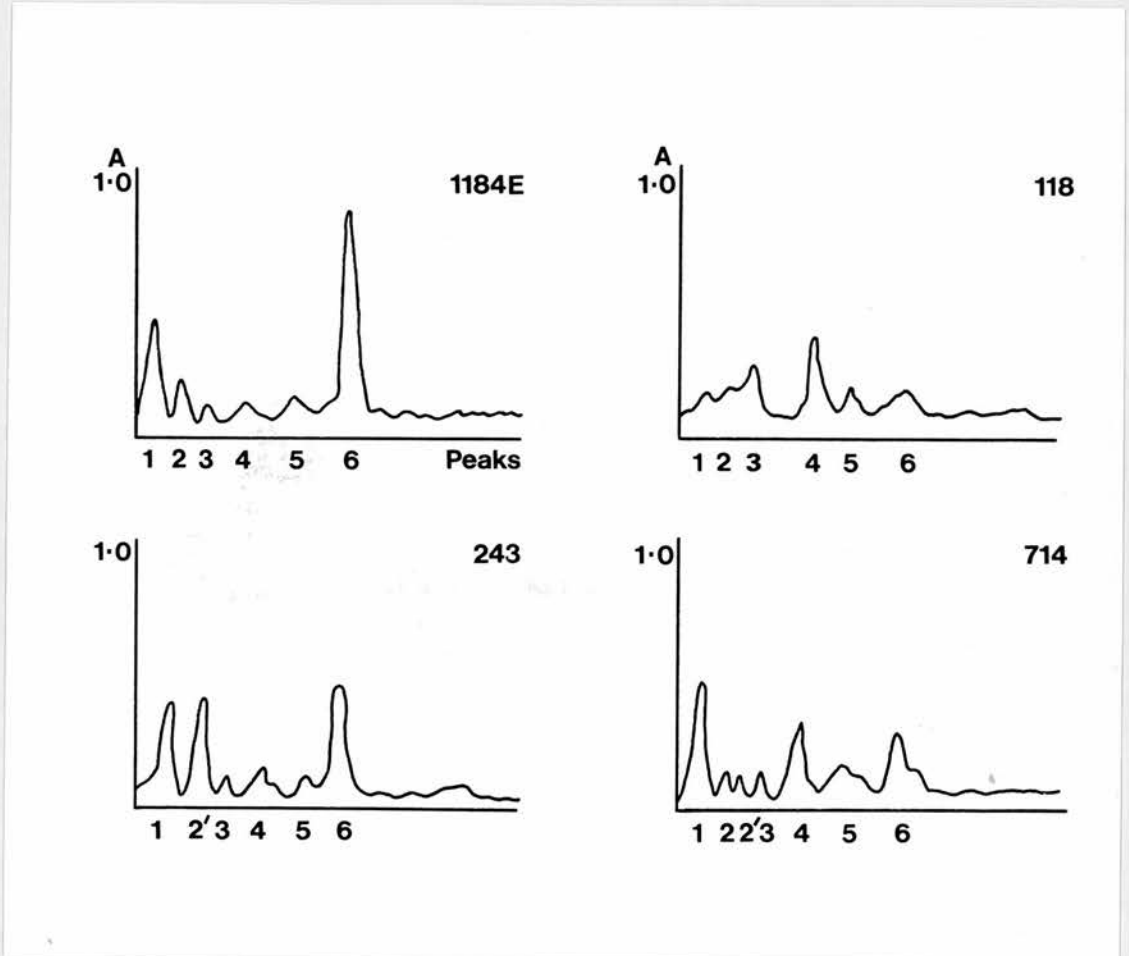
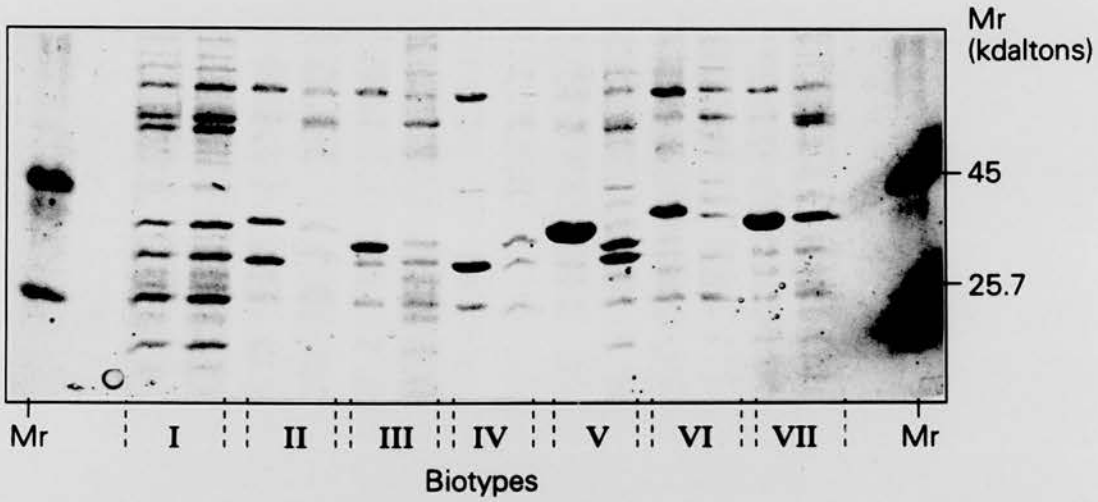


Figure 46

SDS-PAGE of outer membrane proteins (OMPs) of paired H. influenzae standard biotype strains.



respective biotype standards (Figure 47). Strain 118 produced an additional OMP of molecular size 33 k daltons when compared to the biotype VI standard. The three biotype II ampicillin resistant isolates (156, 564 and 684) gave very similar OMP profiles to one another, but different from the biotype II standard strains.

It would be necessary to carry out transformation experiments and compare isogenic strains to confirm that ampicillin resistance did result from OMP or PBP changes.

This study has established that ß-lactamase enzymes are not the sole mechanism of ampicillin resistance in the H. influenzae population and resistance may also result from PBP and OMP alterations.

Table 40 summarises the ampicillin resistance mechanisms identified in the H. influenzae population.

Figure 47

SDS-PAGE of outer membrane proteins (OMPs) of ampicillin resistant H. influenzae compared to those of the respective Haemophilus influenzae standard biotype strains.

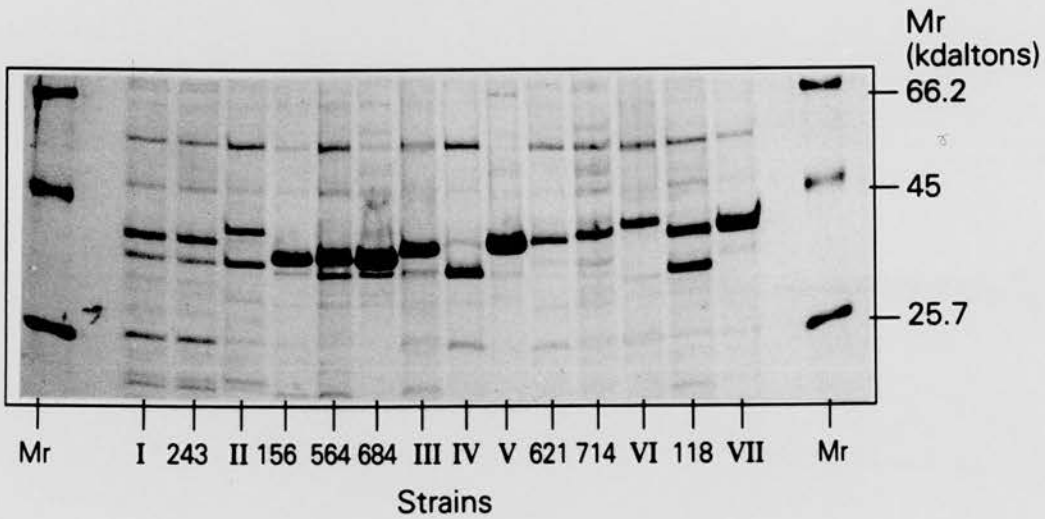


Table 40

Summary of H. influenzae resistance mechanisms

Strain No.	β-lactamase activity	Ampicillin resistance in the presence of CLAV*	Changes in PMPs	Changes in OMPs	Possible source of β-lactam resistance
52	+	-	ND	ND	β-lactamase
74	+	-	ND	ND	"
572	+	-	ND	ND	"
584	+	-	ND	ND	"
585	+	-	ND	ND	"
588	+	-	ND	ND	"
639	+	-	ND	ND	"
733	+	-	ND	ND	"
714	+	+	+	-	β-lactamase/ PBPs
118	-	+	+	+	PBPs/OMPs
156	-	+	-	+	OMPs
243	-	+	+	-	PBPs
564	-	+	-	+	OMPs
621	-	+	-	-	Not identified
684	-	+	-	+	OMPs

* CLAV = Clavulanic acid
+ = difference from "normal" ampicillin sensitive strain
ND = Not done

IV. DISCUSSION

The studies described in this thesis were undertaken to identify and characterize novel genetic and biochemical aspects of ß-lactam resistance in Gram negative bacteria. It has been generally accepted that ß-lactamase enzymes are the most common resistance mechanism to the ß-lactam antibiotics (Richmond and Sykes 1973, Sykes and Matthew 1976, Medeiros 1984). As a result, several surveys have been carried out examining the types of ß-lactamase enzyme produced by ampicillin resistant populations of Gram-negative Enterobacteria (Simpson et al 1980, Roy et al 1983, Stobberingh et al 1985, Simpson et al 1986).

Recently, the use of cephalosporins has increased considerably and thus the survey conducted in this thesis was arranged to monitor specifically the resistance to this group of ß-lactam antibiotics. Therefore, in contrast with previous surveys, resistance to a cephalosporin, cephaloridine, was used to identify resistant Gram negative strains. The subtle change in selection pressure isolated a bacterial population with a different distribution of ß-lactamases from those found in previous studies. The population also contained strains with novel ß-lactam resistance mechanisms.

Bacterial strains were isolated from three centres: Dundee, Edinburgh and Glasgow. The incidence of ampicillin resistance in each centre was higher than the incidence of resistance to cephaloridine. Between 50 and 63% of the ampicillin resistant strains from each centre were cephaloridine resistant. On the other hand, almost all the cephaloridine resistant strains were also ampicillin resistant. The incidence of cephaloridine and ampicillin

resistance in strains isolated in Dundee and Edinburgh are similar to those reported by Gruneberg (1984) for London (between 45.2% and 48.4% ampicillin resistance and 22.6% and 28.4% cephaloridine resistance). However, the Glasgow survey revealed 71.9% of strains as ampicillin resistant and 45.1% as cephaloridine resistant. This level of ampicillin resistance is believed to be unprecedented in the UK (Simpson et al 1980, Gruneberg 1984).

Two hundred and thirty four cephaloridine resistant strains were collected from the three centres. The selection of resistant strains on cephaloridine, rather than ampicillin, resulted in the production of only species specific chromosomal β-lactamases by the majority of strains (50.8%); these were mainly the class I cephalosporinases identified by Richmond and Sykes (1973). β-lactamases typically associated with plasmids were found in 112 strains (47.9%). The most common of these was the TEM-1 β-lactamase (41.9% of all the strains tested) while TEM-2 was produced by only 3.5% of strains. Only four strains isolated produced OXA class β-lactamases. This contrasts with the results of previous surveys where the plasmid-mediated β-lactamases (principally TEM-1) predominated amongst ampicillin resistant isolates (Simpson et al 1980, Roy et al 1983, Stobberingh et al 1985, Simpson et al 1986).

The plasmid β-lactamases generally hydrolyse cephaloridine less efficiently than ampicillin (Medeiros et al 1974, Medeiros 1984). The most effective β-lactamases active against cephaloridine are the chromosomal class I β-lactamases (Richmond and Sykes 1973). These two facts may provide the explanation for the lower incidence of plasmid-mediated β-lactamases found in this present survey.

In previous surveys, the designation of plasmid-mediated ß-lactamase enzyme was made purely on the basis of biochemical results (Matthew 1979, Simpson et al 1980, Roy et al 1983, Stobberingh et al 1985, Simpson et al 1986). However, the results of this thesis show this to be a misnomer. Genetic experiments were carried out to confirm the location of the ß-lactamases which were predicted to be plasmid-mediated. Selection in all cases was made for the transfer of ampicillin resistance, the principal enzyme substrate. One hundred and twelve strains produced "plasmid mediated ß-lactamases", but only 78 strains transferred the ß-lactamase gene in question. The ß-lactamase gene from a further 15 strains transferred after mobilisation. However, a high proportion of strains, 19 out of 112 (17%), could not transfer their ß-lactamase genes. Fifteen of these strains produced the TEM-1 ß-lactamase. It appears therefore, that genetic classification made on the basis of biochemical evidence is far from definitive.

Subsequent surveys of ß-lactamases within bacterial populations should, therefore, include genetic tests along with the biochemical analysis. Such comprehensive surveys would also reveal whether genetic changes were taking place within the population, such as a move towards the chromosomal carriage of ß-lactamase genes. This has already been observed with two ß-lactamase transposons: the PSE-4 transposon (Tn2521) in P. aeruginosa (Sinclair and Holloway 1982) and the TEM-1 transposon (Tn2) in H. influenzae (Murphey-Corb et al 1984). The migration of resistance genes, normally associated with plasmids, into the bacterial chromosome is becoming more

prevalent and has occurred on a large scale with other resistances, notably trimethoprim (Amyes et al 1986).

The determination of the genetic locus of resistance genes can be time consuming and more recently genetic probes have been used to identify the location and types of ß-lactamase and other resistance genes (Sinclair and Holloway 1982, Cooksey et al 1985, Elwell and Falkow 1986). However, although they are highly sensitive, they do have limitations as reported by Cooksey et al (1985) who identified a silent copy of the TEM ß-lactamase on the OXA-2 plasmid R46 with a TEM-1 probe. The presence of repressed or mutated genes among target DNA may make the interpretation of gene hybridisations difficult. Therefore, in epidemiological studies, it is essential to include biochemical investigations in parallel with gene probing.

Frequently, there is no correlation between the specific activity of the ß-lactamase present in the cell and the ß-lactam resistance level (Richmond and Curtis 1973, Medeiros 1984). The ß-lactamases isolated from the cephaloridine resistant strains hydrolysed cephaloridine at variable rates. One group, comprising 72 E. coli producing the TEM-1 ß-lactamase, were studied in detail. The specific enzyme activity varied by 650 fold with the majority (85%) in the range of 1 to 35nM cephaloridine hydrolysed $minute^{-1}$ mg^{-1} protein. Hedges et al (1974) also found that the TEM-type enzyme was heterogeneous in the levels of ß-lactamase produced. Quantitative differences in the levels of ß-lactamase production can result from mutations in the promoter region of the ß-lactamase gene, resulting in more efficient transcription (Yamamoto et al 1982). Gene amplification or multiple transposition also have a

quantitative effect, increasing the amount of ß-lactamase produced (Uhlin and Nordström 1977). The study also found that variation in TEM-1 expression, even within one species (E. coli) was considerable. When the plasmids carrying the TEM-1 ß-lactamase were transferred into the isogenic background of E. coli J62-2, variation in the specific enzyme activity was still observed.

The relative specific activities of the plasmid enzymes in the clinical hosts, however, was not mirrored in the transconjugants. In addition, the specific activities did not correlate with the resistance level of the transconjugant strains. In the transconjugants the variation in specific activity was around 500-fold, but all the strains were resistant to high levels of ampicillin (MIC $\geqslant$1000mgL^{-1}). On the other hand, most of the transconjugants (45/62) were sensitive to cephaloridine (MIC $\leqslant$16mgL^{-1}), even though the clinical E. coli parent strains were resistant. Therefore, it appears that the TEM-1 ß-lactamase is not the principal mechanism of resistance to cephaloridine in these clinical strains.

Two significant biochemical differences between cephaloridine and ampicillin may provide an explanation for the different sensitivies observed. Firstly, the TEM enzymes have a much lower affinity for cephaloridine (16-20 fold) than for ampicillin (Medeiros 1974, Bush and Sykes 1984). Secondly, the crypticity value of cephaloridine is 1 for E. coli K12 indicating that this strain presents no barrier to the entry of this drug whereas ampicillin has a comparatively high crypticity value of 65 (Richmond and Sykes 1973), indicating that

ampicillin enters the periplasmic space more slowly. In combination, these differences in permeability to cephalosporins and penicillins as well as the differences in the TEM-1 affinity for the substrates may explain why the *E. coli* K12 J62-$_2$ transconjugants are susceptible to cephaloridine but highly resistant to ampicillin. Although *in vitro* the Vmax values for both substrates are generally high, *in vivo* the hydrolysis of cephaloridine is not rapid enough to inactivate sufficient β-lactam and thus the level of cephaloridine rises to a lethal concentration for *E. coli* K12.

The remaining *E. coli* J62-$_2$ transconjugants (17/62) were more resistant to cephaloridine and had MICs of cephaloridine of 16 or 32mgL^{-1}. The enzyme specific activity of these strains was, on average, just over two fold higher than that found in the cephaloridine sensitive transconjugants. This two fold increase in enzyme production could provide greater protection against the incoming cephaloridine in these strains. However, one strain with a very low specific activity (0.63mM cephaloridine hydrolysed minute^{-1} mg protein^{-1}) had an MIC of cephaloridine of 32mgL^{-1}. Thus, increases in enzyme production alone cannot explain the cephaloridine resistance of these strains.

A further explanation is that the plasmid DNA transferred to *E. coli* J62-$_2$ may have had an effect on the outer membrane of the recipient strains, resulting in an alteration in permeability and thus the strains' susceptibility to cephaloridine. There have been two reports of plasmids which have an effect on the composition of the outer membrane of *E. coli*. In the first, plasmids of the N incompatibility group, when introduced into *E. coli* B/r reduced the

amount of the outer membrane protein (the OmpF equivalent). However, no similar effect was observed when the plasmid was introduced into E. coli K12 (Iyer et al 1978). In the second, R124 an incFIV plasmid reduced the level of expression of OmpF in E. coli K12 and influenced the sensitivity of the organism to a wide variety of inhibitory agents (Rossouw and Rowbury 1984). Both these plasmids confer tetracycline resistance which, interestingly, is also co-transferred with TEM-1 to the E. coli J62-$_2$ cephaloridine resistant strains of the present work. Significantly, E. coli K12 strains harbouring a plasmid (related to R124), which produced an even greater reduction in the amount of OmpF, had an increase in resistance to cephaloridine (Rossouw and Rowbury 1984). A similar transferable impermeability barrier may be present on some of the TEM-1 plasmids. A resistance mechanism of this nature, genetically linked to a β-lactamase gene would significantly increase the resistance of the strains to a variety of β-lactams.

During the β-lactamase survey, a K. pneumoniae strain was isolated which produced a β-lactamase of novel pI. The bacterial isolate was itself unusual as it produced three β-lactamase enzymes, TEM-1, SHV-1 and the novel enzyme. The strain transferred all three β-lactamase genes to E. coli J62-$_2$ on a single plasmid, pUK702.

The novel β-lactamase enzyme had a pI of 6.5 which was unlike that of any previously described plasmid-mediated β-lactamase (Table 2). It had a molecular size of 19,000 daltons, similar to the size of TLE-1 which is 19,800 daltons (Medeiros et al 1985). Conventional substrate hydrolysis assays and inhibition profiles showed the novel

enzyme to have similar properties to the TEM enzymes which has a broad spectrum of activity. In addition, it was most effective against benzyl penicillin and ampicillin, and sensitive to inhibition by cloxacillin, clavulanic acid and pCMB.

The classification of ß-lactamase enzymes is largely dependent on their substrate and inhibition profiles (Richmond and Sykes 1973, Sykes and Matthew 1976). The substrate profile of the novel ß-lactamase was established with the parameter relative efficiency of hydrolysis (Bush et al 1982) which takes account of the Vmax and the Km of the ß-lactamase. This index provides more information about the enzyme than relative rates of hydrolysis (which may, or may not, be carried out at the Vmax of the ß-lactamase).

Evaluation of the kinetics of hydrolysis revealed that, unlike TEM and SHV type enzymes, the novel ß-lactamase did not obey first order kinetics during the hydrolysis of cephaloridine (Bush and Sykes 1984, Kliebe et al 1985). It was also apparent that even at the high substrate concentrations used the enzyme did not reach its Vmax of hydrolysis of cephaloridine.

The RSAI (James 1983) is a measure of the affinity of the enzyme for a wide variety of ß-lactam antibiotics. The number and variety of compounds which can be tested allows the differentiation of even closely related ß-lactamase types. The novel enzyme was differentiated from TEM-1 and SHV-1 by its extremely high affinity for cefsulodin. As the ß-lactamase was very similar in other respects to the TEM-type ß-lactamases it was given the designation TLE-2 (TEM-like enzymes). There is a great deal of diversity of ß-

lactamase enzymes and the discovery of TLE-2 brings the number of plasmid-mediated ß-lactamases to 28.

One group of plasmid-mediated ß-lactamases are the Pseudomonas specific enzymes (PSE). Two of the cephaloridine resistant enterobacteria produced PSE-4. This was the first identification of this ß-lactamase outwith P. aeruginosa. Previously, PSE-1 (Medeiros et al 1982) and PSE-2 (Livermore et al 1984) ß-lactamases had been found in enterobacterial strains and with the isolation of PSE-4 in enterobacteria, the designation "Pseudomonas specific" is misleading and the enzymes would be better classified as carbenicillin hydrolysing enzymes. The PSE-4 ß-lactamase determinants dal1 and dal2 transferred from both the original clinical strains to E. coli $J62\text{-}_2$ and P. aeruginosa PA08.

When the expression of the enzyme in various host bacteria was examined it was found that the biochemical and biophysical properties of the ß-lactamase were host dependent. Subtle changes in the enzyme's isoelectric point and hydrolysis kinetics were found to take place when dal1 or dal2 were transferred from enterobacterial strains to P. aeruginosa. However, when the determinants were transferred from P. aeruginosa to E. coli the enzyme maintained the characteristic Pseudomonas biochemical and biophysical profile. In P. aeruginosa the structural gene for the production of PSE-4 may have been altered slightly, resulting in a protein which differed from that found in the enterobacterial isolates. A few changes in the amino acid composition of an enzyme can change the tertiary structure of the protein, thereby affecting such properties as the pI. This has been observed with TEM-1 and

TEM-2 which differ by one amino acid (Matthew *et al* 1975). Brive *et al* (1977) also noted changes in the IEF patterns of plasmid-mediated ß-lactamases from different host strains. Changes in the folding of the enzyme may also alter the ß-lactamases' ability to process ß-lactam substrates and thus increase or reduce the turnover rate. Variation in Vmax was observed with the PSE-4 ß-lactamases isolated from different hosts. Alterations in the enzyme folding would be less likely to affect the affinity of the ß-lactamase for the substrate, and indeed it was confirmed that there was little alteration in the Km values of the various PSE-4 ß-lactamases. Alteration in the nucleic acid sequence of the PSE-4 determinants could have occurred when the gene integrated into the *P. aeruginosa* chromosome, perhaps removing a terminal region. Chromosomal integration has also been observed with the PSE-4 transposon (Tn2521) in natural isolates of *P. aeruginosa* (Sinclair and Holloway 1982). Restriction enzyme analysis of the *dal*2 determinant was very similar to that found for Tn2521 (Sinclair and Holloway 1982). This, together with the translocation of *dal*1 into the *P. aeruginosa* chromosome, strongly suggests that the PSE-4 determinants *dal*1 and *dal*2 are carried on transposons similar or identical to Tn2521.

Apart from the two isolations in enterobacteria reported in this thesis, PSE-4 does appear to be restricted to *P. aeruginosa*. Livermore *et al* (1985) examined a group of PSE-4 producing *P. aeruginosa* strains and found that there was an association between the serotype O:16 and this ß-lactamase. They speculated that the PSE-4 gene may be transferred *via* a serotype specific phage. The PSE-4 transposon Tn2521 and the *dal*1 and *dal*2

determinants have a predeliction for insertion into the P. aeruginosa chromosome and they consequently become "trapped". P. aeruginosa may thus act as a reservoir for such transposons.

It is difficult to establish if the Pseudomonas reservoir has been the source of the PSE-4 genes in the two enterobacterial strains from the survey. The results of this thesis show that chromosomal insertion of PSE-4 into Pseudomonas is accompanied by an alteration in the gene product. This change persists even when the gene is mobilised from Pseudomonas into an enterobacterial strain. The permanent alteration argues against Pseudomonas as the source of the enzyme.

The use of incP-1 plasmids to study the genetics and expression of β-lactamase genes is commonplace (Hedges and Jacob 1975, Sinclair and Holloway 1982, Seeberg et al 1983). The initial studies of dal2 were carried out with R751 as the mobilising plasmid; however, the plasmid was found to induce mutations in the E. coli J62 host strain. The mutations resulted in resistance to carbenicillin and ampicillin and a variety of other antimicrobial compounds. In isogenic strains which lacked the R751 plasmid, chromosomal mutations occurred at a very low frequency. The E. coli R751-containing strains which were resistant to carbenicillin/ampicillin were found to produce the β-lactamase SHV-1.

It was not clear whether this β-lactamase was encoded by the R751 plasmid or was situated on the E. coli K12 chromosome, and was, in some way, derepressed by the plasmid. The SHV-1 β-lactamase itself appears to have originated in Klebsiella spp. (Matthew 1979, Nugent

and Hedges 1979) and the R751 plasmid was originally isolated from a rare K. aerogenes strain (Jobanputra and Datta 1974).

The early evidence suggested that the R751 plasmid may possess a "silent" SHV-1 gene which had originated in the K. aerogenes strain. This would be similar to the carriage by the R46 plasmid of a silent copy of the TEM β-lactamase, as has been previously described (Cooksey et al 1985). However, it was found that the SHV-1 β-lactamase was produced by strains which had been cured of the R751 plasmid. This evidence strongly suggests that the β-lactamase is not plasmid-mediated. However, the presence of the plasmid was an absolute requirement for the initial expression of the β-lactamase. The high frequency with which the SHV-1 β-lactamase was found in the cured strains also ruled out transposition of the SHV-1 gene from the R751 plasmid to the E. coli chromosome. It was concluded therefore, that the β-lactamase gene resides on the E. coli J62 chromosome but its initial expression is dependent on the presence of the R751 plasmid.

The mechanism by which R751 affected chromosomal genes is unclear. Resistance plasmids have been reported to increase the mutation frequency of host bacterial strains (Ginoza and Painter 1964, McCann et al 1975, Amyes and Smith 1977). McCann et al (1975) postulated that the plasmid enhanced an error-prone recA dependent DNA repair mechanism thus increasing the mutation rate of the plasmid-bearing strain. In the present study, increased mutation rates to carbenicillin resistance were observed in E. coli PB1150 recA ruling

out a recA dependent system or the integration of R751 into the host chromosome as the cause of mutations.

Insertion sequences are mutagenic (Kleckner et al 1975) at frequencies of 1-4 x 10^{-4} (Foster 1976) and the inverted complementary base pair sequences which flank some transposons may have a similar effect. Such regions may flank the trimethoprim transposon (Tn402) on R751 (Shapiro and Sporn 1977), or IS sequences may be present elsewhere on the plasmid. Alternatively, the R751 plasmid may produce a protein which switches on or off a variety of regulatory systems in the bacterial cell.

During the survey of cephaloridine resistant strains, six isolates were identified which transferred resistance to β-lactams not associated with the production of a β-lactamase. The β-lactam resistance transferred to E. coli recipient strains at a low frequency. In one case this was associated with the presence of a plasmid (pUK703); however, in the remaining five cases, no plasmid DNA was isolated. It is possible that the DNA isolation techniques used may have broken up any large plasmids as the resolution of the techniques used is in the region of 200kb. Alternatively, it is possible, but less likely, that no plasmid DNA transferred in these strains but the β-lactam resistance was carried by a conjugative transposon such as those identified by Franke and Clewell (1981).

The β-lactam resistance mechanism of the strains was examined and three of the six E. coli $J62\text{-}2$ transconjugants were found to have changes in their outer membrane protein profiles. There have been two other reports (previously described) of resistance plasmids

affecting the permeability of the outer membrane (Iyer et al 1978, Rossouw and Rowbury 1984). The three transconjugants were found to have reduced amounts of porin protein OmpF. This was similar to the effect of the plasmids reported by Iyer et al (1978) and Rossouw and Rowbury (1984). However, in the cases reported by Rossouw and Rowbury (1984) the plasmids conferred resistance to cephaloridine. In contrast the transconjugants described here were sensitive to cephaloridine.

In the remaining three transconjugants, including the strain containing the plasmid pUK703, no changes in the outer membrane proteins were observed. In addition, no changes were seen in the affinity of the penicillin binding proteins for ampicillin. It has been observed that some types of resistance mechanism may only be expressed under certain types of growth conditions. This variation has been found with the low affinity PBP of S. aureus (Fontana 1985) which may also be the case with these strains. Alternatively, the resistance may arise from a mechanism such as enzymic modification of the β-lactam, which is distinct from hydrolysis. Bacterial amidases remove the acyl side chain, of principally the penicillins forming the less active aminopenicillanic acid (Batchelor et al 1961, Hamilton-Miller 1966). However, the role of such enzymes in resistance is unclear.

In H. influenzae, ampicillin resistance is generally attributed to the production of the TEM-1 β-lactamase. The present study of this species has established that non-β-lactamase mediated resistance mechanisms acting either alone or in combination are equally prevalent.

The nitrocefin spot test identified those strains which produced a β-lactamase. However, determination of the ampicillin MICs in the presence and absence of the β-lactamase inhibitor, clavulanic acid yielded further information and separated the 15 ampicillin resistant strains into three groups: (i) eight strains owing their resistance primarily to TEM-1 β-lactamase activity, (ii) strain 714 whose resistance depended on TEM-1 β-lactamase activity and some other mechanism(s), and (iii) six strains whose resistance resulted from factors other than β-lactamase activity. The levels of ampicillin resistance seen with non-β-lactamase producing strains were generally less than for the β-lactamase producing strains.

There was no clear correlation between the specific activity of the β-lactamase and the MIC of ampicillin. The MIC of ampicillin of strain 714 was not reduced to fully sensitive levels in the presence of clavulanic acid, indicating a major involvement of other resistance factors. This conclusion was further supported by the strain's usually high resistance to cefoxitin, a compound stable to hydrolysis by TEM-1 β-lactamase (Onishi et al 1974).

Strain 714 and strains 118 and 243 showed marked differences in the mobilities of PBPs in the 69 to 86 kdalton region relative to the ampicillin sensitive control strain 1185E. The PBP2 of H. influenzae is the major target site with the highest affinity for all of the β-lactams except mecillinam (Makover et al 1981). Changes in this PBP would have the greatest effect on the susceptibility of the bacteria to β-lactams. However, the significance of these mobility differences or the appearance of

additional (^{14}C) benzyl penicillin labelled proteins in this region remains unclear.

Alterations in the outer membrane proteins of H. influenzae can also result in increased β-lactam resistance. Permeability changes in one H. influenzae strain have been found associated with the loss of a 27,000 dalton protein from the outer membrane (Mendelman et al 1984). Examination of the OMP profiles of seven pairs of ampicillin sensitive H. influenzae biotype standard strains revealed a high degree of biotype specificity. Indications of such a correlation have been previously reported for non-typable and Type b H. influenzae strains (Barenkamp et al 1981, van Alphen et al 1983). Porras et al (1986) examined the OMP profiles of non-typable strains of five H. influenzae biotypes and found variation in the OMPs produced by biotypes I and II. The antimicrobial drug susceptibility of the strains was not examined however, and no account was taken of possible mutations having occurred in OMPs which would result in an altered profile.

In the present study there was a non-specificity of biotype IV and V strains which may reflect a greater variation within these biotypes than previously acknowledged.

Four of the six β-lactamase negative strains gave different OMP profiles from their respective biotype standards. This infers mutation in the omp genes gives a more resistant phenotype. It would be necessary to transform these genes into an isogenic background to confirm this. It was apparent, however, that β-lactamase enzymes are not the sole mechanism of ampicillin

resistance in the H. influenzae population and resistance may also result from PBP and OMP alterations.

It can be concluded from the studies carried out in this thesis that β-lactamase enzymes are an important mechanism of β-lactam resistance in Gram negative bacteria. However, it can also be seen that this resistance mechanism is inextricably interwoven with a variety of other host factors which affect the cells' susceptibility. It has been shown from the studies of TEM-1 β-lactamase in E. coli that in vitro studies of β-lactamase efficiency do not necessarily give an indication of the cells' in vivo β-lactam resistance. Caution must therefore be observed when extrapolating from results obtained in test tubes.

New plasmid-mediated β-lactamases such as TLE-2 continue to be found, and they are obviously of importance. Of more worrying significance is the discovery of a transferable β-lactam resistance mechanism not attributable to a β-lactamase. In some strains, this mechanism was identified as the loss of the OmpF porin, and in another instance the R751 plasmid was found to induce generalised resistance mutations. These mechanisms may produce cross resistance to a variety of β-lactams and possibly other groups of antimicrobial drugs. Similar, non-β-lactamase, resistance mechanisms were also found in H. influenzae although the genetic location of the resistance mechanism was not examined. It may only be a matter of time before such 'alternative' β-lactam resistance mechanisms become transferable in this species.

It is apparent that β-lactam resistance in Gram negative bacteria is a multifactorial defence system which continues to evolve. Developments in chemotherapy must evolve at a similar pace if we are to maintain our lead in the battle against bacterial infections.

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VI. APPENDIX

1. Cephaloridine Resistant Strains - Identifications and Sources.

No	Date	Source	Infection	Organism	H/No.	API	Resistances
1	831129	G.P.	UTI	Escherichia coli	864	5044552	ApCer
6	831213	C-G.P.	UTI	Escherichia coli	3128	7144532	ApCer
8	831214	C-140P	UTI	Escherichia coli	3270	5144532	ApCer
12	831214	C-18B	UTI	Klebsiella pneumonia	3348	5215773	ApCer
13	831214	C-G.P.	UTI	Escherichia coli	3349	5144512	ApCer
15	831212	C-2	UTI	Klebsiella pneumonia	2895	5215773	ApCer
19	831213	C-G.P.	UTI	Citrobacter freundii	3106	3604572	ApCer
21	831212	C-G.P.	UTI	Escherichia coli	2972	5144512	ApCer
23	831212	C-G.P.	UTI	Klebsiella oxytoca	3002	5245773	ApCer
28	831213	C-G.P.	UTI	Escherichia coli	3160	5144112	ApCer
36	831214	C-G.P.	UTI	Proteus vulgaris	3407	0476021	ApCer
43	831215	R-20	Chest	Enterobacter aerogen	6357	5305773	ApCer
44	831215	R-17	Chest	Pseudomonas aerugino	6316	2202004	ApCer
49	840114	R-19	Chest	Pseudomonas aerugino	5107	2202004	ApCer
50	831215	R-19	Chest	Proteus mirabilis	6379	0536000	ApCer
52	831219	Qu.Ho.	Chest	Haemophilus influenz	47		ApCer
54	831217	R-SCBU	Oral secret.	Escherichia coli	7352	5144572	ApCer
55	831216	R-52	Eye swab	Pseudomonas aerugino	7075	2202004	ApCer
60	831216	C-G.P.	UTI	Escherichia coli	3617	5144572	ApCer
64	840214	DRI-5	UTI	Klebsiella oxytoca	11705	5255773	ApCer
67	831216	C-G.P.	UTI	Pseudomonas aerugino	3644	2206006	ApCer
72	840215	NW-1	UTI	Escherichia coli	11887	1044562	ApCer
73	831212	C-G.P.	UTI	Escherichia coli	2974	5144572	ApCer
74	831220	R-30	Chest	Haemophilus influenz	8322		ApCer
85	831221	R-33	Gut	Escherichia coli	9003	5144552	Cer
93	840114	R-35	UTI	Pseudomonas fluor gp	4984	6206004	ApCer
94	831220	R-15	UTI	Enterobacter cloacae	8260	7305573	ApCer
99	831218	R-51	Gut	Hafnia alvei	7592	4104102	Cer
100	840116	R-16	UTI	Proteus morganii	5265	0374000	ApCer
101	831219	R-13	Wound swab	Enterobacter cloacae	8101	3305573	ApCer
102	831218	R-17	Blood	Enterobacter sakazak	7604	3304173	ApCer
103	831220	R-SCBU	CSF	Enterobacter cloacae	8601	7305573	ApCer
104	831219	R-53	UTI	Escherichia coli	8026	7044562	ApCer
107	840112	R-SCBU	Eye swab	Enterobacter cloacae	4112	3305573	Cer
108	831219	R-19	Chest	Pseudomonas aerugino	7749	2206004	ApCer
110	840103	R-9	UTI	Enterobacter cloacae	149	7305573	ApCer
115	831224	R-19	Chest	Pseudomonas fluor gp	9851	6202004	ApCer
118	840104	R-45	Chest	Haemophilus influenz	588		ApCer
122	840104	SMMP	Wound swab	Enterobacter cloacae	809	7305573	ApCer
126	840104	R-16	UTI	Enterobacter cloacae	818	3305573	ApCer
131	840104	R-33	UTI	Citrobacter freundii	620	3204573	ApCer
132	840104	R-6	UTI	Escherichia coli	636	7144532	ApCer
134	840105	R-6	UTI	Escherichia coli AD	76	5044542	ApCer
135	840105	R-6	UTI	Escherichia coli	976	5144572	ApCer
138	840104	R-10	Wound swab	Proteus mirabilis	900	0736000	ApCer
140	831219	R-16	UTI	Proteus mirabilis	7736	4336000	ApCer
141	840116	R-24	Wound swab	Pseudomonas aerugino	5607	2606000	ApCer
144	831219	R-24	UTI	Escherichia coli	7825	5044500	ApCer
150	831222	R-24	UTI	Escherichia coli	9128	1144552	ApCer
151	831222	R-2	UTI	Escherichia coli	9262	7144533	ApCer
156	840109	R-Haem	Chest	Haemophilus influenz	2874		ApCer
163	840105	R-10	UTI	Pseudomonas maltophi	1361	4002000	ApCer

No	Date	Source	Infection	Organism	H/No.	API	Resistances
167	840107	R-52	Pacemaker	Escherichia coli	2127	5144572	ApCer
175	840109	R-20	Chest	Klebsiella pneumonia	2390	5216773	ApCer
179	840102	C-2	UTI	Pseudomonas aerugino	5298	2602000	ApCer
180	831231	C-8A	UTI	Pseudomonas	5238	2602000	ApCer
188	840104	C-18A	UTI	Pseudomonas	5594	2602000	ApCer
189	840105	C-G.P.	UTI	Escherichia coli	5723	5144572	ApCer
193	840106	C-G.P.	UTI	Escherichia coli AD	5947	5044542	ApCer
196	840106	C-5	UTI	Proteus mirabilis	5866	0536000	ApCer
198	840105	C-G.P.	UTI	Escherichia coli	5806	7144532	ApCer
199	840105	C-G.P.	UTI	Escherichia coli	5804	5144552	ApCer
201	840105	C-G.P.	UTI	Escherichia coli	5777	5144572	ApCer
216	840106	C-G.P.	UTI	Escherichia coli	5971	5144532	ApCer
219	840106	C-G.P.	UTI	Escherichia coli	5958	7144532	ApCer
220	840110	C-16	Vaginal swab	Escherichia coli	6440	7144532	ApCer
221	840109	C-G.P.	UTI	Proteus mirabilis	6277	2736000	ApCer
230	840110	C-G.P.	UTI	Klebsiella ozaenae	6415	7306573	ApCer
235	840110	C-G.P.	UTI	Escherichia coli	6423	5144562	ApCer
241	840112	R-23	Throat swab	Klebsiella pneumonia	4245	5205773	ApCer
243	840111	R-29	Chest	Haemophilus influenz	3775		ApCer
253	840110	R-52	Wound swab	Serratia odorifera	3147	5344573	ApCer
259	840112	R-49	Blood	Citrobacter freundii	3971	1404572	ApCer
261	840111	R-ENT	Ear swab	Proteus mirabilis	3861	0776021	ApCer
264	840111	C-140P	UTI	Escherichia coli	6598	5144552	ApCer
288	840112	C-G.P.	UTI	Enterobacter spp	6846	3305121	ApCer
294	840113	C-G.P.	UTI	Escherichia coli	6961	7044553	ApCer
296	840115	C-8	UTI	Proteus mirabilis	7161	0536000	ApCer
297	840113	C-G.P.	UTI	Escherichia coli	7014	5044512	ApCer
301	840113	C-G.P.	UTI	Escherichia coli	6963	5144572	ApCer
303	840113	C-16A	UTI	Klebsiella pneumonia	6928	5205773	ApCer
307	840215	DRI-6	UTI	Pseudomonas aerugino	12041	2202004	ApCer
308	840112	C-G.P.	UTI	Escherichia coli	6991	1144512	ApCer
326	840117	C-G.P.	UTI	Escherichia coli	7332	5144532	ApCer
330	840117	C-16A	UTI	Escherichia coli	7379	5044552	ApCer
334	840117	C-G.P.	UTI	Escherichia coli	7417	5144552	ApCer
337	840117	C-G.P.	UTI	Escherichia coli	7461	5144512	ApCer
339	840117	C-15	UTI	Klebsiella oxytoca	7523	5244773	ApCer
340	840118	C-19B	UTI	Pseudomonas spp	7558	2716004	ApCer
342	840119	R-33	Gut	Proteus mirabilis	6701	0536000	Cer
344	840120	R-10	Gut	Proteus mirabilis	7026	0736000	Cer
345	840120	R-13	Wound swab	Serratia marcescens	7180	5307761	ApCer
347	840121	R32	Gut	Pseudomonas aerugino	7443	2206004	ApCer
348	840121	R-16	UTI	Escherichia coli	7447	5144512	ApCer
350	840121	R-16	UTI	Escherichia coli	7518	5044512	ApCer
352	840122	R-6	Gut	Enterobacter cloacae	7599	3305573	ApCer
353	840122	R-6	Gut	Pseudomonas aerugino	7602	2202004	ApCer
357	840122	R-2	UTI	Escherichia coli	7674	7144573	ApCer
358	840122	R-2	UTI	Escherichia coli	7678	5144572	ApCer
360	840123	R-16	Drain fluid	Pseudomonas aerugino	7744	6302004	ApCer
361	840123	R-16	Nephrostomy	Enterobacter cloacae	7747	3105573	ApCer
363	840123	R-16	UTI	Enterobacter cloacae	7802	3305573	ApCer
364	840123	R-10	Gut	Enterobacter aerogen	7821	5305773	ApCer
365	840123	R-6	UTI	Klebsiella ozaenae	8063	5004552	ApCer

No	Date	Source	Infection	Organism	H/No.	API	Resistances
367	840123	R-R/O	UTI	Escherichia coli	8223	5144512	ApCer
369	840123	R-5	Drain fluid	Pseudomonas aerugino	8237	6202004	ApCer
372	840123	R-ENT	Ear swab	Acinetobacter calco.	8349	0004040	Cer
373	840119	C-8	UTI	Escherichia coli AD	7779	5044502	ApCer
374	840119	C-G.P.	UTI	Escherichia coli	7821	5144572	ApCer
378	840119	C-G.P.	UTI	Escherichia coli AD	7851	5044542	ApCer
380	840119	C-G.P.	UTI	Escherichia coli	7853	5044512	ApCer
391	840123	R-28	Gut	Escherichia coli	7948	5144572	ApCer
394	840123	R-53	Wound swab	Serratia odorifera	8082	5345773	ApCer
395	840126	R-CCU	UTI	Proteus mirabilis	9390	0736000	Cer
398	840201	GRI	UTI	Escherichia coli	7	7144572	ApCer
401	840201	GRI	UTI	Escherichia coli	12	1144532	ApCer
402	840201	GRI	UTI	Escherichia coli	173	5144542	ApCer
404	840201	GRI	UTI	Escherichia coli AD	25	5044542	ApCer
407	840102	GRI	UTI	Escherichia coli	35	5044512	ApCer
408	840201	GRI	UTI	Escherichia coli	122	5144572	ApCer
410	840201	GRI	UTI	Escherichia coli	126	5144572	ApCer
411	840201	GRI	UTI	Pseudomonas aerugino	129	2202505	ApCer
413	840201	GRI	UTI	Escherichia coli	141	7144552	ApCer
416	840201	GRI	UTI	Escherichia coli	105	1144532	ApCer
419	840201	GRI	UTI	Escherichia coli AD	92	5044542	ApCer
420	840201	GRI	UTI	Pseudomonas	5280	6302000	ApCer
421	840201	GRI	UTI	Pseudomonas aerugino	5314	6300004	ApCer
423	840201	GRI	UTI	Escherichia coli	6054	5144572	ApCer
427	840201	GRI	UTI	Escherichia coli	6174	5044552	ApCer
430	840201	GRI	UTI	Pseudomonas aerugino	421	2300004	ApCer
432	840201	GRI	UTI	Escherichia coli	36	1144572	ApCer
434	840201	GRI	UTI	Proteus morganii	38	0374000	ApCer
435	840201	GRI	UTI	Escherichia coli	39	5144572	ApCer
437	840201	GRI	UTI	Pseudomonas aerugino	44	6302020	ApCer
438	840201	GRI	UTI	Acinetobacter calco.	46	2206000	ApCer
439	840201	GRI	UTI	Escherichia coli	47	5144572	ApCer
441	840201	GRI	UTI	Escherichia coli	49	5144512	ApCer
446	840201	GRI	UTI	Proteus morganii	59	0374000	ApCer
450	840201	GRI	UTI	Pseudomonas fluor.gp	65	5300004	ApCer
451	840201	GRI	UTI	Escherichia coli	66	5044552	ApCer
456	840201	GRI	UTI	Escherichia coli	76	5044512	ApCer
458	840201	GRI	UTI	Escherichia coli	100	5144542	ApCer
461	840201	GRI	UTI	Escherichia coli	113	5044552	ApCer
463	840201	GRI	UTI	Escherichia coli	120	5144572	ApCer
465	840201	GRI	UTI	Citrobacter freundii	4960	1004532	ApCer
466	840201	GRI	UTI	Escherichia coli	92	5144512	ApCer
470	840202	GRI	UTI	Enterobacter cloacae	78	7305573	ApCer
474	840202	GRI	UTI	Klebsiella oxytoca	38	7375573	ApCer
475	840202	GRI	UTI	Escherichia coli	45	5144532	ApCer
479	840202	GRI	UTI	Escherichia coli	9	5144512	ApCer
480	840202	GRI	UTI	Escherichia coli	11	5144512	ApCer
490	840212	NW-12	UTI	Proteus morganii	11025	0174000	ApCer
492	840213	DRI-5	UTI	Proteus mirabilis	11441	0776000	ApCer
494	840215	NW-GP	UTI	Escherichia coli	11913	5144572	ApCer
500	840214	DRI-5	UTI	Klebsiella oxytoca	11703	5255773	ApCer
510	840215	NW-GP	UTI	Pseudomonas aerugino	11967	6300004	ApCer

No	Date	Source	Infection	Organism	H/No.	API	Resistances
514	840211	NW-15	UTI	Proteus morganii	10881	0174000	ApCer
519	840214	DRI-5	UTI	Enterobacter cloacae	11720	7305573	ApCer
525	840215	NW-GP	UTI	Escherichia coli	11994	7144532	ApCer
530	840214	NW-GP	UTI	Citrobacter freundii	11661	3764133	ApCer
531	840215	NW-BCU	UTI	Escherichia coli	12015	5144572	ApCer
535	840214	NW-GP	UTI	Pseudomonas aerugino	11681	2302004	ApCer
542	840214	NW-4	UTI	Enterobacter cloacae	11026	7305573	ApCer
543	840213	RVH	UTI	Enterobacter cloacae	11381	3305573	ApCer
546	840215	NW-5	UTI	Escherichia coli	11807	5044562	ApCer
548	840215	RVH-7	UTI	Escherichia coli	11851	1044152	ApCer
555	840212	KXH-1	UTI	Escherichia coli	11121	5156572	ApCer
559	840214	Ash-14	UTI	Enterobacter cloacae	11492	7345573	ApCer
560	840213	KXH-6	UTI	Escherichia coli	11123	5576512	ApCer
564	830105	C-8A	Chest	Haemophilus influenz	53610		ApCer
572	830122	C-8A	Chest	Haemophilus influenz	56090		ApCer
584	830126	C-8	Chest	Haemophilus influenz	56571		ApCer
585	830126	C-19A	Chest	Haemophilus influenz	56650		ApCer
588	830128	C-6	Chest	Haemophilus influenz	56970		ApCer
621	830212	C-8A	Chest	Haemophilus influenz	59572		ApCer
639	830223	C-GP	Chest	Haemophilus influenz	61350		ApCer
684	830616	C-GP	Chest	Haemophilus influenz	77415		ApCer
714	830810	C-GP	Chest	Haemophilus influenz	84832		ApCer
733	830913	C-5A	Chest	Haemophilus influenz	89805		ApCer

No	Date	Source	Infection	Organism	H/No.	API	Resistances
A006	830302	R-2	UTI	Proteus vulgaris	16-4	0676020	ApCer
A015	830303	R-2	UTI	Enterobacter cloacae	26-2	3305573	ApCer
A055	820218	GRI	UTI	Enterobacter cloacae	9026	3304573	ApCer
A056	820223	GRI	UTI	Proteus morganii	10083	0154000	ApCer
A057	820319	GRI	UTI	Escherichia coli	14999	5144552	ApCer
A058	820420	GRI	UTI	Enterobacter cloacae	21005	3305773	ApCer
A059	821019	GRI	UTI	Enterobacter cloacae	53624	3305523	ApCer
A060	821019	GRI	UTI	Serratia sp.	53771	5317763	ApCer
A061	811211	GRI	UTI	Escherichia coli	64135	5144772	ApCer
A062	830201	GRI	UTI	Escherichia coli	5265	5144572	ApCer
A063	820525	GRI	UTI	Providencia stuartii	27580	0034020	ApCer
A064	820322	GRI	UTI	Escherichia coli	15293	5144572	ApCer
A066	820125	GRI	UTI	Serratia marscesens	3855	5316561	ApCer
A067	820206	GRI	UTI	Serratia marscesens	6655	5317721	ApCer
A068	820304	GRI	UTI	Escherichia coli	11916	5144572	ApCer
A069	811201	GRI	UTI	Escherichia coli	61959	5144572	ApCer
A070	811019	GRI	UTI	Proteus vulgaris	53706	0076021	ApCer
A071	811125	GRI	UTI	Escherichia coli	60747	5044552	ApCer
A072	820423	GRI	UTI	Escherichia coli	21418	1040542	ApCer
A073	820315	GRI	UTI	Klebsiella oxytoca	13802	5254773	ApCer
A074	811201	GRI	UTI	Escherichia coli	62070	5144572	ApCer
A075	811019	GRI	UTI	Proteus morganii	51764	0154000	ApCer
A076	811208	GRI	UTI	Klebsiella oxytoca	62997	5254773	ApCer
A077	820125	GRI	UTI	Escherichia coli	3862	5144572	ApCer
A078	820000	GRI	Chest	Serratia marcescens	2677-8	5306763	ApCer
A079	820000	GRI	Chest	Serratia marscesens	2677-8	5306763	ApCer
A080	840109	NW	UTI	Klebsiella oxytoca	01535	5254773	ApCer
A081	840111	NW-GP	Wound swab	Klebsiella pneumonia	01562	4214773	ApCer
A083	840107	NW-10	Wound swab	Proteus vulgaris	01563	0476020	ApCer
A084	840109	NW-GP	UTI	Escherichia coli	01606	1044552	ApCer
A085	840109	NW-UR	UTI	Escherichia coli	01760	1044552	ApCer
A088	840110	NW-GP	UTI	Enterobacter agglome	02075	1044532	ApCer
A089	840110	NW-GP	UTI	Escherichia coli	02079	1044552	ApCer
A091	840110	NW-GP	Wound swab	Enterobacter cloacae	02217	3304573	Cer
A092	840110	NW-GP	Wound swab	Klebsiella ozaenae	02217	7204572	ApCer
A094	840111	NW-GP	UTI	Enterobacter cloacae	02755	3305573	ApCer
A095	840112	NW-11	Wound swab	Escherichia coli	02788	5144512	ApCer
A096	840112	NW-11	Wound swab	Klebsiella pneumonia	02789	5254773	ApCer
A097	840112	DRI-5	UTI	Klebsiella oxytoca	02971	5254773	ApCer
A099	840113	DRI-5	Wound swab	Klebsiella oxytoca	03129	5254773	ApCer
A101	840113	NW	Wound swab	Enterobacter cloacae	03461	3305575	ApCer
A102	840113	NW-15	Chest	Citrobacter freundii	03457	3604573	ApCer
A103	840114	NW-40	ET Tube	Klebsiella oxytoca	03481	5246572	ApCer
A104	840113	NW-GP	CAPD Fluid	Enterobacter cloacae	03126	7305573	ApCer
A109	840116	NW-28	Wound swab	Escherichia coli	04042	5144512	ApCer
A111	840116	NW-GP	Wound swab	Klebsiella oxytoca	04137	5244773	ApCer
A113	840118	NW-6	Wound swab	Enterobacter cloacae	04459	3307573	ApCer
A114	840119	NW-GP	UTI	Escherichia coli	04713	5144572	ApCer
A116	840121	NW-24	Eye swab	Proteus morganii	05429	0374000	ApCer
A117	840121	NW-7	Wound swab	Escherichia coli	5466	7144572	ApCer
A119	840120	NW-GP	UTI	Citrobacter freundii	05622	3104573	ApCer
A121	840123	NW-GP	Wound swab	Proteus morganii	05717	0374000	ApCer

No	Date	Source	Infection	Organism	H/No.	API	Resistances
A123	840125	NW-GP	Wound swab	Escherichia coli	06323	7144512	ApCer
A125	840127	NW-GP	CAPD-Fluid	Serratia marsescens	06758	5317763	ApCer
A128	840130	NW-GP	UTI	Escherichia coli	07795	4144402	ApCer
A131	840203	NW-16	Wound swab	Escherichia coli	08752	5144572	ApCer
A134	840206	NW	CAPD Fluid	Serratia marsescens	09263	5317763	ApCer
A136	840207	NW-40	Chest	Escherichia coli	09529	5144572	ApCer
A137	840206	NW-7	Wound swab	Klebsiella pneumonia	09550	7215773	ApCer
A138	840206	NW-7	Wound swab	Escherichia coli	09550	5146572	ApCer
A139	840206	NW-Ped	Wound swab	Escherichia coli	09603	5044572	ApCer
A141	840207	NW-GP	Wound swab	Proteus mirabilis	09886	0536000	ApCer
A142	840207	DRI-15	Wound swab	Escherichia coli	09923	5476533	ApCer
A143	840208	NW-GP	Wound swab	Proteus spp.	10045	5044532	ApCer
A182	830202	E-2	UTI	Escherichia coli	00012	5144520	ApCer
A261	841203	E-2	Wound swab	Proteus vulgaris	00150	0076021	ApCer
A263	841212	R-2	UTI	Proteus vulgaris	00151	0076021	ApCer
A264	841123	R-2	Wound swab	Escherichia coli	00153	5144520	ApCer
A265	841013	R-2	UTI	Proteus vulgaris	00153	0076021	ApCer
A266	830202	R-2	UTI	Proteus vulgaris	00154	0076021	ApCer

2. Strain Sensitivities.

Acinetobacter calcoaceticus

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
372	64	125	64	2.0	32.0	8.0	4.00	2.00	16.0	2.00	4.0	4.0	8.0	4.00	CoCmNf
438	500	500	1000	32.0	125.0	64.0	16.00	8.00	125.0	16.00	2000.0	500.0	250.0	8.00	GmSmSuKmCmTcMxNfHg

Citrobacter freundii

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
19	16	8	64	2.0	2.0	64.0	0.25	0.50	0.5	0.25	16.0	64.0	16.0	0.25	Hg
131	16	125	500	4.0	16.0	64.0	0.25	0.25	0.5	0.25	2000.0	250.0	250.0	0.25	GmSmTpSuKmCmTcHg
259	2000	1000	500	64.0	125.0	250.0	16.00	32.00	64.0	32.00	1000.0	125.0	64.0	8.00	Hg
465	2000	1000	500	64.0	64.0	64.0	1.00	4.00	32.0	4.00	1000.0	125.0	8.0	0.50	RfSmTpSuCmTc
530	250	64	250	2.0	2.0	4.0	0.25	0.25	0.5	0.25	4.0	64.0	8.0	0.25	RfCoCmTcHg
A102	16	2	32	2.0	0.5	32.0	0.25	0.50	0.5	0.25	8.0	64.0	4.0	0.25	RfHg
A119	2000	2000	500	125.0	250.0	250.0	16.00	4.00	64.0	16.00	1000.0	64.0	64.0	4.00	

Enterobacter aerogenes

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
43	8	4	16	2.0	4.0	4.0	0.25	0.50	0.5	0.25	1000.0	250.0	125.0	0.25	RfSmTcHg
364	2000	500	500	64.0	125.0	250.0	4.00	8.00	32.0	8.00	500.0	125.0	32.0	4.00	Hg

Enterobacter agglomerans

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
A088	16	2	4	2.0	1.0	8.0	0.25	0.25	0.5	0.25	8.0	64.0	2.0	0.25	

Enterobacter cloacae

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
94	125	1000	1000	4.0	2.0	125.0	0.25	0.25	64.0	0.25	500.0	125.0	4.0	0.25	Hg
101	64	64	250	4.0	2.0	1000.0	0.25	0.50	1.0	0.25	64.0	125.0	4.0	0.25	CoCmHg
103	64	125	250	4.0	2.0	500.0	0.25	0.50	1.0	0.25	64.0	125.0	4.0	0.25	CoCmHg
107	16	8	250	4.0	1.0	500.0	0.25	0.25	0.5	0.25	8.0	64.0	4.0	0.25	CoHg
110	16	4	125	4.0	2.0	250.0	0.25	0.25	0.5	0.25	8.0	64.0	2.0	0.25	Hg
122	16	16	125	4.0	1.0	250.0	0.25	0.25	0.5	0.25	16.0	125.0	2.0	0.25	CoHg
126	2000	2000	500	250.0	500.0	125.0	16.00	8.00	64.0	32.00	2000.0	250.0	500.0	4.00	NaGmSmTpSuKmCmNfHg
352	1000	2000	1000	8.0	8.0	250.0	0.25	0.50	125.0	0.25	500.0	125.0	4.0	0.25	CmHg
361	2000	16	125	8.0	8.0	500.0	0.50	0.50	0.5	0.25	16.0	125.0	4.0	0.25	Hg
363	2000	2000	1000	8.0	8.0	250.0	0.25	0.50	125.0	0.25	500.0	250.0	4.0	0.25	Hg
470	2000	2000	1000	500.0	500.0	250.0	4.00	8.00	125.0	8.00	2000.0	500.0	16.0	1.00	RfSmTpSuCmMxHg
519	16	4	125	8.0	2.0	250.0	0.25	0.50	0.5	0.25	8.0	125.0	8.0	0.25	CmHg
542	125	8	16	2.0	8.0	8.0	0.25	0.50	0.5	0.25	1000.0	250.0	125.0	0.25	SmTpSuTcHg
543	500	2000	500	8.0	4.0	125.0	0.25	0.50	125.0	0.25	500.0	125.0	2.0	0.25	Sm
559	2000	2000	1000	32.0	64.0	125.0	1.00	1.00	64.0	1.00	500.0	250.0	8.0	0.25	CmHg
A015	2000	2000	2000	500.0	1000.0	250.0	32.00	32.00	125.0	125.00	2000.0	500.0	125.0	8.00	SmTpSuMxRfHg
A055	2000	2000	2000	250.0	125.0	64.0	2.00	1.00	32.0	2.00	1000.0	125.0	8.0	0.25	Hg
A058	2000	2000	2000	1000.0	1000.0	1000.0	64.00	32.00	1000.0	125.00	2000.0	500.0	500.0	32.00	GmSmTpSuCoCmTcMxHg
A059	16	4	64	2.0	1.0	250.0	0.25	0.25	0.5	0.25	64.0	64.0	4.0	0.25	SmSuCoHg
A091	8	2	16	1.0	0.5	64.0	0.25	0.25	0.5	0.25	4.0	64.0	4.0	0.25	SmHg
A094	500	2000	1000	8.0	8.0	250.0	0.25	0.50	125.0	0.25	500.0	250.0	4.0	0.25	TcSmTpSuCmHg
A101	64	16	250	4.0	2.0	500.0	0.25	0.25	1.0	0.25	16.0	125.0	2.0	0.25	CoCmHg
A104	32	32	500	8.0	2.0	125.0	0.25	0.50	0.5	0.25	16.0	125.0	4.0	0.25	Hg
A113	125	64	250	4.0	2.0	250.0	0.25	0.25	2.0	0.25	64.0	125.0	4.0	0.25	Hg

Enterobacter sakazakii

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
102	2000	2000	1000	1000.0	1000.0	250.0	250.00	125.00	125.0	250.00	2000.0	500.0	500.0	64.00	GmSmTpSuKmCmTcMxHg

Enterobacter spp

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
288	250	500	250	8.0	4.0	1000.0	0.25	0.50	500.0	0.25	250.0	250.0	4.0	0.25	Co

Escherichia coli

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
1	8	4	8	2.0	2.0	4.0	0.25	0.25	0.5	0.25	250.0	64.0	16.0	0.25	SmTcHg
6	8	4	16	2.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	Hg
8	64	16	64	2.0	8.0	16.0	0.25	0.25	0.5	0.25	250.0	250.0	8.0	0.25	SmTpSuCmHg
13	8	4	8	4.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	64.0	125.0	0.25	SmSuHg
21	8	8	32	4.0	8.0	16.0	0.25	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	TcHg
28	8	8	32	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	250.0	125.0	0.25	SmSuCmHg
54	16	2	4	2.0	1.0	125.0	0.25	0.25	0.5	0.25	8.0	125.0	2.0	0.25	Hg
60	1000	64	125	64.0	16.0	64.0	2.00	8.00	32.0	1.00	500.0	125.0	32.0	4.00	SmTpSuCmTcHg
72	16	8	32	2.0	8.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	NaSmTpSuNfHg
73	16	8	32	4.0	8.0	4.0	0.25	1.00	0.5	0.25	2000.0	250.0	125.0	0.25	SmTpSuCmTcHg
85	16	16	64	4.0	1.0	125.0	0.25	1.00	0.5	0.25	4.0	32.0	4.0	0.25	SmSuHg
104	8	4	32	2.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	Tc
132	8	4	4	4.0	1.0	4.0	0.25	0.25	0.5	0.25	4.0	64.0	2.0	0.25	Hg
134	8	2	8	4.0	2.0	4.0	0.50	0.25	0.5	0.25	500.0	64.0	32.0	0.25	SmSuHg
135	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	32.0	4.00	250.0	64.0	32.0	2.00	NaKmCmHg
144	8	8	16	2.0	8.0	4.0	0.50	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	Hg
150	8	8	32	2.0	16.0	4.0	0.50	0.50	0.5	0.25	2000.0	250.0	250.0	0.25	SmSuKmHg
151	8	2	8	2.0	2.0	4.0	0.50	0.25	0.5	0.25	500.0	125.0	32.0	0.25	SmSuTc
167	1000	32	64	64.0	16.0	64.0	2.00	8.00	8.0	1.00	250.0	64.0	16.0	4.00	TcHg
189	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	32.0	2.00	2000.0	250.0	125.0	1.00	NaSmSuKm
193	2000	4	16	125.0	4.0	8.0	2.00	0.50	1.0	1.00	1000.0	125.0	125.0	1.00	SmSuHg
198	8	4	16	2.0	4.0	4.0	0.50	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	
199	16	8	32	8.0	16.0	8.0	0.50	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	NaCmTcNfHg
201	8	1	8	2.0	1.0	4.0	0.50	0.25	0.5	0.25	4.0	64.0	2.0	0.25	Su
216	8	4	4	2.0	0.5	4.0	0.25	0.25	0.5	0.25	4.0	64.0	2.0	0.25	Hg
219	8	4	16	2.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	Hg
220	16	64	125	8.0	64.0	4.0	0.25	1.00	0.5	0.25	2000.0	500.0	500.0	0.50	NaGmSmTpSuCmTcNfHg
235	16	8	32	2.0	8.0	8.0	0.25	0.50	0.5	0.25	1000.0	125.0	125.0	0.25	SmSuTcHg
264	16	8	32	4.0	8.0	8.0	0.25	0.50	0.5	0.25	2000.0	250.0	125.0	0.25	SmTpSuKmCmTcHg
294	2000	2000	2000	1000.0	1000.0	1000.0	64.00	4.00	32.0	16.00	500.0	64.0	16.0	8.00	NaTpSuKmCmTcMxNfHg
297	8	4	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	250.0	0.25	Hg
301	1000	125	125	64.0	32.0	125.0	4.00	8.00	16.0	1.00	500.0	125.0	32.0	4.00	SmTpSuCmTcHg
308	8	4	16	4.0	8.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	CmHg
326	8	4	16	4.0	4.0	2.0	0.25	0.25	0.5	0.25	1000.0	64.0	125.0	0.25	TpSuTcHg
330	8	8	16	4.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	500.0	125.0	0.25	Hg
334	16	8	32	2.0	4.0	8.0	0.25	0.25	0.5	0.25	250.0	125.0	16.0	0.25	SmSuCmTcHg
337	8	2	8	2.0	1.0	4.0	0.25	0.25	0.5	0.25	4.0	64.0	1.0	0.25	Hg
348	8	4	16	2.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	Hg
350	16	8	32	2.0	2.0	4.0	0.25	0.25	0.5	0.25	500.0	64.0	32.0	0.25	TcHg
357	8	4	16	2.0	8.0	8.0	0.25	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	SmSuTc
358	500	16	64	125.0	8.0	32.0	1.00	8.00	4.0	1.00	250.0	64.0	16.0	8.00	SmSuTcHg
367	125	8	16	16.0	4.0	32.0	0.50	1.00	2.0	0.25	500.0	125.0	32.0	1.00	SmSuHg
373	8	2	8	4.0	2.0	4.0	0.25	0.25	0.5	0.25	500.0	125.0	32.0	0.25	SmSuHg
374	16	16	64	8.0	16.0	8.0	0.25	1.00	0.5	0.25	2000.0	500.0	500.0	0.25	SmSuKmCmTcHg
378	8	2	8	2.0	2.0	4.0	0.25	0.25	0.5	0.25	500.0	125.0	32.0	0.25	SmSuHg
380	8	4	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	SmSuHg
391	16	32	125	4.0	32.0	4.0	0.25	1.00	0.5	0.25	2000.0	500.0	500.0	0.25	SmTpSuKmTcHg
398	16	16	64	4.0	16.0	4.0	0.25	0.50	0.5	0.25	2000.0	250.0	500.0	0.25	CoTcHg
401	125	16	16	32.0	4.0	32.0	1.00	4.00	4.0	0.50	250.0	32.0	16.0	4.00	SmTc
402	8	8	32	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	250.0	250.0	0.25	SmTpSuTc
404	8	8	16	4.0	8.0	8.0	0.25	0.50	0.5	0.25	2000.0	125.0	125.0	0.25	Hg
407	8	4	16	4.0	4.0	8.0	0.25	0.25	0.5	0.25	500.0	125.0	64.0	0.25	SmSuHg
408	16	4	8	4.0	1.0	4.0	0.25	0.25	0.5	0.25	8.0	64.0	4.0	0.25	CoTcHg
410	8	4	16	4.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	125.0	125.0	0.25	SmTpSuKmCmHg
413	1000	250	500	16.0	2.0	32.0	0.25	0.25	1.0	0.25	250.0	125.0	16.0	0.25	CoTcHg
416	125	8	16	32.0	4.0	32.0	1.00	4.00	4.0	0.25	250.0	32.0	16.0	2.00	SmTc
419	8	4	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	250.0	125.0	0.25	Hg
423	16	8	32	8.0	16.0	16.0	0.25	0.50	0.5	0.25	1000.0	250.0	125.0	0.25	NaSmTpSuCmNfHg

427	16	8	32	8.0	16.0	8.0	0.25	0.25	0.5	0.25	2000.0	500.0	125.0	0.25	SmTpSuCmHg
432	16	1000	32	8.0	32.0	125.0	2.00	1.00	1.0	0.25	2000.0	250.0	250.0	0.25	NaSmTpSuKmCmTcNfHg
435	16	4	8	16.0	2.0	4.0	0.25	0.25	0.5	0.25	8.0	125.0	4.0	0.25	Hg
439	16	8	64	16.0	16.0	32.0	0.25	0.50	0.5	0.25	2000.0	500.0	125.0	0.25	NaSmTpSuCmNfHg
441	16	8	32	16.0	16.0	16.0	0.25	1.00	0.5	0.25	2000.0	125.0	125.0	0.25	CmHg
451	8	4	8	8.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	SmSuHg
456	8	4	16	8.0	4.0	4.0	0.25	0.50	0.5	0.25	1000.0	125.0	125.0	0.25	Hg
458	8	4	16	2.0	8.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	64.0	0.25	SmSuHg
461	8	4	16	4.0	4.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	SmSuTcHg
463	1000	32	64	2.0	16.0	64.0	2.00	8.00	8.0	1.00	250.0	64.0	16.0	4.00	SmSuCmTcHg
466	16	8	16	4.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	125.0	250.0	0.25	Hg
475	16	8	64	4.0	16.0	4.0	0.25	0.25	0.5	0.25	2000.0	125.0	125.0	0.25	SmHg
479	8	4	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	250.0	0.25	Hg
480	8	8	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	250.0	0.25	Hg
494	16	8	32	2.0	8.0	8.0	0.25	0.25	0.5	0.25	2000.0	250.0	125.0	0.25	
525	64	8	125	2.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	250.0	125.0	0.25	SmCoCmTcHg
531	16	4	16	2.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	250.0	125.0	0.25	SmTpSuCmTcHg
546	16	8	64	2.0	8.0	4.0	0.25	0.25	0.5	0.25	2000.0	125.0	250.0	0.25	SmTpSu
548	8	4	16	2.0	4.0	4.0	0.25	0.25	0.5	0.25	2000.0	125.0	125.0	0.25	TcHg
555	64	8	16	4.0	4.0	8.0	0.25	0.25	0.5	0.25	1000.0	125.0	125.0	0.25	SmSuCoCmTcHg
560	16	8	32	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	250.0	250.0	0.25	CoCmTcHg
A057	1000	32	125	64.0	16.0	64.0	4.00	8.00	8.0	2.00	500.0	125.0	32.0	2.00	SmSu
A061	1000	32	64	64.0	16.0	64.0	2.00	8.00	8.0	1.00	500.0	64.0	32.0	8.00	
A062	250	16	32	32.0	8.0	32.0	1.00	4.00	4.0	0.50	125.0	125.0	16.0	2.00	SmSuKmCmTcHg
A064	1000	32	64	64.0	8.0	64.0	2.00	8.00	8.0	1.00	250.0	64.0	16.0	4.00	SmTpSuTc
A068	250	16	32	32.0	8.0	32.0	1.00	4.00	4.0	0.50	125.0	125.0	8.0	1.00	SmSuKmCmTc
A069	500	16	32	32.0	8.0	32.0	1.00	4.00	4.0	0.50	125.0	64.0	8.0	2.00	TcSmSuKmCmHg
A071	64	4	8	4.0	2.0	8.0	0.25	0.50	0.5	0.25	500.0	125.0	32.0	0.25	Hg
A072	64	8	32	2.0	8.0	16.0	0.25	0.25	0.5	0.25	500.0	125.0	32.0	0.25	TcNaSmNfHg
A074	32	4	64	4.0	4.0	4.0	0.25	2.00	1.0	0.25	1000.0	250.0	125.0	0.25	SmTpSuCoCmTc
A077	16	8	8	2.0	4.0	32.0	0.25	0.25	2.0	0.25	1000.0	64.0	2.0	0.25	NaNfHg
A084	500	16	64	32.0	8.0	32.0	2.00	8.00	4.0	1.00	500.0	125.0	32.0	4.00	TcSmTpSuKmCm
A085	16	32	125	2.0	64.0	4.0	0.25	1.00	0.5	0.25	2000.0	500.0	500.0	0.25	TcSmTpSu
A089	16	2	4	4.0	1.0	8.0	0.25	0.50	0.5	0.25	8.0	32.0	4.0	0.25	CmHg
A095	250	2	4	32.0	1.0	32.0	8.00	0.50	1.0	16.00	32.0	32.0	16.0	0.25	CmTcHg
A109	8	2	4	0.5	0.5	4.0	0.25	0.25	0.5	0.25	4.0	64.0	2.0	0.25	
A114	1000	32	64	64.0	16.0	64.0	2.00	8.00	8.0	1.00	1000.0	250.0	125.0	4.00	SmTpSuTc
A117	8	2	8	4.0	2.0	4.0	0.25	0.50	0.5	0.25	500.0	125.0	32.0	0.25	SmSu
A123	8	4	16	4.0	8.0	4.0	0.25	0.50	0.5	0.25	2000.0	125.0	250.0	0.25	SmSuTc
A128	1000	32	64	32.0	16.0	64.0	2.00	8.00	8.0	1.00	250.0	64.0	16.0	4.00	SmSuTc
A131	1000	1000	500	64.0	16.0	64.0	2.00	8.00	8.0	1.00	500.0	125.0	32.0	4.00	RfCoHg
A136	16	8	32	8.0	16.0	8.0	0.25	0.50	0.5	0.25	2000.0	250.0	250.0	0.25	SmCmHg
A138	125	8	16	2.0	4.0	16.0	0.50	2.00	2.0	0.25	125.0	64.0	8.0	1.00	SmSuKmCmTc
A139	8	4	16	8.0	8.0	8.0	0.25	0.50	0.5	0.25	2000.0	250.0	125.0	0.25	SmSuCmHg
A142	500	250	500	64.0	32.0	4.0	0.25	0.25	0.5	0.25	250.0	125.0	2.0	0.25	SmTpSuCoTcHg
A182	16	4	8	1.0	0.5	1.0	0.25	0.15	0.2	0.25	4.0	32.0	2.0	0.15	Tc

Haemophilus influenzae

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
52	16	32	32	0.5	1.0	8.0	0.01	0.12	2.0	0.01	16.0	32.0	4.0	0.06	
74	16	16	16	0.5	1.0	4.0	0.01	0.25	1.0	0.01	8.0	32.0	4.0	0.06	CoGmTpTcNfSxNaKmSm
118	125	32	64	4.0	4.0	4.0	0.06	0.50	8.0	0.01	8.0	125.0	0.0	0.50	
156	16	1	16	0.5	0.5	2.0	0.01	0.12	1.0	0.01	2.0	32.0	0.0	0.06	NfSx
243	64	32	32	1.0	2.0	4.0	0.01	0.12	2.0	0.01	4.0	64.0	0.5	0.06	Nf
564	16	32	16	4.0	0.5	8.0	0.01	0.12	1.0	0.01	16.0	8.0	0.2	0.06	Nf
572	8	1	16	0.5	0.5	4.0	0.01	0.12	1.0	0.01	125.0	8.0	32.0	0.06	Nf
584	250	16	32	2.0	4.0	8.0	0.01	0.25	2.0	0.01	500.0	32.0	16.0	0.06	NfSx
585	250	16	32	0.5	1.0	4.0	0.01	0.06	1.0	0.01	250.0	32.0	64.0	0.06	Sx
588	64	8	16	1.0	2.0	4.0	0.01	0.25	2.0	0.01	16.0	64.0	0.2	0.06	Nf
621	16	1	16	1.0	0.5	4.0	0.01	0.12	1.0	0.01	16.0	64.0	0.0	0.06	NfSx
639	8	125	32	0.5	1.0	2.0	0.01	0.06	1.0	0.01	500.0	32.0	32.0	0.06	NfSxNa
684	64	32	32	2.0	1.0	8.0	0.01	0.06	2.0	0.01	4.0	32.0	0.0	0.06	Nf
714	16	32	125	4.0	4.0	32.0	0.01	0.12	2.0	0.01	64.0	32.0	64.0	0.06	TcNf
733	16	16	32	0.5	1.0	4.0	0.01	0.12	2.0	0.01	125.0	32.0	2.0	0.06	NfSx

Hafnia alvei

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
99	8	8	32	2.0	1.0	4.0	0.25	1.00	0.5	0.25	4.0	8.0	4.0	0.25	CoHg

Klebsiella oxytoca

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
23	8	125	64	32.0	16.0	2.0	0.25	0.25	0.5	1.00	2000.0	125.0	500.0	4.00	Hg
64	125	500	250	125.0	250.0	4.0	1.00	0.50	0.5	4.00	2000.0	500.0	500.0	8.00	GmSmTpSuKmHg
339	8	8	8	2.0	8.0	2.0	0.25	0.25	0.5	0.25	64.0	64.0	8.0	0.25	Hg
474	500	250	500	32.0	4.0	16.0	0.25	1.00	0.5	0.25	250.0	250.0	32.0	0.25	RfCoHg
500	125	500	250	250.0	500.0	8.0	1.00	0.50	0.5	8.00	2000.0	500.0	500.0	64.00	GmSmTpSuKmHg
A073	64	500	250	64.0	125.0	2.0	0.50	1.00	0.5	4.00	2000.0	500.0	500.0	8.00	RfHg
A076	64	500	250	64.0	125.0	2.0	0.50	0.50	0.5	4.00	2000.0	250.0	500.0	8.00	Hg
A080	125	500	250	125.0	250.0	4.0	1.00	0.50	0.5	8.00	2000.0	500.0	500.0	64.00	GmSmTpSuKmHg
A097	125	500	250	125.0	250.0	8.0	1.00	0.50	0.5	8.00	2000.0	500.0	500.0	32.00	GmSmTpSuKm
A099	125	500	250	125.0	250.0	8.0	1.00	1.00	0.5	8.00	2000.0	500.0	500.0	64.00	GmSmTpSuKm
A103	1000	2000	500	64.0	64.0	32.0	0.25	0.50	1.0	0.25	2000.0	250.0	250.0	0.25	RfSmCoCmTcHg
A111	125	1000	250	500.0	500.0	4.0	1.00	1.00	0.5	8.00	2000.0	500.0	500.0	32.00	

Klebsiella ozaenae

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
230	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	500.0	4.00	2000.0	32.0	32.0	4.00	NaSmTpKmCmHg
365	16	500	250	64.0	125.0	125.0	4.00	8.00	32.0	8.00	500.0	64.0	32.0	4.00	SmKmHg
A092	64	16	64	2.0	0.5	64.0	0.25	0.50	0.5	0.25	16.0	32.0	2.0	0.25	NfHg

Klebsiella pneumoniae

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
12	8	2	4	4.0	2.0	8.0	0.25	0.50	0.5	0.25	64.0	64.0	8.0	0.25	RfNaTpSuCmNfHg
15	8	16	64	2.0	8.0	4.0	0.25	0.50	0.5	0.25	1000.0	500.0	125.0	0.25	Hg
175	2000	2000	500	500.0	1000.0	125.0	16.00	4.00	32.0	4.00	2000.0	250.0	500.0	4.00	NaGmSmTpSuKmCmTcHg
241	16	32	64	4.0	64.0	2.0	1.00	1.00	0.5	1.00	2000.0	500.0	250.0	0.25	GmSmTpSuKmCmTcHg
303	8	16	64	4.0	16.0	8.0	0.25	1.00	0.5	0.25	2000.0	500.0	500.0	0.25	GmSmTpSuKmCmTcHg
A081	8	4	4	1.0	0.5	2.0	0.25	0.25	0.5	0.50	64.0	125.0	4.0	0.25	Hg
A096	8	2	4	2.0	1.0	4.0	0.25	0.25	0.5	0.25	64.0	125.0	8.0	0.25	Hg
A137	8	2	4	1.0	0.5	2.0	0.25	0.25	0.5	0.25	16.0	64.0	8.0	0.25	RfHg

Proteus mirabilis

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
50	64	64	250	4.0	2.0	500.0	0.25	0.50	4.0	0.25	64.0	125.0	4.0	0.25	CoCmTcHg
138	1000	500	500	64.0	125.0	64.0	16.00	8.00	125.0	16.00	64.0	64.0	32.0	32.00	SmTpSuCoCmTcMxNfHg
140	500	500	500	32.0	125.0	64.0	16.00	8.00	125.0	16.00	1000.0	250.0	125.0	16.00	GmSmSuKmCmTcMxNfHg
196	2000	16	125	125.0	8.0	8.0	2.00	0.25	0.5	1.00	1000.0	125.0	125.0	0.25	TpSuKmCoTcHg
221	16	8	16	2.0	2.0	4.0	0.25	0.25	0.5	0.25	250.0	64.0	8.0	0.25	SmTpSuCoTcHg
261	32	64	32	4.0	2.0	4.0	0.25	0.25	0.5	0.25	8.0	125.0	1.0	0.25	SmCoTcHg
296	16	4	16	2.0	1.0	2.0	0.25	0.25	0.5	0.25	250.0	64.0	8.0	0.25	CoTcHg
342	16	8	8	2.0	4.0	4.0	0.25	0.25	0.5	0.25	2.0	64.0	1.0	0.25	CmTc
344	16	4	8	2.0	1.0	4.0	0.25	0.25	0.5	0.25	2.0	64.0	1.0	0.25	CoTcHg
395	16	8	8	2.0	2.0	4.0	0.25	0.25	0.5	0.25	4.0	64.0	2.0	0.25	CoCmTcHg
492	32	4	16	8.0	8.0	4.0	0.25	0.50	0.5	0.25	4.0	64.0	1.0	0.25	CoCmTcHg
A141	64	8	16	8.0	4.0	8.0	0.25	0.25	0.5	0.25	8.0	125.0	2.0	0.25	CoCmTcHg

Proteus morganii

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
100	1000	500	1000	64.0	125.0	125.0	16.00	8.00	125.0	16.00	1000.0	250.0	250.0	32.00	GmSmTpSuKmCoCmTcMxNfH
434	1000	250	500	32.0	1.0	16.0	0.25	0.25	0.5	0.25	125.0	125.0	1.0	0.25	CoCm
446	1000	500	500	32.0	4.0	32.0	0.25	0.25	0.5	0.25	250.0	125.0	2.0	0.25	CoCmHg
490	1000	500	1000	32.0	1.0	32.0	0.25	0.25	0.5	0.25	125.0	250.0	1.0	0.25	CoHg
514	1000	250	500	32.0	2.0	16.0	0.25	0.25	0.5	0.25	250.0	125.0	1.0	0.25	KmCoCmHg
A056	1000	250	500	32.0	1.0	8.0	0.25	0.25	0.5	0.25	125.0	125.0	2.0	0.25	Co
A075	1000	250	500	32.0	1.0	16.0	0.25	0.25	0.5	0.25	125.0	125.0	1.0	0.25	CoHg
A116	500	250	250	32.0	1.0	8.0	0.25	0.25	1.0	0.25	125.0	125.0	1.0	0.25	Co
A121	500	2000	500	64.0	64.0	32.0	0.25	0.50	1.0	0.25	1000.0	125.0	8.0	0.25	RfCoTc

Proteus spp.

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
A143	64	8	16	8.0	2.0	8.0	0.25	0.50	2.0	0.25	125.0	125.0	4.0	0.50	CoTc

Proteus vulgaris

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
36	125	125	500	64.0	4.0	4.0	0.25	0.25	0.5	0.25	250.0	250.0	0.5	0.25	CoHg
A006	1000	500	1000	125.0	64.0	64.0	2.00	4.00	8.0	4.00	500.0	125.0	16.0	8.00	GmSmTpKmCoNf
A070	1000	500	1000	1000.0	64.0	4.0	0.25	0.25	0.5	0.25	1000.0	125.0	2.0	0.25	SmHg
A083	1000	500	500	250.0	64.0	4.0	0.25	0.25	0.5	0.25	500.0	125.0	2.0	0.25	CoHg
A261	32	16	32	32.0	4.0	16.0	4.00	2.00	4.0	2.00	16.0	64.0	32.0	2.00	TcSp
A263	4	2	16	16.0	2.0	4.0	0.50	0.25	2.0	0.50	16.0	64.0	32.0	0.50	Tc
A264	8	16	32	16.0	2.0	16.0	0.50	0.25	0.5	0.25	8.0	64.0	64.0	64.00	TcSp
A265	2	2	16	8.0	1.0	2.0	0.25	0.12	2.0	0.25	16.0	32.0	32.0	0.12	
A266	16	4	8	16.0	2.0	4.0	0.12	0.12	2.0	0.12	16.0	64.0	32.0	0.12	Sp

Providencia stuartii

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
A063	2000	2000	2000	1000.0	1000.0	8.0	8.00	0.50	0.5	8.00	2000.0	500.0	16.0	0.25	CoCmHg

Pseudomonas

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
44	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	32.0	4.00	250.0	2.0	8.0	4.00	NaTpSuKmCmNfHg
49	2000	2000	2000	500.0	1000.0	1000.0	16.00	1.00	32.0	4.00	250.0	2.0	8.0	4.00	NaTpSuKmCmMxNfHg
55	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	64.0	8.00	500.0	2.0	8.0	4.00	RfNaSmTpSuKmCmMxNfHg
67	2000	2000	2000	500.0	1000.0	1000.0	8.00	1.00	500.0	4.00	2000.0	2.0	8.0	4.00	NaSmTpSuKmCmHg
93	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	32.0	2.00	250.0	2.0	8.0	4.00	NaTpKmCmHg
108	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	32.0	4.00	125.0	32.0	8.0	4.00	NaTpKmCmNfHg
115	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	64.0	4.00	125.0	2.0	8.0	4.00	NaSmTpKmCmHg
141	2000	2000	2000	250.0	1000.0	500.0	8.00	1.00	64.0	4.00	250.0	2.0	4.0	4.00	NaTpKmCmHg
163	250	250	250	250.0	64.0	64.0	8.00	4.00	64.0	8.00	500.0	32.0	32.0	8.00	GmSmTpSuKmCmMxNfHg
179	2000	2000	2000	500.0	1000.0	1000.0	8.00	2.00	64.0	4.00	500.0	2.0	8.0	4.00	NaTpKmCmTcHg
180	2000	2000	2000	250.0	1000.0	1000.0	4.00	1.00	500.0	4.00	1000.0	1.0	4.0	2.00	NaTpKmCmHg
188	2000	2000	2000	500.0	1000.0	1000.0	8.00	1.00	125.0	4.00	500.0	2.0	8.0	4.00	NaSmTpKmCmHg
307	2000	500	64	125.0	500.0	32.0	2.00	0.50	4.0	1.00	16.0	0.5	2.0	0.50	NaNfHg
340	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	32.0	2.00	125.0	64.0	4.0	1.00	NaKmCoTcHg
347	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	64.0	4.00	250.0	2.0	4.0	2.00	NaSmTpKmCmHg
353	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	32.0	2.00	125.0	1.0	2.0	1.00	NaTpKmCmHg
360	2000	1000	125	500.0	1000.0	125.0	16.00	2.00	16.0	4.00	125.0	4.0	8.0	8.00	NaSmTpSuKmCmMxTcNfHg
369	2000	2000	2000	250.0	1000.0	500.0	8.00	1.00	64.0	4.00	500.0	2.0	4.0	4.00	NaTpKmCmHg
411	2000	1000	125	250.0	500.0	64.0	8.00	2.00	8.0	4.00	64.0	4.0	4.0	4.00	NaSmTpKmCmNfHg
420	2000	2000	2000	500.0	1000.0	1000.0	16.00	2.00	125.0	4.00	1000.0	4.0	8.0	4.00	NaSmTpSuKmCmTcMxNfRfHg
421	2000	2000	2000	500.0	1000.0	1000.0	8.00	2.00	64.0	4.00	1000.0	2.0	8.0	4.00	NaSmTpKmCmTcNfRfHg
430	2000	2000	2000	250.0	1000.0	1000.0	8.00	2.00	64.0	4.00	500.0	2.0	8.0	4.00	NaSmTpSuKmCmTcNfRfHg
437	2000	2000	2000	250.0	1000.0	500.0	4.00	1.00	32.0	2.00	125.0	1.0	4.0	1.00	NaSmTpKmCmHg
450	2000	2000	2000	1000.0	1000.0	500.0	32.00	4.00	125.0	8.00	500.0	8.0	16.0	8.00	NaSmTpSuKmCmTcMxNfRfHg
510	2000	2000	125	250.0	1000.0	500.0	8.00	2.00	32.0	4.00	125.0	32.0	16.0	8.00	NaTpSuCmNfMxHg
535	2000	2000	1000	500.0	1000.0	250.0	16.00	2.00	32.0	8.00	250.0	125.0	32.0	8.00	NaTpSuCmNfMxHg

Serratia

No.	CED	CFZ	CER	CXM	CMD	CFX	CTX	CAZ	CTN	CTR	AMP	CFS	PIP	AZT	Other resistances
253	8	4	64	4.0	1.0	125.0	0.25	0.25	0.5	0.25	4.0	64.0	4.0	0.25	Hg
345	250	2000	250	32.0	16.0	16.0	0.25	0.25	0.5	0.25	64.0	125.0	4.0	0.25	RfCoHg
394	16	4	32	4.0	1.0	125.0	0.25	0.25	2.0	0.25	64.0	125.0	4.0	0.25	SmSuTc
A060	500	2000	500	64.0	32.0	32.0	0.50	1.00	1.0	0.25	125.0	125.0	4.0	0.25	CoCmTcRf
A066	1000	2000	1000	64.0	64.0	32.0	0.50	1.00	1.0	0.50	64.0	125.0	4.0	0.25	CmTcRfHg
A067	1000	2000	500	64.0	64.0	32.0	0.50	1.00	1.0	0.25	125.0	125.0	8.0	0.25	CoCmTcRf
A078	125	500	125	16.0	16.0	8.0	0.25	0.25	0.5	0.25	16.0	125.0	2.0	0.25	RfCoCmTcHg
A079	2000	2000	2000	1000.0	1000.0	64.0	4.00	2.00	16.0	16.00	500.0	250.0	125.0	2.00	GmRfSmKmCoCmTcMx
A125	1000	2000	1000	64.0	64.0	32.0	0.25	1.00	1.0	0.25	1000.0	125.0	8.0	0.25	RfCoCmTc
A134	1000	2000	1000	64.0	64.0	32.0	0.25	0.50	1.0	0.25	1000.0	125.0	8.0	0.25	RfCoCmTc

3. B-lactamase Profiles.

No.	Organism	B-lactamase	Chromosomal pI	Sp Act/ mg protein
1	Escherichia coli	TEM-1	0.00	0.02144
6	Escherichia coli	TEM-1	7.50	0.03907
8	Escherichia coli	TEM-1	7.50	0.02147
12	Klebsiella pneumonia		7.00	0.00020
13	Escherichia coli	TEM-1	7.50	0.00846
15	Klebsiella pneumonia		7.80	0.01688
19	Citrobacter freundii		8.45	0.00036
21	Escherichia coli	TEM-1	0.00	0.01625
23	Klebsiella oxytoca		7.40	0.00778
28	Escherichia coli	TEM-1	7.60	0.03036
36	Proteus vulgaris		0.00	0.00048 x
43	Enterobacter aerogen		7.41	0.03120
44	Pseudomonas	S&A	7.6	0.00114
49	Pseudomonas	S&A	8.21	0.00201
50	Proteus mirabilis		8.3	0.00036
52	Haemophilus influenz	TEM-1	0.00	0.00477
54	Escherichia coli		7.50	0.00048
55	Pseudomonas	S&A	7.2	0.00012
60	Escherichia coli	TEM-1	8.60	0.00160
64	Klebsiella oxytoca		K1	2.27907
67	Pseudomonas	S&A	7.2	0.00112
72	Escherichia coli	TEM-1	8.60	0.00374
73	Escherichia coli	OXA-3	8.60	0.09023
74	Haemophilus influenz	TEM-1	7.50	0.02101
85	Escherichia coli	TEM-1	8.60	0.00034
93	Pseudomonas	S&A	8.30	0.00050 x
94	Enterobacter cloacae	Enzyme A	8.50	0.00094
99	Hafnia alvei	OXA-1	8.25	0.01690
100	Proteus morganii	TEM-1	7.30	0.02115
101	Enterobacter cloacae		P99	0.00034
102	Enterobacter sakazak	TEM-1	P99	0.56237
103	Enterobacter cloacae	Enzyme A	8.60	0.07054
104	Escherichia coli	TEM-1	7.73	0.00012
107	Enterobacter cloacae	OXA-2	8.2	0.00168
108	Pseudomonas	S&A	7.25	0.00033
110	Enterobacter cloacae		7.92	0.00097
115	Pseudomonas	S&A	7.60	0.00011
118	Haemophilus influenz	None	0.00	0.00000
122	Enterobacter cloacae	Enzyme A	8.20	0.00163
126	Enterobacter cloacae	TEM-1	8.20	0.04946
131	Citrobacter freundii	TEM-1	8.20	0.04651
132	Escherichia coli		8.50	0.00030
134	Escherichia coli	TEM-1	7.60	0.00397
135	Escherichia coli	TEM-1	9.00	0.00032
138	Proteus mirabilis		9.00	0.00161
140	Proteus mirabilis	TEM-1	9.00	0.00030
141	Pseudomonas	TEM-1	8.20	0.00428
144	Escherichia coli	TEM-1	8.60	0.00594
150	Escherichia coli	TEM-1	7.49	0.01627
151	Escherichia coli	TEM-1	0.00	0.00337
156	Haemophilus influenz	None	0.00	0.00000
163	Pseudomonas	TEM-1	8.00	0.36345
167	Escherichia coli	TEM-1	8.30	0.00018
175	Klebsiella pneumonia	TEM-1,S-1	7.60	0.07843
179	Pseudomonas	S&A	8.32	0.00039
180	Pseudomonas	S&A	7.80	0.00142
188	Pseudomonas	S&A	7.80	0.00031
189	Escherichia coli	TEM-1	8.70	0.05375
193	Escherichia coli	TEM-1	9.00	0.02022
196	Proteus mirabilis	TEM-2	0.00	0.00016
198	Escherichia coli	TEM-1	7.40	0.01204
199	Escherichia coli	TEM-1	7.40	0.07002
201	Escherichia coli	TEM-1	8.70	0.00226
216	Escherichia coli	TEM-1	8.70	0.00132
219	Escherichia coli	TEM-1	7.40	0.02040
220	Escherichia coli	TEM-1	7.60	0.08199
221	Proteus mirabilis	TEM-1	0.00	0.00023
230	Klebsiella ozaenae	TEM-1,S-1	7.60	0.00464
235	Escherichia coli	TEM-1	7.40	0.00819
241	Klebsiella pneumonia	PSE-4	7.20	0.01611
243	Haemophilus influenz	None	0.00	0.00000

No.	Organism	B-lactamase	Chromosomal pI	Sp Act/ mg protein
253	Serratia		8.00	0.00054
259	Citrobacter freundii		8.60	0.24680
261	Proteus mirabilis	TEM-1	0.00	0.00059
264	Escherichia coli	TEM-1	8.60	0.01963
288	Enterobacter spp		8.20	0.01744
294	Escherichia coli		7.40	0.00029
296	Proteus mirabilis	TEM-1	0.00	0.00152
297	Escherichia coli	TEM-1	7.40	0.00573
301	Escherichia coli		8.59	0.09582
303	Klebsiella pneumonia	TEM-1	8.50	0.04207
307	Pseudomonas	TEM-1	8.20	0.00321
308	Escherichia coli	TEM-1	8.50	0.00524
326	Escherichia coli	TEM-1	8.59	0.02826
330	Escherichia coli	TEM-1	8.24	0.00157
334	Escherichia coli	TEM-1	8.59	0.00221
337	Escherichia coli		8.50	0.00093
339	Klebsiella oxytoca	TEM-1	0.00	0.00987
340	Pseudomonas	S&A	8.33	0.00027
342	Proteus mirabilis	None	0.00	0.00026 x
344	Proteus mirabilis	None	0.00	0.00017 x
345	Serratia		8.40	0.00211
347	Pseudomonas	S&A	8.00	0.00094
348	Escherichia coli	TEM-1	7.80	0.01138
350	Escherichia coli	TEM-2	7.80	0.00466
352	Enterobacter cloacae		8.70	0.00039
353	Pseudomonas	S&A	8.22	0.00126
357	Escherichia coli	TEM-1	8.70	0.00300
358	Escherichia coli		8.75	0.00454
360	Pseudomonas	S&A	8.80	0.00970 x
361	Enterobacter cloacae		8.20	0.00460
363	Enterobacter cloacae		8.63	0.00066
364	Enterobacter aerogen		8.22	0.11127
365	Klebsiella ozaenae		8.63	0.00070
367	Escherichia coli	TEM-1	8.75	0.00247
369	Pseudomonas	S&A	8.50	0.11030 x
372	Acinetobacter calco.		9.00	0.00503
373	Escherichia coli	TEM-1	8.30	0.01126
374	Escherichia coli	TEM-1	9.33	0.20840
378	Escherichia coli	TEM-1	8.60	0.00212
380	Escherichia coli	TEM-1	8.80	0.04724
391	Escherichia coli	TEM-1	9.4	0.10365
394	Serratia		8.60	0.00272
395	Proteus mirabilis		8.75	0.00123
398	Escherichia coli	TEM-1	8.80	0.01642
401	Escherichia coli		8.90	0.01178
402	Escherichia coli	TEM-1	9.50	0.02121
404	Escherichia coli	TEM-1	8.68	0.00626
407	Escherichia coli	TEM-1	8.80	0.01584
408	Escherichia coli		8.85	0.00082
410	Escherichia coli	TEM-1	9.33	0.02736
411	Pseudomonas		0.00	0.00159 x
413	Escherichia coli	TEM-1	8.75	0.00432
416	Escherichia coli		8.60	0.02481
419	Escherichia coli	TEM-1	8.40	0.01006
420	Pseudomonas	S&A	8.20	0.00022
421	Pseudomonas	S&A	8.30	0.00118
423	Escherichia coli	TEM-1	9.33	0.07140
427	Escherichia coli	TEM-1	8.46	0.02666
430	Pseudomonas	S&A	8.20	0.00031
432	Escherichia coli	TEM-1	8.60	0.07360
434	Proteus morganii		7.80	0.00075
435	Escherichia coli		8.60	0.00053
437	Pseudomonas	S&A	7.90	0.00073
438	Acinetobacter calco.	TEM-1	9.00	0.06442
439	Escherichia coli	TEM-1	8.60	0.00790
441	Escherichia coli	TEM-1	7.50	0.03101
446	Proteus morganii		7.70	0.00094
450	Pseudomonas	S&A	7.60	0.00045
451	Escherichia coli	TEM-1	7.40	0.00122
456	Escherichia coli	TEM-1	8.40	0.00197
458	Escherichia coli	TEM-1	8.40	0.01043

No.	Organism	B-lactamase	Chromosomal pI	Sp Act/ mg protein	
461	Escherichia coli	TEM-1	8.60	0.02018	
463	Escherichia coli		8.90	0.01562	
465	Citrobacter freundii		8.80	0.03849	
466	Escherichia coli	TEM-1	7.60	0.02527	
470	Enterobacter cloacae		P99	0.24806	
474	Klebsiella oxytoca		7.40	0.00154	
475	Escherichia coli	TEM-1	8.62	0.00705	
479	Escherichia coli	TEM-1	7.49	0.01422	
480	Escherichia coli	TEM-1	8.60	0.03245	
490	Proteus morganii		7.60	0.00025	
492	Proteus mirabilis	None	0.00	0.00899	X
494	Escherichia coli	TEM-1	8.50	0.00734	
500	Klebsiella oxytoca		K1	0.05554	
510	Pseudomonas	S&A	8.30	0.00036	
514	Proteus morganii		7.60	0.00035	
519	Enterobacter cloacae	TEM-1	8.00	0.00083	
525	Escherichia coli	TEM-1	9.50	0.01305	
530	Citrobacter freundii		5.00	0.00029	
531	Escherichia coli	TEM-1	9.00	0.00417	
535	Pseudomonas	S&A	8.20	0.01370	
542	Enterobacter cloacae	TEM-1	9.00	0.01063	
543	Enterobacter cloacae		8.37	0.00074	
546	Escherichia coli	TEM-1	9.40	0.03186	
548	Escherichia coli	TEM-1	8.40	0.00694	
555	Escherichia coli	TEM-1	8.40	0.00422	
559	Enterobacter cloacae		8.40	0.00097	
560	Escherichia coli	TEM-1	9.40	0.00869	
564	Haemophilus influenz	None	0.00	0.00000	
572	Haemophilus influenz	TEM-1	0.00	0.00540	
584	Haemophilus influenz	TEM-1	0.00	0.01206	
585	Haemophilus influenz	TEM-1	0.00	0.00739	
588	Haemophilus influenz	TEM-1	0.00	0.00116	
621	Haemophilus influenz	None	0.00	0.00000	
639	Haemophilus influenz	TEM-1	0.00	0.01418	
684	Haemophilus influenz	None	0.00	0.00000	
714	Haemophilus influenz	TEM-1	0.00	0.01255	
733	Haemophilus influenz	TEM-1	0.00	0.00802	
A006	Proteus vulgaris		7.00	0.00223	X
A015	Enterobacter cloacae	TEM-1	P99	0.13517	
A055	Enterobacter cloacae	TEM-2	8.60	0.02224	
A056	Proteus morganii		6.50	0.00055	
A057	Escherichia coli		7.00	0.15821	
A058	Enterobacter cloacae	TEM-2	8.60	0.02215	
A059	Enterobacter cloacae	TEM-2	9.37	0.00182	
A060	Serratia		8.90	0.00173	
A061	Escherichia coli		8.60	0.01730	
A062	Escherichia coli		8.60	0.01161	
A063	Providencia stuartii		6.65	0.00058	
A064	Escherichia coli		9.00	0.01488	
A066	Serratia		9.10	0.00276	
A067	Serratia		9.10	0.00183	
A068	Escherichia coli		9.00	0.00692	
A069	Escherichia coli		9.00	0.00400	
A070	Proteus vulgaris		9.10	0.00117	
A071	Escherichia coli	TEM-1	8.70	0.00213	
A072	Escherichia coli	TEM-1	8.80	0.00184	
A073	Klebsiella oxytoca		K1	0.07128	
A074	Escherichia coli	TEM-1	0.00	0.00398	
A075	Proteus morganii	TEM-1	7.70	0.00516	
A076	Klebsiella oxytoca	TEM-2	K1	0.20682	
A077	Escherichia coli	TEM-2	8.89	0.00145	
A078	Serratia		8.80	0.02597	
A079	Serratia		9.00	0.00127	
A080	Klebsiella oxytoca		K1	0.00644	
A081	Klebsiella pneumonia		7.60	0.00087	
A083	Proteus vulgaris		8.20	0.00025	X
A084	Escherichia coli	TEM-1	7.80	0.05858	
A085	Escherichia coli	TEM-1	7.80	0.05224	
A088	Enterobacter agglome		7.70	0.00118	
A089	Escherichia coli		7.80	0.00045	
A091	Enterobacter cloacae		7.54	0.00145	

No.	Organism	β-lactamase	Chromosomal pI	Sp Act/ mg protein
A092	Klebsiella ozaenae		9.20	0.00104
A094	Enterobacter cloacae		7.54	0.00081
A095	Escherichia coli		7.80	0.00228
A096	Klebsiella pneumonia		7.68	0.00219
A097	Klebsiella oxytoca		K1	0.01184
A099	Klebsiella oxytoca		K1	0.00401
A101	Enterobacter cloacae	Enzyme A	7.00	0.00422
A102	Citrobacter freundii		9.00	0.00182
A103	Klebsiella oxytoca	TEM-1	9.3	0.00161
A104	Enterobacter cloacae		P99	0.00182
A109	Escherichia coli		7.00	0.00046
A111	Klebsiella oxytoca		K14	1.12132
A113	Enterobacter cloacae	PSE-4	9.30	0.00733
A114	Escherichia coli	TEM-1	7.60	0.01462
A116	Proteus morganii		6.80	0.00024
A117	Escherichia coli	TEM-1	7.49	0.00265
A119	Citrobacter freundii	TEM-1	8.50	0.45102
A121	Proteus morganii		7.20	0.00064
A123	Escherichia coli	TEM-1	7.6	0.02506
A125	Serratia		9.30	0.02080
A128	Escherichia coli		8.50	0.00260
A131	Escherichia coli		8.50	0.05226
A134	Serratia	OXA-3	9.30	0.07561
A136	Escherichia coli	TEM-1	7.50	0.03261
A137	Klebsiella pneumonia		7.20	0.00030
A138	Escherichia coli		9.00	0.01861
A139	Escherichia coli	TEM-1	9.00	0.00838
A141	Proteus mirabilis	TEM-2	9.00	0.00032
A142	Escherichia coli		9.00	0.00008
A143	Proteus spp.		9.00	0.00012
A182	Escherichia coli		9.00	0.00010
A261	Proteus vulgaris		8.90	0.00020
A263	Proteus vulgaris		8.90	0.00010
A264	Proteus vulgaris		6.90	0.00010
A265	Proteus vulgaris		6.90	0.00010
A266	Proteus vulgaris		8.90	0.00010

+, Culture required induction before β-lactamase was visualised on IEF.

S&A, P.aeruginosa Sabbath and Abraham (1964) β-lactamase.

4. E.coli TEM-1 Strains and their Transconjugants.

No.	CLINICAL STRAINS				TRANSCONJUGANTS			
	Specific Enzyme Activity	CER MIC	AMP MIC		Specific Enzyme Activity	CER MIC	AMP MIC	
1	0.02144	8.0	250.0	SmTcHg	0.02100	16.0	2000.0	Tc
8	0.02147	64.0	250.0	SmTpSuCmHg	0.01170	16.0	2000.0	SuSmTcSp
13	0.00846	8.0	1000.0	SmSuHg	0.03460	<8.0	2000.0	SpSmTpSu
21	0.01625	32.0	2000.0	TcHg	0.03400	8.0	2000.0	Tc
28	0.03036	32.0	2000.0	SmSuCmHg	0.02030	<8.0	2000.0	SuSm
72	0.00374	32.0	1000.0	NaSmTpSuNfHg	0.01550	<8.0	2000.0	SuSm
85	0.00034	64.0	4.0	SmSuHg	0.01490	<8.0	2000.0	
104	0.00012	32.0	1000.0	Tc	0.04650	16.0	2000.0	
134	0.00397	8.0	500.0	SmSuHg	0.01659	<8.0	2000.0	SuSm
135	0.00032	2000.0	250.0	NaKmCmHg	0.00640	<8.0	2000.0	
144	0.00594	16.0	1000.0	Hg	0.00190	<8.0	2000.0	
150	0.01627	32.0	2000.0	SmSuKmHg	0.02810	16.0	2000.0	Km
151	0.00337	8.0	500.0	SmSuTc	0.01300	<8.0	2000.0	TcSmSu
193	0.02022	16.0	1000.0	SmSuHg	0.01910	<8.0	2000.0	SuSm
198	0.01204	16.0	1000.0		0.01290	<8.0	1000.0	
199	0.07002	32.0	2000.0	NaCmTcNfHg	0.01445	<8.0	2000.0	Tc
201	0.00226	8.0	4.0	Su	0.01200	<8.0	2000.0	
216	0.00132	4.0	4.0	Hg	0.01050	<8.0	2000.0	
219	0.02040	16.0	1000.0	Hg	0.07430	16.0	2000.0	Tc
220	0.08199	125.0	2000.0	NaGmSmTpSuCmTcNfHg	0.05430	16.0	2000.0	SuGmCmTpTc
235	0.00819	32.0	1000.0	SmSuTcHg	0.03980	<8.0	2000.0	SuSm
297	0.00573	16.0	2000.0	Hg	0.00064	16.0	2000.0	
308	0.00524	16.0	1000.0	CmHg	0.00061	32.0	2000.0	
326	0.02826	16.0	1000.0	TpSuTcHg	0.02590	<8.0	2000.0	Tc
330	0.00157	16.0	2000.0	Hg	0.00165	<8.0	2000.0	
334	0.00221	32.0	250.0	SmSuCmTcHg	0.00085	<8.0	2000.0	
357	0.00300	16.0	2000.0	SmSuTc	0.02376	<8.0	2000.0	
367	0.00247	16.0	500.0	SmSuHg	0.01230	<8.0	2000.0	SuSm
373	0.01126	8.0	500.0	SmSuHg	0.00620	<8.0	2000.0	
374	0.20840	64.0	2000.0	SmSuKmCmTcHg	0.18990	16.0	2000.0	KmTcSmSu
378	0.00212	8.0	500.0	SmSuHg	0.06076	<8.0	1000.0	SuSm
380	0.04724	16.0	2000.0	SmSuHg	0.00512	<8.0	2000.0	
398	0.01642	64.0	2000.0	CoTcHg	0.02980	16.0	2000.0	SuSmTc
402	0.02121	32.0	2000.0	SmTpSuTc	0.02270	<8.0	2000.0	Tc
407	0.01584	16.0	500.0	SmSuHg	0.01790	<8.0	2000.0	SuSm
410	0.02736	16.0	2000.0	SmTpSuKmCmHg	0.00458	<8.0	2000.0	SuSm
413	0.00432	500.0	250.0	CoTcHg	0.19650	8.0	2000.0	
419	0.01006	16.0	2000.0	Hg	0.00190	<8.0	1000.0	
427	0.02666	32.0	2000.0	SmTpSuCmHg	0.01140	<8.0	2000.0	
432	0.07360	32.0	2000.0	NaSmTpSuKmCmTcNfHg	0.02500	16.0	2000.0	TcKmSm
439	0.00790	64.0	2000.0	NaSmTpSuCmNfHg	0.00145	8.0	2000.0	
441	0.03101	32.0	2000.0	CmHg	0.00069	<8.0	1000.0	
451	0.00122	8.0	1000.0	SmSuHg	0.05890	<8.0	2000.0	
456	0.00197	16.0	1000.0	Hg	0.01071	16.0	2000.0	
458	0.01043	16.0	1000.0	SmSuHg	0.00940	<8.0	2000.0	
461	0.02018	16.0	1000.0	SmSuTcHg	0.00170	16.0	2000.0	KmSm
475	0.00705	64.0	2000.0	SmHg.	0.01141	<8.0	2000.0	
479	0.01422	16.0	2000.0	Hg	0.01510	4.0	2000.0	TcSuTpSm
480	0.03245	16.0	2000.0	Hg	0.00214	<8.0	2000.0	
494	0.00734	32.0	2000.0		0.01010	<8.0	2000.0	
546	0.03186	64.0	2000.0	SmTpSu	0.04070	<8.0	2000.0	
548	0.00694	16.0	2000.0	TcHg	0.00840	<8.0	2000.0	
555	0.00422	16.0	1000.0	SmSuCoCmTcHg	0.02350	<8.0	1000.0	
1071	0.00213	8.0	500.0	Hg	0.00320	<8.0	2000.0	
1074	0.00398	64.0	1000.0	SmTpSuCoCmTc	0.00460	<8.0	2000.0	
1084	0.05858	64.0	500.0	TcSmTpSuKmCm	0.01385	<8.0	2000.0	SuSmCmTcSp
1085	0.05224	125.0	2000.0	TcSmTpSu	0.00167	<8.0	2000.0	
1114	0.01462	64.0	1000.0	SmTpSuTc	0.01420	<8.0	2000.0	SuSm
1117	0.00265	8.0	500.0	SmSu	0.01650	<8.0	2000.0	SuSm
1123	0.02506	16.0	2000.0	SmSuTc	0.28580	16.0	2000.0	Tc
1136	0.03261	32.0	2000.0	SmCmHg	0.06240	16.0	2000.0	Sm
1139	0.00838	16.0	2000.0	SmSuCmHg	0.00880	32.0	2000.0	SuSm

ANTIMICROB. AGENTS CHEMOTHER., Aug. 1986, p. 245–247
0066-4804/86/080245-03$02.00/0

Plasmid Penicillin Resistance in *Vibrio cholerae*: Identification of New β-Lactamase SAR-1

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Received 3 January 1986/Accepted 5 May 1986

Two strains of *Vibrio cholerae* biotype El Tor, isolated in Tanzania, possessed a single IncC resistance plasmid of 113 kilobases. Both plasmids encoded the production of a novel β-lactamase, SAR-1, which was 33,700 daltons in size and was able to hydrolyze carbenicillin as well as penicillin G. The SAR-1 β-lactamase was quite distinct from all other plasmid β-lactamases by virtue of its unusually low isoelectric point and a combination of its size, substrate profile, and inhibition properties. This enzyme is only the second β-lactamase identified in *V. cholerae* species and the first to be reported in *V. cholerae* strains isolated in Southern Africa.

The most common resistance mechanism to β-lactam antibiotics is the production of a β-lactamase enzyme which hydrolyzes the drug and renders it inactive. Most of the plasmid-encoded resistance is manifested by one β-lactamase, TEM-1 (7, 18), and there are more than 20 other plasmid-determined β-lactamases which account for the remainder (12). Transferable resistance to β-lactam antibiotics has been slow to develop in *Vibrio cholerae*; and the first stable resistance plasmid (R plasmid) carrying ampicillin resistance, which could successfully be transferred to and maintained in *Escherichia coli*, was obtained from a strain of *V. cholerae* isolated in Algeria (16). Transferable ampicillin resistance emerged in Asia in the late 1970s (5, 20), and only the TEM-1 β-lactamase was identified (5). This β-lactamase also accounted for the plasmid-mediated ampicillin resistance found in a recent *V. cholerae* strain isolated from a patient who had recently returned to France from a stay in Algeria (4).

Ampicillin resistance has been described in *V. cholerae* strains isolated in Tanzania (13), and some was shown to be mediated by R plasmids (21). Here we report the first identification of new plasmid-determined β-lactamases from *V. cholerae* which were isolated from patients in Tanzania.

MATERIALS AND METHODS

During a recent outbreak of *V. cholerae* in Tanzania, four ampicillin-resistant strains were identified in the Department of Microbiology, Muhimbili Medical Centre, Dar es Salaam. Two of these strains (DT137 and DT139) possessed a single plasmid encoding the production of the TEM-1 β-lactamase. The other two strains (DT136 and DT138) each possessed a single IncC plasmid of 113 kilobases (pUK657 and pUK658, respectively) (22). Strains DT136 and DT138 were kindly typed at the Division of Enteric Pathogens at the Central Public Health Laboratory, Colindale, England, and shown to be biotype El Tor serotype O1 *inaba*. The rifampin-resistant *E. coli* K-12 strain J62-2 *his pro trp* (3) was used as the recipient in all conjugation experiments.

Antibacterial drug susceptibility and plasmid transfer. Susceptibility tests and MICs of antibacterial drugs were determined on solid medium as described previously (1). Plasmid transfer was performed by the method of Amyes and Gould (1).

β-Lactamase preparation and purification. β-Lactamase extracts for isoelectric focusing were prepared from bacteria grown on nutrient agar slopes (6). For further purification of the enzyme, 500-ml overnight broth cultures (brain heart infusion broth CM 225; Oxoid Ltd., Basingstoke, England) were concentrated and disrupted by ultrasonication. Ammonium sulfate was added to the cleared lysates to 35% saturation. After centrifugation and removal of the pellet, additional ammonium sulfate was added to 70% saturation. Following centrifugation, the pellet was suspended in a minimum volume of 50 mM sodium phosphate buffer (pH 7.0) and applied to a Sephadex G-75 gel filtration column (2 cm^2 by 90 cm) and eluted with the same buffer at 9 ml/h. Molecular size determinations were performed by gel filtration on a Sephadex G-75 column with internal standards (2).

β-Lactamase activity and isoelectric focusing. Enzyme activity was determined by spectrophotometric assays (14), and the effect of the β-lactamase inhibitors was examined by same assay (14). All inhibitor studies were performed with nitrocefin (100 μM) as the substrate. The inhibitor was added at the start of the assay at various concentrations ranging from 10 to 100 nM. The effect of the inhibitors was expressed as the concentration required for 50% inhibition (ID_{50}) of enzyme activity. The K_m and V_{max} values were obtained by measuring the rate of hydrolysis at limiting substrate concentrations and by plotting the reciprocal of the substrate concentration against the reciprocal of the rate by the Lineweaver-Burk method. The V_{max} values were normalized with respect to penicillin G, i.e., relative V_{max} = [(V_{max} per μl of enzyme) of substrate/ (V_{max} per μl of enzyme) of penicillin G] × 100. The efficiency of hydrolysis of the enzyme was obtained with the same parameter as Pollock physiological efficiency, as outlined by Sykes et al. (19), in which efficiency of hydrolysis = V_{max} per μl of enzyme/K_m. The relative efficiency of hydrolysis was also normalized with respect to penicillin G, i.e., relative efficiency = (efficiency of hydrolysis of substrate/efficiency of hydrolysis of penicillin G) × 100.

The isoelectric points of the β-lactamases were determined by the method of Matthew et al. (8). The pH gradient was formed by mixing equal amounts of pH 4 - 6 (catalog no. 1809-116) and pH 3.5 - 10 (catalog no. 1809-101) ampholines (LKB, Bromma, Sweden).

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TABLE 1. Resistance profile of *V. cholerae* strains and their transconjugants

Strain	MIC (mg/liter)			
	Ampicillin	Carbenicillin	Cephaloridine	Cephradine
V. cholerae DT136(pUK657)	>1,000	>1,000	16	16
E. coli J62-2(pUK657)	>1,000	>1,000	<8	<8
E. coli J62-2(pUK657a)	<8	<8	<8	<8
V. cholerae DT138(pUK658)	>1,000	>1,000	16	16
E. coli J62-2(pUK658)	>1,000	>1,000	<8	<8
E. coli J62-2	<8	<8	<8	<8

RESULTS

V. cholerae DT136 and DT138 and their respective *E. coli* transconjugants were all highly resistant to both ampicillin and carbenicillin (Table 1). Although the original *V. cholerae* showed a slight degree of resistance to cephradine and cephaloridine, this was not evident in the transconjugant strains (Table 1). Both *V. cholerae* strains were resistant to sulfamethoxazole, streptomycin, chloramphenicol, and trimethoprim, as well as ampicillin; and all these resistance markers transferred at the same high frequency during a 5-h conjugation experiment to *E. coli* (frequency, 10^{-3} transconjugants per donor strain). In each case, a single plasmid was found in the recipient, and when selection was made on ampicillin, all the other resistance markers were present in the transconjugant strain. However, ampicillin resistance was not always found in transconjugants selected on trimethoprim-containing plates (pUK657a, Table 1), which infers that this ampicillin resistance gene may be unstable.

Isoelectric focusing. The sonicated extracts from both *V. cholerae* DT136 and DT138 and their *E. coli* transconjugants revealed a single β-lactamase band with a pI corresponding to 4.9 (Fig. 1). This novel β-lactamase was designated SAR-1. The extracts from these *V. cholerae* strains showed no additional β-lactamase bands, and thus, *V. cholerae* seemed to possess no demonstrable chromosomal β-lactamase. Therefore, strain DT136 was used for further purification of the SAR-1 enzyme, as it lacked any contaminating β-lactamase.

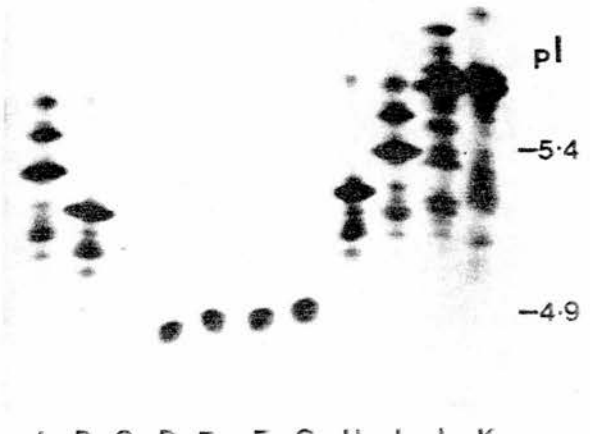

FIG. 1. Isoelectric focusing pattern of the SAR-1 β-lactamase. β-Lactamase standards: TEM-1 (pI = 5.4), lane A; PSE-4 (pI = 5.3), lane B; HMS-1 (pI = 5.2), lane C; PSE-4, lane H; TEM-1, lane I; TEM-2 (pI = 5.6), lane J; PSE-1 (pI = 5.7), lane K. SAR-1 β-lactamase samples: *V. cholerae* DT136(pUK657), lane D; *E. coli* J62-2(pUK657), lane E; *V. cholerae* DT138(pUK658), lane F; *E. coli* J62-2(pUK658), lane G

Purification and molecular weight determination. The SAR-1 β-lactamase was not completely precipitated by 50% saturation with ammonium sulfate. Indeed, considerably less than half of the enzyme was found in this fraction. However, a fraction between 35 and 70% saturation precipitated 64% of the available enzyme. Separation of this enzyme by gel filtration resulted in a purification of over 80-fold. When the enzyme was run through a calibrated column, the enzymatic peak corresponded to a molecular weight of 33,700.

Substrate profile and Michaelis-Menten kinetics. The SAR-1 β-lactamase is primarily a penicillin-hydrolyzing enzyme and had a reduced effect against the cephalosporins. This enzyme hydrolyzes ampicillin and carbenicillin at approximately the same rate as penicillin G (Table 2) and had a similar profile to that of the PSE-1 β-lactamase (10). The relative efficiencies of hydrolysis of ampicillin and carbenicillin by the SAR-1 enzyme were less than that of penicillin G, but they were not noticeably different from each other. However, this similarity resulted, in the case of carbenicillin, from a high K_m value and, in the case of ampicillin, from a lowered V_{max} (Table 2). In addition, the relative efficiency of hydrolysis of SAR-1 with cephaloridine was low, which resulted from its low relative V_{max}. The enzyme was sensitive to inhibition by cloxacillin (ID_{50} = 7 μM) and clavulanic acid (ID_{50} = 5 nM) but was resistant to inhibition by pCMB (ID_{50} > 100 μM). Therefore, although SAR-1 showed a similar substrate profile to those of PSE-1 and CARB-4, its inhibition profile was more like that of TEM-1 and, to a lesser extent, HMS-1 (10, 15).

DISCUSSION

The SAR-1 β-lactamase is a novel plasmid-mediated enzyme with a unique combination of biochemical properties. It has a low pI value in the same region as that reported recently for CARB-4 (pI = 4.3) (15). In our hands, both enzymes appeared to have similar, but discrete, pI bands. Before the identification of SAR-1 and CARB-4, the β-lactamase which had the lowest pI was HMS-1, with a published value of 5.2 (9). The relative rates of hydrolysis of penicillin G and carbenicillin confirmed that this enzyme is not HMS-1. Its relative efficiency of hydrolysis suggests that SAR-1 is primarily a penicillinase, and therefore, it differs from the broad-spectrum TEM enzymes and HMS-1. In substrate profile this enzyme resembles the so-called *Pseudomonas*-specific enzymes (PSE) (7, 11; A. J. Reid, I. N. Simpson, P. B. Harper, and S. G. B. Amyes, Abstr. 14th Int. Congr. Chemother., Kyoto, Japan, 1985). Indeed, one enzyme in this group (PSE-4) is the only plasmid-directed enzyme that has a molecular size similar to that of SAR-1.

TABLE 2. K_m values and relative efficiency of hydrolysis of SAR-1 prepared from *V. cholerae* DT136

Substrate[a]	K_m (μM)	Relative V_{max}[b]	Relative efficiency[b]
Penicillin G	42	100	100
Ampicillin	68	63	39.3
Carbenicillin	190	122	27.5
Cephaloridine	93	21	9.6
Cefamandole	95	53	23
Nitrocefin	83	89	91

[a] There was no detectable hydrolysis of methicillin, cloxacillin, or cefotaxime by the spectrophotometric method.

[b] Expressed as a percentage of the value for penicillin G

However, the inhibition profile of SAR-1 is quite unlike that of the PSE or CARB-4 β-lactamases (10, 15).

These two *V. cholerae* plasmids are unique not only because they are the first such plasmids to harbor the gene for the trimethoprim-resistant type I dihydrofolate reductase (22) but also because we have now shown that they carry a gene encoding a new β-lactamase. The only β-lactamase previously described in *V. cholerae* strains has been TEM-1 (4, 5), for no chromosomal enzyme has been demonstrated in this species. The identification of SAR-1 in the *V. cholerae* population is analogous to the discovery of ROB-1 in *Haemophilus influenzae*, in that TEM-1 had been the only plasmid-borne β-lactamase previously found in the species (17). It is not clear why a new penicillin-hydrolyzing enzyme should emerge in *V. cholerae* strains in Tanzania, especially when the TEM-1 enzyme has been found in other strains from the same outbreak and that treatment for infection by this organism has relied heavily on antibacterial drugs other than β-lactam antibiotics.

ACKNOWLEDGMENTS

We thank F. S. Mhalu, Department of Microbiology, Muhimbili Medical Centre, Dar es Salaam, and the Tanzanian National Scientific Research Council for providing research facilities.

We are grateful to the Carnegie Trust and Roche Products Ltd. for providing funds for S.G.B.A. to work in Tanzania. We also acknowledge the Science and Engineering Research Council CASE studentship, in collaboration with Glaxo Group Research Ltd., to A.J.R.

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OFFERED PAPERS: GENERAL (ORAL)

Wednesday, 18 September

Lecture Theatre 1

To be chaired by Dr K.J. TOWNER

0900 *An Unusual Resistance Plasmid Conferring Delayed Resistance to Trimethoprim in Complex Media*
H-K. YOUNG and S.G.B. AMYES (Department of Bacteriology, University of Edinburgh)

The unusual plasmid pUK1123, isolated in India, confers moderate resistance to trimethoprim (minimum inhibitory concentration = 160 mg/l) on solid minimal medium but no resistance on Isosensitest agar. However, growth experiments in Isosensitest broth show that pUK1123 enables the host organism to remain viable in 10 mg/l trimethoprim and eventually provides a mechanism which allows the inhibitory effect of trimethoprim to be overcome.

0915 *Resistance to Gentamicin and Related Aminoglycosides in Salmonella typhimurium Endemic in Cattle in Britain*
E.J. THRELFALL, J.L. FERGUSON, L.R. WARD and B. ROWE (Central Public Health Laboratory, London)

Plasmid profile visualization and plasmid characterization have been used to study gentamicin-resistant strains of *Salmonella typhimurium* phage type 204c isolated from outbreaks in cattle and humans in Britain. The study has demonstrated the existence of three lines of gentamicin-resistant strains, two of which have been found only in cattle and one in cattle and humans.

0930 *Expression of PSE-4 β-Lactamase in Different Hosts*
A.J. REID, I.N. SIMPSON*, P.B. HARPER* and S.G.B. AMYES (Department of Bacteriology, University of Edinburgh and *Glaxo Group Research, Greenford)

The genetic determinant for PSE-4 β-lactamase has now been found in Enterobacteria. The *dal*1 gene is carried on a self-transferable plasmid (pUK700) in *Klebsiella pneumoniae* 241. This plasmid carrier is lost on transfer to *Pseudomonas aeruginosa* PAO8. Expression of the *dal*1 gene product in *P. aeruginosa* is not only higher than in the original clinical isolate but displays modified biochemical properties.

0945 *An Automated in vitro Model to Investigate the Activity of Multiple Antibiotic Combinations*
D.H. STOKES, B. SLOCOMBE, R. SUTHERLAND and A.R. WHITE (Beecham Pharmaceuticals Research Division, Betchworth, Surrey)

The bactericidal effects produced by combinations of antibiotics with differing pharmacokinetic characteristics were measured in a computer-controlled kinetic model in which concentrations simulating those obtained in man were reproduced *in vitro*. Synergistic activity was obtained with simulated doses of a formulation of ticarcillin and clavulanic acid combined with gentamicin or tobramycin against ticarcillin/aminoglycoside-resistant bacteria.

1000 *Inactivation of Ticarcillin by Bacterial β-Lactamase in Mixed Culture and Protection by Clavulanic Acid in an in vitro Kinetic Model*
C.E. COOPER, R. SUTHERLAND and A.R. WHITE (Beecham Pharmaceuticals Research Division, Betchworth, Surrey)

The bactericidal activity of simulated doses of ticarcillin and ticarcillin/clavulanic acid was determined in a kinetic model against a mixed culture of *Pseudomonas aeruginosa* (ticarcillin-sensitive) and a β-lactamase-positive *Staphylococcus aureus*. Ticarcillin was inactivated by *S. aureus* β-lactamase and was ineffective against *P. aeruginosa*; in the presence of clavulanic acid the β-lactamase was inhibited and ticarcillin/clavulanic acid was effective against both organisms.

1015 *The Effect of Iron-Stress on Streptomycin Uptake in Escherichia coli*
G. MORRIS and S.M. HAMMOND (Department of Microbiology, University of Leeds)

Iron-stressed *E. coli* showed decreased uptake of dihydrostreptomycin compared with iron-replete cells. Induced phenotypic changes in the outer membrane of iron-stressed cells are not responsible for the differential uptake as verified by uptake studies using spheroplasts. The role of induced changes in the cytoplasmic membrane and to the transmembrane electrochemical gradient will be discussed with regard to streptomycin uptake.

1030 *Inhibition of Sterol Metabolism by N-Imidazole Antifungals*
R.O. NICHOLAS and D. KERRIDGE (Department of Biochemistry, University of Cambridge)

The *N*-imidazole antifungals partially inhibit the growth of *Candida albicans* over a wide concentration range. This has lead to problems in determining the conventional parameter, minimal inhibitory concentration. The most sensitive target site of these compounds is apparently cytochrome P450-dependent sterol demethylase, and there is a direct correlation between the inhibition of growth and the interference with sterol metabolism.

Reprinted from

RECENT ADVANCES IN CHEMOTHERAPY

Antimicrobial Section

Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985

Edited by Joji Ishigami

University of Tokyo Press, 1985

Discovery of PSE-4 β-Lactamase in Non-*Pseudomonas* Species

A.J. Reid*, I.N. Simpson**, P.B. Harper**, and S.G.B. Amyes*

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This communication reports the first discovery of the Pseudomonas specific β-lactamase PSE-4 in two clinical enterobacteria and the transfer of genetic determinants for this enzyme (Dal 1 and Dal 2) to other genera by conjugation. In Klebsiella pneumoniae 241, the Dal 1 gene is carried on a self transmissable plasmid (pUK700) which is lost upon transfer of Dal 1 into Pseudomonas aeruginosa. In Enterobacter cloacae 1113, no plasmid carrier for the Dal 2 gene was found. Dal 1 and Dal 2 express much higher levels of PSE-4 production in P.aeruginosa than in the Enterobacteriaceae. The isoelectric points, substrate profile and level of expression of the PSE-4 gene products from Dal 1 and Dal 2 appear to be host modified.

The pseudomonas specific enzymes (PSE) were originally believed to be mediated by genetic determinants confined to Pseudomonas aeruginosa (1). Recently the production of PSE-1 and PSE-2 has been reported in the Enterobacteriaceae (2,3). No similar migration has been observed with PSE-4 even though it is the most common plasmid-mediated β-lactamase produced by P.aeruginosa (4).

RESULTS

Two PSE-4 producing isolates, Klebsiella pneumoniae 241 and Enterobacter cloacae 1113 were discovered during a β-lactamase survey of 228 cephaloridine resistant strains isolated from hospital patients in Scotland, U.K. Initial isoelectric focusing (IEF) studies (pH3.5-10.0) indicated that both strains produced the PSE-4 'Dalgleish' β-lactamase. Closer examination (expanded pH4.0-6.0 range) revealed minor differences between the newly isolated 'PSE-4' enzymes and the original Dalgleish enzyme.

Genetic Location of the PSE-4 Determinants

The genetic determinant for PSE-4 β-lactamase in K.pneumoniae 241 (Dal 1) was freely transferable to Escherichia coli J62-2 and P.aeruginosa PA08. K.pneumoniae 241 contained two large plasmids - 132 kilobases (kb) and ca 290kb. As only the 132kb plasmid transferred to E.coli J62-2 it must carry the gene for PSE-4 production. This plasmid was not seen in the PA08 transconjugants, which may reflect difficulty found in extracting large intact plasmids from Pseudomonas. The PSE-4 determinant however, did not transfer from the PA08 host to either P.aeruginosa or E.coli suggesting that the gene had integrated into the bacterial chromosome. This hypothesis is supported by loss of some pUK700 genetic markers which accompanies its transfer into PA08.

The genetic determinant for PSE-4 production in Ent.cloacae 1113 (Dal 2) was not self-transferable, but following mobilisation with R751 was transferred to E.coli J62-2 and P.aeruginosa PA08. No plasmids could be distinguished in Ent.cloacae 1113.

Expression of PSE-4 β-lactamase

Following transfer to P.aeruginosa both Dal 1 (pUK 700) and Dal 2 mediated PSE-4 enzymes which were biochemically identical to the Dalgleish PSE-4 enzyme. Levels of β-lactamase activity were increased over 100 fold in the recipient (Table 1) which was accompanied by a greater than 20 fold rise in minimum inhibitory concentration (MIC) of carbenicillin. Following transfer to E.coli J62-2, Dal 1 and Dal 2 continued to mediate low levels of enzymes biochemically similar to those produced by the clinical strains; in both cases the carbenicillin MIC was increased by greater than 1000 fold. In these enterobacterial hosts, PSE-4 has different isoelectric points (pI) and substrate profiles from those found in P.aeruginosa.

Table 1

Enzyme source	Activity*	Relative Rates of Hydrolysis				CARB MIC*	pI
		CARB	AMP	PENG	CER		
P.aeruginosa Dalgeish	485.0	100	98	102	4	>1000	5.20
K.pneumoniae 241 Dal 1	5.3	100	766†	936	304	>1000	5.25
E.coli J62-2 Dal 1	5.0	100	128	680	404	>1000	5.25
P.aeruginosa Dal 1	652.0	100	90	107	7	>1000	5.20
Ent.cloacae 1113 Dal 2	6.1	100	161	433	120	32	5.30
E.coli J62-2(R751) Dal 2	6.0	100	102	667	350	>1000	5.30
P.aeruginosa PAO8(R751)Dal 2	697.0	100	75	99	11	>1000	5.20
E.coli J62-2	<1.0	-	-	-	-	1	-
P.aeruginosa PAO8	<1.0	-	-	-	-	64	-

CARB-carbenicillin ; AMP-ampicillin ; PENG-penicillin G; CER-cephaloridine.
* nMoles of carbenicillin hydrolysed/minute/mg protein. * mg/L
† Relative rate inflated by presence of chromosomal enzyme.

CONCLUSIONS

This is the first identification of the PSE-4 β-lactamase in clinical enterobacteria. In K.pneumoniae 241, the Dal 1 gene is carried on a self transferable plasmid (pUK700). In Ent.cloacae 1113, no plasmid carrier for the Dal 2 gene could be found. The PSE-4 determinants both transfer to other genera and transfer of Dal 1 into P.aeruginosa is followed by loss of the pUK700 plasmid. Dal 1 and Dal 2 express much higher levels of PSE-4 production in P.aeruginosa than in the Enterobacteriaceae. Differences observed in pI and specific activity of PSE-4 in the different strains suggests that the biochemical and biophysical properties of the PSE-4 enzyme are host dependent.

The PSE-4 β-lactamase is no longer confined to P.aeruginosa and is emerging as a resistance mechanism in the Enterobacteriaceae.

AJR is supported by a SERC CASE studentship.

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