NOVEL ASPECTS OF TRANSFERABLE RESISTANCE TO BETA-LACTAM ANTIBIOTICS

by

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Thesis for the degree of Doctor of Philosophy

University of Edinburgh

1990

"The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact".

Thomas Henry Huxley 1825-1895

in Biogenesis and Abiogenesis.

Abstract

Until 1983, third generation cephalosporins (3GCs) were thought to be resistant to hydrolysis by all plasmid-mediated beta-lactamases. However, TEM and SHV derived beta-lactamases have recently evolved which can confer transferable resistance to 3GCs.

Six novel plasmid beta-lactamases, which confer transferable resistance to 3GCs have been identified and characterised. They have been compared directly to the other 3GC hydrolysing beta-lactamases discovered elsewhere. The criteria implemented to distinguish these different beta-lactamases were: Km and Vmax values for the hydrolysis of different substrates, molecular weight, isoelectric focusing point (pI) and susceptibility to inhibitors. The beta-lactamases TEM-E1, TEM-E2, TEM-E3, and TEM-E4, were all TEM derived and, although they conferred a greater transferable resistance to ceftazidime than cefotaxime, they hydrolysed both these substrates with similar efficiencies. TEM-E2 was produced by an organism which was isolated in Liverpool in 1982 and is, consequently, the first example of transferable 3GC resistance. TEM-E3 was produced by clinical isolates from two different London hospitals, and found to be the same as TEM-10 which was discovered in the USA after the characterisation of TEM-E3.

TEM-E1 and TEM-E2 like enzymes could be obtained spontaneously from a TEM-1 producing E.coli and, in the same way, TEM-E4 like enzyme could be obtained from a TEM-2 producing organism. Utilising the same methodology a beta-lactamase, which conferred transferable resistance to ceftazidime, was obtained from PSE-4, although such an enzyme has not yet been reported in clinical isolates.

The fifth novel beta-lactamase, DJP-1 (pI 7.9), was produced by an organism isolated in India which also produced TEM-1 and CAZ-6. This was the first example of transferable 3GC resistance in Asia and the first report of two transferable 3GC hydrolysing beta-lactamases being encoded by the same plasmid. The characteristics of the sixth novel enzyme, BIL-1, suggest that it was originally an E.coli chromosomal beta-lactamase. Analysis of this strain revealed that the beta-lactamase (pI greater than 8.2) conferred transferable resistance to all beta-lactam antibiotics and clavulanic acid.

Finally, two novel methods for the isolation and purification of beta-lactamases were developed during these studies. Electro-dialysis of beta-lactamases from polyacrylamide gel allowed rapid purification of TEM-E2 from TEM-1. Fast Protein Liquid Chromatography System was employed to separate beta-lactamase DJP-1 from the other beta-lactamases produced by the host strain. In addition it was illustrated that different beta-lactam molecules can induce a variety of different beta-lactamase satellite bands which can be visualized by isoelectric focusing. Moreover, it was also verified that these satellite bands were variants of the main beta-lactamase protein.

Contents

Declaration.

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

Acknowledgements.

I would like to thank Sebastian Amyes for his constant encouragement and valuable advice throughout the duration of my PhD. I am also indebted to Michael Marriott for providing such a cooperative collaboration with Glaxo Group Research Ltd. I must also thank Chris Christodoulou for his expert supervision during the PCR and probing experiments, and I am grateful to Professor Collee for his continued support.

I thank the Photographic Department at Glaxo Group Research Ltd for the production of the figures in this Thesis, and I would like to express thanks to both the SERC and Glaxo Group Research Ltd for the financial assistance which has made this research possible.

Lastly, I must thank all the personnel of the Bacteriology Research Laboratory for providing such a enjoyable and stimulating atmosphere for the duration of this chapter in my life.

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Abbreviations

AMP	ampicillin
bp	base pairs
CARB	carbenicillin
CAZ	ceftazidime
CED	cephradine
CER	cephaloridine
cfu	colony forming units
clav	clavulanic acid
CTX	cefotaxime
CXM	cefuroxime
DM	Davis and Mingioli
FPLC System	Fast Protein Liquid Chromatography System*
his	histidine
IEF	isoelectric focusing
inc	incompatibility group
Kb	Kilobases
Kd	kilodaltons
Km	kanamycin
L	litres
M	Molar
Mdal	megadaltons
met	methionine
MF	mating frequency
MIC	minimum inhibitory concentration
mM	milliMolar
nmole	nanomole
ND	not done
NMH	North Middlesex Hospital, London.
NS	not specified
Omp	outer membrane protein
PBP	penicillin binding protein
PCR	polymerase chain reaction
pmol	picomole
pro	proline
Rf	rifampicin
RMH	Royal Marsden Hospital, London.
SDM	stably derepressed mutants
SDS	sodium dodecyl sulphate
sulb	sulbactam
trp	tryptophan
ul	microlitres
um	micometers
uM	micromolar
UM	unmeasurable
3GC	"third generation cephalosporin"

* Registered trade mark of Pharmacia LKB Biotechnology.

1.0. INTRODUCTION

A world without antibiotics is now unimaginable. A host of once untreatable infections are no longer regarded with concern. Mortality from bacterial endocarditis was 100% before the advent of antibiotics; effective treatment with antibiotics has now reduced this to between 15% and 30%. Antibiotics have been the major medical discovery of all time and have accelerated the advancement of medicine, notably organ transplantations, hip replacements, major abdominal and cardio-thoracic surgery, and the treatment of cancer (Dryden, 1987).

Beta-lactam antibiotics have played a key role in reducing the impact of life threatening diseases. However, over the last 30 years the bacteria causing these infections have become increasingly resistant to beta-lactam antibiotics. The main mechanism causing this resistance has resulted from the production of beta-lactamases capable of hydrolysing these drugs and rendering them inactive. The pharmaceutical industry has constantly responded to the emergence of these enzymes by developing new antibiotics of greater sophistication and better beta-lactamase stability. However, bacteria have a remarkable ability eventually to counteract any type of beta-lactam antibiotic, and this Thesis will demonstrate how this group of

microorganisms has now overcome a group of beta-lactam antibiotics which were believed to be, in terms of sophistication and design, the "ultimate" group of antibiotics - the third generation cephalosporins.

1.1. The discovery and development of penicillins.

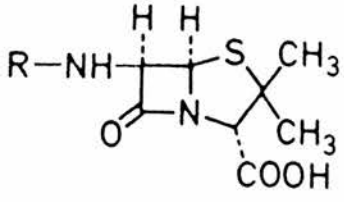
The growth of this group of antibiotics began with Alexander Fleming's astute observation, that a contaminating mould was inhibiting the growth of staphylococcal colonies which had previously been inoculated onto the agar plate (Fleming, 1929). This mould was identified as Penicillium notatum. Fleming realised that a small molecular weight compound was diffusing through the agar and was responsible for the antibacterial properties. He predicted that this compound, which he called penicillin, was of clinical significance so he tried to obtain penicillin extracts by fermenting Penicillium notatum, and crudely removing the whole cells. At that time further investigation was generally impeded by the current enthusiasm for the newly introduced sulphonamides. However, Fleming continued to study the effects of the crude mixture and, in 1932, he administered the crude antibiotic preparation to cure a pneumococcus eye infection which was preventing one of his medical students from participating in his rifle team. This was possibly the first successful clinical use of a beta-lactam antibiotic. Further work on penicillin was conducted by Heatley, Florey and Chain at Oxford which to led to their first publication on the properties of penicillin in 1940 (Chain, et al.,

1940), followed by a fuller account with impressive clinical evidence by Abraham et al. (1941). However, it was the clinical trials conducted on injured soldiers in the North African campaign of the war (Pulvertaft, 1943) which acted as a further stimulus to enhance penicillin production at Oxford and by several British pharmaceutical companies. In the USA pharmaceutical companies had started collaborative work on the production of penicillin. This vastly increased penicillin output and marked the foundation of the modern pharmaceutical industry.

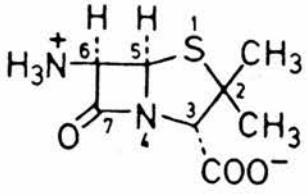
1.1.1. Semi-synthetic penicillins.

In 1959 Beechams Research Laboratories in England developed the technology for obtaining large amounts of 6-amino-penicillanic acid (6APA), the core structure of penicillin. This opened up almost infinite prospects of new antibiotic derivatives by simple acylation substitution of the 6-Carbon of the beta-lactam ring of 6APA (Batchelor., et al. 1959) (fig. 1). Acylation of the 6APA, the penicillin nucleus, with different carboxylic acid, chlorides or mixed anhydrides has permitted the semi-synthetic modification of the penicillin side chain. Consequently, thousands of semi-synthetic penicillins have since been prepared, mainly with a view to increase the spectrum of these drugs and widen the groups of bacteria able to be treated with penicillins, but only a few have proved to be of real therapeutic value.

Fig. 1. Structure of penicillins and 6-aminopenicillanic acid.

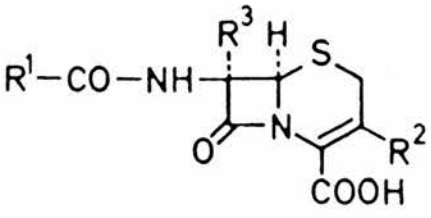


Penicillins

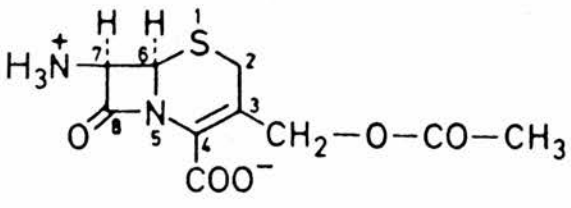


6-Aminopenicillanic acid (6-APA)
[$C_8H_{12}N_2O_3S$ (216.26)]

Fig. 2. Structure of cephalosporins and aminocephalosporanic acid.



Cephalosporins



Aminocephalosporanic acid (7-ACA)
[$C_{10}H_{12}N_2O_5S$ (272.28)]

1.1.2. Beta-lactamases and the success of penicillins.

At the start of the 1940s Abraham and Chain discovered that Escherichia coli, produced penicillinase which could inactivate penicillin by hydrolysis (Abraham and Chain, 1940). This discovery identified a resistance mechanism which has been an important consideration throughout the subsequent development of beta-lactam antibiotics.

From 1945 to 1960 the increased use of penicillin led to the emergence of penicillin resistant staphylococci. Over the subsequent years these levels of resistance proliferated. Neu (1985) showed that more than 95% of hospital isolates and 85% of community isolates of S.aureus produce beta-lactamases conferring resistance to a wide range of beta-lactam antibiotics.

1.1.3. Beta-lactamase stable penicillins.

In order to combat the increasing problem of penicillin resistance Beechams released the semi-synthetic penicillin, methicillin, which was highly resistant to staphylococcal penicillinases (Rolinson et al., 1960). This was followed by a number of other penicillin derivatives which were resistant to staphylococcal penicillinases i.e. oxacillin, cloxacillin, flucloxacillin. However, like methicillin the antibacterial activity of these drugs was limited to Gram-positive bacteria.

1.1.4. The future of penicillins.

One of the main factors which hindered the development of the penicillin group of antibiotics was that the side chain determining the identity of the penicillin is always at the same point on the molecule (fig. 1). Consequently, there is far less scope for chemical modification. This appears to be one of the reasons why it has not been possible to design a penicillin which has both penicillinase stability and a broad spectrum of activity. Therefore, the penicillin group of antibiotics has not yet reached the sophisticated levels obtained by the modern cephalosporin antibiotics (see next section). This has since been overcome by the use of beta-lactamase inhibitors in combination with a penicillin. An example of this is Augmentin which is a combination of amoxycillin and clavulanic acid (Brogden et al., 1981).

1.2. The discovery and development of cephalosporins.

The injectable antibiotic market in the USA grossed $1.3 billion in sales in 1985, and 56% of these sales were for cephalosporins (Quenzer, 1987). The development and growth of this group of antibiotics stems from Professor Giuseppe Brotzu's observation in 1945, whilst Rector in the Sicilian University of Cagliari, Sardinia, that sea water from a sewage outlet appeared to be self-purifying. He hypothesised that this may be the result of bacterial antagonism and, on further investigation, he isolated

a fungus which was classified as Cephalosporium acremonium. Fluid cultures of this organism exhibited anti-bacterial activity and Brotzu had limited success when treating typhoid and brucellosis with crude extracts of the fungus. Unfortunately, no established scientific journal would accept his findings, so in 1948 he formed a journal called the Lavori dell'istituto d'Igiene di Cagliari which published his work in 1948. Brotzu's work may easily have been lost in the reams of obscure scientific literature, had it not been for Dr C.O.S. Blyth Brook, a former public health officer in Sardinia during the war, who wrote to H.W. Florey telling him of Brotzu's discovery.

1.2.1. The discovery of cephalosporin C.

In August 1948 a culture of the fungus was sent to Oxford, further research revealed that the fungus produced not one, but seven different antibiotics. Cephalosporins P1, P2, P3, P4, and P5 were isolated by Burton and Abraham (1951) and were later found to have a steroid structure similar to fusidic acid (Godtfredsen et al., 1965). Cephalosporin N was found to yield penicillamine on acid hydrolysis (Newton and Abraham, 1954) and was renamed adicillin. Newton and Abraham (1955) identified another antibiotic from the culture fluids of Cephalosporium acremonium and this was designated cephalosporin C.

1.2.2. The importance of cephalosporin C.

The discovery of cephalosporin C received great attention, mainly because

it was equally effective against strains of penicillin resistant and penicillin sensitive staphylococci, but also because it had a wide range of anti-bacterial activity. Further work illustrated that cephalosporin C was highly resistant to hydrolysis by the staphylococcal penicillinase and it competitively inhibited the action of penicillinase on benzylpenicillin (Abraham and Newton, 1956). This prototype cephalosporin was also non toxic to mice and cured a streptococcal infection when given subcutaneously (Florey, 1955). At this particular time the occurrence of penicillinase producing strains of bacteria had begun to diminish the effectiveness of penicillin and the vast potential of the "penicillinase resistant" cephalosporin antibiotics was realized. Consequently, research into cephalosporins proliferated in an eager attempt to obtain better yields of the antibiotic and once this was achieved, increased anti-bacterial performance by chemical modification.

1.3. Chemical modification of cephalosporins.

The cephalosporin nucleus contains several positions where chemical modification can be made (fig. 2) and as a result of this many thousands of cephalosporin antibiotics have been produced by various pharmaceutical companies worldwide (Williams & Williams, 1980).

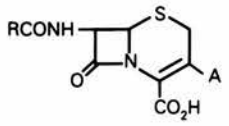
1.3.1. The first cephalosporins.

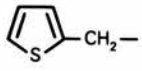
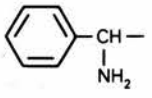
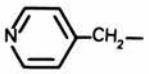
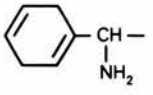
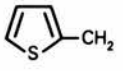
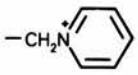
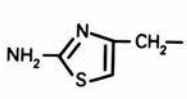
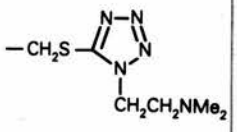
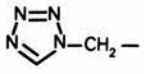
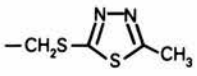
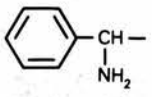
Cephalosporins are derivatives of 7-aminocephalosporic acid (7-ACA) (fig. 2), which is prepared by chemical cleavage of the side chain of cephalosporin C. The first cephalosporin, cephalothin, became available in the United States in 1964, this was a semi-synthetic cephalosporin and developed by Chauvette et al. (1962). At approximately the same time cephaloridine was introduced into the United Kingdom by Glaxo (Muggleton et al., 1964) (fig. 3).

1.3.2. Primary design initiatives of cephalosporins.

Most of the early modifications, such as cephacetrile, cephalothin, or cephapirin, were directed at the 7-acyl group as it was soon realized that this was the substituent which had a strong influence on anti-bacterial activity. All three of these pioneering cephalosporins had acetoxymethyl groups as their 3-substituents. However, such groups were prone to esterases found in the body and cephalothin, cephapirin and cephacetrile (Knusel et al., 1970) were converted into their corresponding deacetyl compound which had an inferior anti-bacterial action. This problem was overcome by replacing the ester group at position 3. This led to the development of cephaloridine (Muggleton, 1964) by Glaxo and cefazolin (Symposium, 1973) which was introduced by Eli Lilley (fig. 3).

Figure 3: The structure of cephalosporins



Parenteral			Oral		
R	Generic name	A	R	Generic name	A
Metabolically unstable			*Metabolically stable*		
	Cephalothin	$-CH_2OCOCH_3$		Cephalexin	$-CH_3$
	Cephapirin	$-CH_2OCOCH_3$		Cephradine	$-CH_3$
$N \equiv C-CH_2-$	Cephacetrile	$-CH_2OCOCH_3$			
Metabolically stable					
	Cephaloridine			Cefotiam	
	Cefazolin				
				Cefaclor	$-Cl$

1.3.3. Design of oral cephalosporins.

Further investigations showed that oral absorption of cephalosporins could be achieved with an alpha amino group on the 7-beta-acyl substituent and with a small uncharged group in position 3 (O'Callaghan, 1975). This led to the introduction of cephalexin (Wick, 1967) by Glaxo which was the first successful orally absorbed cephalosporin. Cephradine was later introduced in 1973 (Gadebusch et al., 1972) followed by cefaclor in 1978 (Neu and Fu, 1978) (fig. 3).

1.3.4. Design of beta-lactamase resistant cephalosporins.

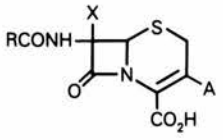
As the future success of some of the earlier cephalosporins appeared to be threatened by their susceptibility to beta-lactamases, the further development of the cephalosporin group of antibiotics is an on-going story of designing beta-lactamase stable molecules with an ever increasing spectrum of anti-bacterial efficacy. Like the penicillins, significant improvement in resistance to beta-lactamase hydrolysis has relied on manipulations which involve steric effects in the vicinity of the 7-beta-acyl position. A lot of the earlier cephalosporins possessed a $-CH_2-$ next to the amide link, and this was accompanied by sensitivity to the beta-lactamases of Gram-negative organisms, (i.e. cephaloridine or cephalothin), but this decreased as more bulky groups were introduced at the 7-beta-acyl position, i.e. cefotiam. The incorporation of a side chain alpha carbon atom into a ring in order to increase steric bulk lowers the general activity of the cephalosporin. Similarly, multiple substitutions

of the alpha carbon atom with alkyl and aryl groups to increase steric bulk have a detrimental effect on activity. This, in general, can be overcome by the introduction of certain polar substituents, which increase resistance to beta-lactamases and increase the spectrum of anti-bacterial activity. Cephalexin, which has an alpha amino substituent, has moderate resistance to beta-lactamases, although it does not have a significant spectrum of anti-bacterial activity. Cefamandole and cefonicid demonstrate that an alpha hydroxyl group is more effective in this position and these two drugs have an increased resistance to beta-lactamases along with a broader spectrum of activity (reviewed by Hoover, 1983) (fig. 4).

1.3.5. Cefuroxime.

The next increase in beta-lactamase stability was achieved with cefuroxime which was introduced by Glaxo (O'Callaghan et al., 1976). This differed in structure from cefamandole but had much the same intrinsic level of anti-bacterial activity against a wide spectrum of organisms (fig. 4). However, cefuroxime has a broader spectrum of resistance to beta-lactamases than cefamandole (Richmond and Wotton 1976) which was achieved by an 2-oxyimino group on the 7-beta-acyl substituent. The furyl ring was shown to have an activity advantage over phenyl and thienyl groups (reviewed by Hoover, 1983). Glaxo have since launched an oral cefuroxime preparation, cefuroxime axetil, which is an esterified derivative of cefuroxime (Williams and Harding, 1984).

Figure 4: The structure of cephalosporins



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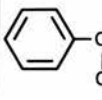
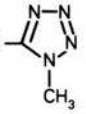
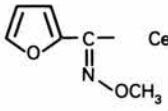
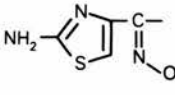
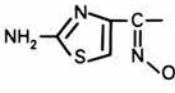
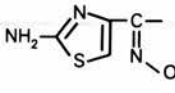
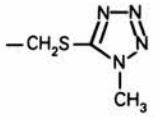
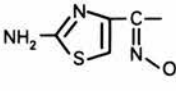
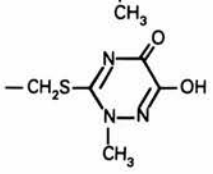
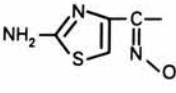
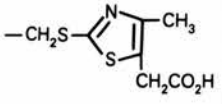
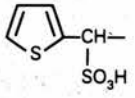
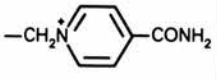
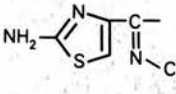
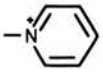
	Cefamandole	
	Cefuroxime	$-CH_2OCONH_2$
Cephamycins ($7\alpha-OCH_3$)		
	Cefoxitin	$-CH_2OCONH_2$

R	Generic name	A
	Cefotaxime	$-CH_2OCOCH_3$
	Ceftizoxime	$-H$
	Cefmenoxime	
	Ceftriaxone	
	Cefodizime	

activity includes Ps. aeruginosa

	Cefsulodin	
	Ceftazidime	

1.3.6. Cephamycins.

Another group of beta-lactamase stable compounds have emerged with similarities to cephalosporins. These beta-lactams are called the cephamycins of which cefoxitin was the first to be used clinically (Birnbaum et al., 1978). These compounds possess a 7-alpha methoxy group which provides stability to the beta-lactam ring from beta-lactamase hydrolysis. Their resistance to beta-lactamases has been demonstrated on many occasions (Fu and Neu, 1978 and 1979). Although cephamycins exhibit high resistance to beta-lactamases their potency has since been found to be limited by the presence of a substituent at the 7-alpha position. (Stapley et al., 1979) (fig. 4).

1.3.7. Design of broad spectrum and beta-lactamase stable cephalosporins.

The next series of cephalosporins to be developed had similar resistances to beta-lactamases as cefoxitin, but these new drugs had a much higher antibacterial activity over an even broader range of bacterial species. The majority of these cephalosporins have a 7-beta-(2-aminothiazol-4-yl) acetylamino side chain, and the alpha-carbon atom is incorporated into a oxyimino grouping. The potential for achieving unusually high activity against Gram-negative bacteria through the use of this 7-beta-heterocyclic substituent was illustrated by cefotiam. The acquisition of beta-lactamase resistance by the incorporation of the alpha carbon atom into a oxyimino grouping was predicted by the structure-activity relationships of

cefuroxime. Cefotaxime, ceftizoxime, cefmenoxime, ceftriaxone, cefodizime, and cefpirome all have the same 7-beta and -alpha carbon substituents. However, their 3-substituents vary enormously from a single hydrogen atom (ceftizoxime) to a methyl-tetrazolethio group (cefmenoxime). These six compounds all exhibit similar in vitro activity and resistance to beta-lactamases, therefore, such properties must be achieved independently of the 3-substituent (reviewed by Hoover, 1983) (fig. 4).

1.3.8. Design of ceftazidime.

Anti-bacterial activity declines with the increase in the bulk of the alkyl group on the alpha carbon of the 7-beta-substituent, however, there is a fairly broad structural latitude. Consequently, ceftazidime, which was introduced by Glaxo in 1981 (Muggleton, 1981), had an alkyl group carrying a carboxyl function at this particular position, which is somewhat similar to carbenicillin. Introduction of a carboxyl group on the 7-beta-side chain lowers Gram-positive activity but, in this case, significantly increased activity against some Gram-negative bacteria including Pseudomonas spp.. Thus, ceftazidime has better activity against clinical isolates of Pseudomonas aeruginosa than azlocillin (Wise et al., 1980). The discovery of ceftazidime was a major breakthrough because it was stable against the majority of the then known beta-lactamases (Simpson et al., 1982). In addition, ceftazidime had a broad spectrum of anti-bacterial activity which included activity against P.aeruginosa (Klingeren, 1981). This organism was resistant to the earlier cephalosporins because of intrinsic rather than enzymic resistance

mechanisms. Only 3% of clinical isolates of Pseudomonas spp. produce beta-lactamases (Williams et al., 1984). Therefore, beta-lactam resistance in most strains was caused by the inability of the drug to penetrate the outer membrane (Yoshimura and Nikaido, 1982). There were some other beta-lactams to rival the anti-pseudomonal activity of ceftazidime. One such antibiotic is cefsulodin, but this drug is extraordinary because it has poor activity against bacteria other than P.aeruginosa (Tsuchiya et al., 1978) (fig. 4).

1.4. The mode of action of cephalosporin antibiotics.

The mode of action of benzylpenicillin has been studied in much detail and because of similarities in structure, it is believed to typify the bactericidal mechanisms exhibited by all the penicillins and cephalosporins. Tipper and Strominger, (1965) observed that S.aureus treated with penicillin contained large amounts of non-crossed linked peptidoglycan units. This was confirmed by Waxman et al. (1980) when they identified linear peptidoglycan in penicillin treated cells. This led to the conclusion that penicillins and cephalosporins inhibited the transpeptidase enzyme, which catalyses the final cross linking of the peptide side chain of the nascent peptidoglycan (Tipper and Strominger, 1965). This cross linked peptidoglycan is essential to the bacterial cell as it provides rigidity and protection. More recent studies (reviewed by Spratt 1980, 1983) showed that, in addition to transpeptidase, both

D-alanine carboxypeptidase and peptidoglycan endopeptidase have been found sensitive to penicillin. It is thus apparent that bacteria possess a number of enzymes that are sensitive to beta-lactam inhibition.

Cooper (1956) observed that radio labelled penicillin binds to the cell membrane and that this attachment was associated with the killing action of beta-lactams. This led to the characterisation of penicillin binding proteins (PBPs). Different species of bacteria possess different types of PBPs (reviewed by Livermore, 1987), but the Escherichia coli PBPs have been studied in the most detail and, so far, seven have been characterised.

1.4.1 Characterisation of E.coli PBPs.

PBPs 4, 5 and 6 are thought to be unconnected with the antibacterial effect of cephalosporins and penicillins. However, PBPs 1-3 produced a variety of morphological effects when bound to beta-lactams. Blockage of PBP 1a and 1b leads to cell lysis. However, bacterial mutants deficient in either one of these PBPs grow normally, presumably the other protein can compensate for its loss. Obstruction of PBP-2 leads to the formation of large osmotically stable round cells which lyse slowly after several hours. Inhibition of PBP-3 induces filamentation of E.coli by specifically inhibiting cell division without affecting cell lysis (reviewed by Spratt, 1983).

1.5 Antibacterial activity of cephalosporins.

Table 1 shows the antibacterial activity of cephalosporin antibiotics. This is only a rough guide as many species show wide strain variations. In some cases, where activity is stated as good there may be a few strains which are resistant. The table avoids minimum inhibitory concentration (MIC) values as these vary slightly from laboratory to laboratory, and can be affected dramatically by the size of the inoculum (Brook et al.,1989). This makes comparisons difficult.

The early cephalosporins, such as cephaloridine, had moderate activity against staphylococcal beta-lactamases, but were unstable against Gram-negative beta-lactamases. Cefazolin had improved antimicrobial activity over cephaloridine against Gram-negative organisms, but cephaloridine had higher activity against Gram-positive bacteria (Wick and Preston, 1972; Acter et al.,1977). The next group of cephalosporins represented by drugs like cephalexin were stable to Gram-positive beta-lactamases and possessed moderate resistance to Gram-negative beta-lactamases. The cephalexin group of drugs had a spectrum of antibacterial activity similar to the cephaloridine-like compounds, but with a somewhat lower degree of activity against most species. However, as these cephalosporins can be oral preparations, cephalexin-like drugs have attained wide popularity (Garrard et al., 1981).

Table 1. Summary of the antibacterial activity of cephalosporins (taken from Greenwood, 1983).

Cephalosporin.	Compounds with similar antibacterial properties.	Staphylococci		Strepto-cocci *	Neiss-eria	Haemo-philus	Enterobacteria		Pseudo-monas	Ana-erobes
		activity	stability				activity	stability		
cephaloridine	cephacetrile cephapirin cephacetrile cefazolin cephalothin	good	fair	fair	fair	poor	variable	variable	no useful activity	variable
cephalexin	cephradine cefaclor	good	good	fair	fair	poor	variable	variable	no useful activity	poor
cefuroxime	cefoxitin cefamandole	good	good	good	good	good	good	good	no useful activity	fair
cefoperazone		good	good	fair	good	good	variable	variable	good	fair
cefsulodin		poor	good	poor	-------no useful activity--------				excellent	poor
cefotaxime	ceftizoxime ceftazidime ceftriaxone	good	good	----------excellent----------					fair	poor

"stability" = stability to beta-lactamases. * = Streptococcus faecalis strains are resistant to all cephalosporins.

These earlier cephalosporins filled the therapeutic gap which resulted from the emergence of staphylococcal beta-lactamases which were capable of inactivating penicillins.

The later cephalosporins such as those grouped with cefuroxime and cefotaxime had a better activity against Streptococci spp., Neisseria spp., and Haemophilus spp. The cefotaxime group of antibiotics were hailed as the ultimate beta-lactams of their time. They had excellent activity against a broad spectrum of bacterial species which included Pseudomonas spp. (Slack, 1981). Cefotaxime and ceftazidime were also resistant to nearly all the known plasmid mediated beta-lactamases of that time (Richmond, 1980; Simpson et al., 1982, respectively). However, such complacency was unjustified, as over the last seven years, there has been an increasing number of reports of plasmid mediated beta-lactamases which have the ability to confer transferable resistance to cephalosporins from both the cefotaxime and the cefuroxime groups of antibiotics.

1.6 Classification of cephalosporins.

Table 2 shows the classification of cephalosporins into three different generations and, although this scheme produces broad generalisations, it is the most widely used method of categorizing the cephalosporin group of anti-bacterial agents. However, the grouping of the drugs into generations suggests that each new generation represents a general advance in all aspects of the cephalosporin characteristics, rather than fairly specific

Table 2. Classification of cephalosporins (taken from Uri and Actor, 1983).

Parenteral beta-lactamase-susceptible - first generation cephalosporins.

Cephalothin
Cephapirin
Cephacetrile
Cephaloridine
Cefazolin
Cephradine

Parenteral beta-lactamase-resistant - second generation cephalosporins.

Cefamandole
Cefoxitin (a)
Cefmetazole (a)
Cefuroxime
Ceforanide
Cefonicid

Parenteral beta-lactamase resistant anti-pseudomonal - third generation cephalosporins.

Cefotaxime
Ceftizoxime
Moxalactam (b)
Cefoperazone
Ceftriaxone
Cefmenoxime
Ceftazidime
Cefotiam
Cefsulodin
Cefodizime

Oral beta-lactamase-susceptible - first generation cephalosporins

Cephalexin
Cephradine
Cefatrizine
Cefaclor
Cefroxadine
Cefadroxil
Cephaloglycin

(a) = cephamycin. (b) = oxacephamycin

† Second generation cephalosporins introduced after 1983 are discussed in text.

modifications to one or more properties (Williams, 1987). Indeed, changes in the chemical structures of cephalosporins which increase activity against one group of organisms normally have an adverse effect on the activity against another species.

To illustrate this the first generation cephalosporins are more active against Gram-positive bacteria. However, the second generation have a greater spectrum of activity and increased resistance to beta-lactamases, and correspondingly they have less potency against Gram-positive bacteria. Similarly, the third generation cephalosporins (3GCs) have a wider spectrum of activity against Gram-negative and Gram-positive bacteria along with greater stability against beta-lactamases, but their in vitro activity against Gram-positive microbes is weaker than the first and perhaps the second generation cephalosporins (Uri and Actor, 1983).

Other classification schemes have been devised. Williams (1987) proposed a more accurate method of categorizing parenteral cephalosporins based on antimicrobial activity, stability to beta-lactamases, stability to mammalian esterases, and variations in pharmacological properties. However, this scheme produces rather artificial distinctions between cephalosporins and has not been widely adopted.

It is probable that if a new cephalosporin is introduced which has significant advantages over the 3GCs, it will be designated as a fourth generation cephalosporin.

1.7. Bacterial resistance to cephalosporins.

Resistance to cephalosporin antibiotics can arise from:

a. Modification of the targets which are responsible for the anti-bacterial action of cephalosporins.

b. Inability of the cephalosporin to reach the microbial target.

c. Inactivation of the cephalosporin by beta-lactamases.

1.7.1. Cephalosporin resistance caused by target modification.

It has been explained previously that cephalosporins must interact with PBPs to exert their anti-bacterial action. However, PBPs can either be replaced or undergo modification to reduce their affinity for cephalosporins and consequently provide protection from these drugs.

a. PBP modification in Streptococci spp.

PBP modification has been shown to be the mechanism of resistance in strains of Streptococcus faecium highly resistant to cephalosporins. The strains of S.faecium were demonstrated to produce PBP-5 which reacts very slowly with beta-lactams (Fontana et al., 1983). Penicillin resistance, mediated by PBP modifications, has also been reported in Streptococcus pneumoniae

(Handwerger and Tomasz, 1986; Zighelboim and Tomasz, 1980).

b. PBP modifications in Haemophilus and Neisseria.

Neisseria gonorrhoeae was found to exhibit intrinsic resistance to cephalosporins and penicillins by decreasing beta-lactam binding to PBP-1 and PBP-2 (Dougherty et al., 1980, 1981). Parr and Bryan (1984) reported that intrinsic resistance in Haemophilus influenzae resulted from reduced affinity of PBP-3a and 3b for benzylpenicillin. Serfass et al. (1986) reiterated these findings, and reported that intrinsic cephalosporin resistance in isolates of H.influenzae was caused by reduced affinity of PBP-5. PBP-5 corresponded to PBP-3b in Parr and Bryan's publication (Livermore, 1987.)

1.7.2. Cephalosporin resistance caused by PBP replacement.

Beta-lactam resistance can also occur where an additional PBP is produced which is unaffected by the presence of penicillins or cephalosporins. It has been shown that methicillin resistant strains of Staphylococcus aureus, which are also resistant to cephalosporins, produce an additional PBP (PBP-2') which is not present in methicillin sensitive isolates (Hartman and Tomasz, 1984; Ubukata et al., 1985). Although many methicillin resistant S.aureus produce penicillinase this contributes little to their resistance to beta-lactamase stable beta-lactam antibiotics (Spratt, 1989). Consequently, the acquisition of PBP-2' appears to be the major mechanism of methicillin resistance which is being observed in increasing numbers of S.aureus isolates, and probably

responsible for the emergence of methicillin resistant S.aureus in many hospitals (Cookson and Phillips, 1988; Kayser, 1975; McGowan, 1988).

1.7.3. Impermeability mediated cephalosporin resistance.

Permeability is often proposed as the mechanism of resistance once all other potential resistance mechanisms have been discounted. However, there is strong evidence to suggest that the outer membrane, in Gram-negative bacteria, can shield PBPs from the actions of cephalosporins. The less sophisticated wall structure of Gram-positive bacteria implies that this layer must be much more permeable to the diffusion of cephalosporins. Indeed, Williamson et al. (1983) showed that the PBPs of Group D streptococci were completely exposed. Consequently, the rest of this section will be concerned with impermeability mediated resistance in Gram-negative bacteria.

The survival of Gram-negative bacteria depends on their ability to absorb nutrients and excrete waste products. However, the bilayer region of the outer membrane provides a relatively impermeable barrier to hydrophobic solutes and presumably to hydrophilic solutes. Consequently, Gram-negative bacteria have developed porins which are a class of proteins that produce water filled non-specific transmembrane diffusion channels (reviewed by Nikaido, 1989). Nikaido et al. (1983) showed that these porin channels were the predominant pathway for the diffusion of beta-lactams into E.coli cells.

a. Porins and cephalosporin resistance.

Porins function either individually or, more often as trimers (Nakae et al., 1979), and many different types of porins have been identified (reviewed by Hammond et al., 1984). Indeed, porins have been characterised in thirty two species of Gram-negative bacteria (Hancock, 1987). The two most significant E.coli porins are Omp F and Omp C (Nikaido et al.,1983), and mutants which produce reduced quantities of these two porins were more resistant to many cephalosporins compared to the wild type strains (Harder et al., 1981; Jaffe et al., 1982; Sawai et al., 1982). As yet there has been no conclusive evidence to suggest that the genes controlling the porin production are transferable between bacterial cells.

b. Impermeability resistance in Pseudomonas aeruginosa.

P.aeruginosa has a broad spectrum of resistance which suggests a non-specific resistance mechanism such as impermeability. The extent of this impermeability is illustrated by the fact that although P.aeruginosa isolates are frequently considered sensitive to third generation cephalosporins such as ceftazidime and cefoperazone, they have MICs up to 100 fold greater than E.coli (Livermore, 1987). However, it appears that this impermeability is independent of the porins present for the following reasons. Firstly, P.aeruginosa produces large amounts of a porin (protein F) which has been shown to form large pores (Hancock et al., 1979). This particular phenomenon may be explained if most of the pores were partially closed (Hancock, 1986). Secondly, resistant and sensitive P.aeruginosa isolates produce similar quantities of protein F (Livermore, 1984) and

thirdly, some porin deficient mutants of P.aeruginosa have been shown to have reduced outer membrane permeability with no corresponding increase in beta-lactam resistance (Gilleland and Farley, 1982; Nicas and Hancock, 1983). Consequently, impermeability mediated cephalosporin resistance in P.aeruginosa can be explained by either a complex control of porin channels and/or an additional barrier separating the outer membrane and PBPs (Livermore, 1987). Godfrey et al. (1984) actually showed that the resistance phenotypes of permeability mutants of P.aeruginosa correlated to changes within the lipopolysaccharide.

1.8. Beta-lactamase mediated resistance to cephalosporins.

The bacterial production of beta-lactamases is considered to be the most prominent and widespread mechanism of resistance to cephalosporin antibiotics.

1.8.1. Richmond and Sykes method of classifying beta-lactamases.

Richmond and Sykes (1973) proposed one of the most widely accepted classification schemes. The beta-lactamases of Gram-negative bacteria were subdivided into five groups by virtue of their substrate specificity (penicillinase or cephalosporinase), genetic derivation (plasmid or chromosomal), and susceptibility to inhibitors. The largest,

and constantly growing group of beta-lactamases are the R-plasmid mediated beta-lactamases. These are classified into Groups III and V. These two groups provide insufficient criteria for the classification of such a large and diverse group of enzymes (Amyes, 1987). This problem was elucidated by the development of analytical isoelectric focusing as a tool for the beta-lactamase identification (Matthew et al., 1975). This distinguished beta-lactamases by their isoelectric focusing point (pI) and consequently revolutionised the classification of plasmid mediated beta-lactamases (Medeiros, 1984). This has led to the publication of many tables which differentiate the plasmid mediated beta-lactamases by listing their pI, molecular weight, substrate profile and susceptibility to inhibitors. However, almost immediately after such tables are published other novel beta-lactamases have been characterised and the tables rapidly become dated (see Amyes, 1989 for the most recent tabulation of the properties of plasmid mediated beta-lactamases).

a. Disadvantages of the biochemical classification of beta-lactamases.

The main criterion used by the Richmond and Sykes scheme is substrate profile which is normally expressed as an enzymic rate of hydrolysis or Vmax related as a percentage to the rate obtained with a standard beta-lactam substrate. The listing of such data has allowed the enzymes to be segregated into groups of carbenicillinases, oxacillinases or broad spectrum enzymes. However, this scheme does not take into account the enzymes affinity for different substrates (Km), and the listing of hydrolysis data without referring to the Km has been criticised on many occasions (Amyes, 1987; Bush and Sykes, 1986; Livermore, 1987).

In addition hydrolysis data determined by different laboratories can show considerable variations. Also as so many beta-lactamases have similar pIs it is becoming increasingly difficult to use isoelectric focusing as a method of differentiating novel beta-lactamases. Lastly, the Richmond and Sykes scheme does not consider sequence homology relationships between different beta-lactamases.

1.8.2. The Ambler method of beta-lactamase classification.

Over the last 10 years, there has been a significant increase in the technology associated with molecular probing, amino acid and nucleotide sequencing which has provided a wealth of information on the amino acid sequences of many beta-lactamases. This information has been utilized in this second method of classification which groups both Gram-positive and Gram-negative beta-lactamases into three or may be five distinct Classes (table 3). Ambler (1980) devised this method of classification and introduced the Class A and Class B beta-lactamases.

a. Class A beta-lactamases.

Class A beta-lactamases have a serine residue at their active site and they have molecular weights of around 29000. These enzymes show significant amino acid homology and are either penicillinases or broad spectrum beta-lactamases. Class A enzymes show considerable homology with D-alanine carboxypeptidase which is one of the target sites of penicillin action in Bacillus spp.. This has led to the theory that the target sites of penicillin action (PBPs) share a common evolutionary origin with

Table 3. Classification of beta-lactamases based on molecular structure (Ambler 1980).

Beta-lactamase		Reference
Class A		
Staphylococcus aureus PC1		Ambler (1980)
Bacillus licheniformis		Ambler (1980)
Bacillus cereus I		Ambler (1980)
Klebsiella pneumonia K-1, (SC10436)		Joris et al. (1987)
Klebsiella pneumonia (LEN-1)		Arakawa et al. (1986)
Klebsiella aerogenes K-1, (1082E)		Emanuel et al. (1986)
Streptomyces albus G		De Meester et al. (1987)
Streptomyces cacaoi		De Meester et al. (1987)
Plasmid mediated	TEM-1	Sutcliffe, (1978)
	TEM-2	Ambler and Scott (1978)
	TEM-3	Sougakoff et al. (1988)
	SHV-1	Barthelemy et al. (1988a)
	SHV-2	Barthelemy et al. (1988b)
	PSE-4	Boissinot and Levesque (1990)
Class B		
Bacillus cereus II		Ambler (1980)
Class C		
Escherichia coli K12		Knott-Hunziker et al. (1982)
Pseudomonas aeruginosa		Knott-Hunziker et al. (1982)
Citrobacter freundii		Lindberg and Normark (1986)
Enterobacter cloacae P99		Joris et al. (1984)
Serratia marcescens		Joris et al. (1986)
Class D		
Plasmid mediated	OXA-1	Ouellette (1988)
	OXA-2	Dale et al. (1985)
	OXA-5	Levesque (1988)
	PSE-1	Jacoby (1988)
	PSE-2	Huovinen et al. (1988a)
Class E		
Xanthomonas maltophilia L-1		Bicknell et al. (1985)

beta-lactamases (Asoh et al., 1986; Waxman et al., 1982; Yocum et al., 1979). This hypothesis has been reinforced by the studies showing that the secondary and tertiary structures of the beta-lactamase of Bacillus licheniformis (another Class A enzyme) is similar to the PBP of Streptomyces R6 despite limited amino acid homology (Kelly et al., 1986; Samraoui et al., 1986). Class A also includes the TEM and SHV-derived beta-lactamases.

b. Class B beta-lactamases.

Class B beta-lactamases include metallo-enzymes such as Bacillus cereus II cephalosporinase which requires a metal cofactor which is normally ZnII, but other ions can substitute (Davies and Abraham, 1974). These enzymes show insignificant amino acid homology with the enzymes from Class A.

c. Class C beta-lactamases.

This group was added by Jaurin and Grundstrom, (1981). These enzymes were serine beta-lactamases but their amino acid sequence around the active site was very different from that of the Class A proteins. Class C beta-lactamases favour cephalosporin hydrolysis, and share no sequence homology with the Class A beta-lactamases (Bergstrom et al., 1982; Jaurin and Grundstrom., 1981; Knott-Hunziker et al., 1982).

d. Class D beta-lactamases.

The nucleotide sequence of the PSE-2 beta-lactamase was determined by

Huovinen et al. (1988a) and this was found to have distinct homology with the sequence of OXA-2 which was deduced by Dale et al. (1985). However, Huovinen et al. (1988a) suggested that as neither of these enzymes exhibited structural similarities with the TEM-1 or amp C beta-lactamases they should be classified into a new Class. In addition to this, Jacoby (1988) has partly sequenced PSE-1 and Ouellette (1988) has sequenced OXA-1. Jacoby (1988) demonstrated that the amino acid sequences of OXA-1/2 and PSE-1/2 showed greater homology between each other than TEM-1. Consequently, the OXA and PSE enzymes appear to be related by amino acid sequence homology but not by substrate specificities. Levesque (1988) has also reported that the sequence of OXA-5 shows greater homology with the previously discussed PSE and OXA enzymes than with TEM-1. Therefore, Jacoby (1988) and Huovinen et al. (1988a) have suggested that OXA-1, 2, 5, and PSE-1, 2 belong to a new Class of beta-lactamases in the Ambler system - Class D. DNA-DNA hybridisation studies suggest that other OXA and PSE enzymes may be allocated to this new Class. Although hybridisation studies have illustrated that PSE-4 shows homology with other PSE enzymes, the recent nucleotide sequencing of this gene has revealed that it has 50% nucleotide homology with TEM-1 (Boissinot and Levesque, 1990). Consequently, PSE-4 appears atypical of the other PSE beta-lactamases as it is more related to the Class A beta-lactamases.

e. Class E beta-lactamases.

It has been suggested that the metallo-enzyme, L-1, produced by Xanthomonas maltophilia should be designated to this new Class. This is because the N-terminal amino acid sequence and the biochemical properties of the L-1 beta-lactamase show no similarity to those of the Bacillus cereus II metallo-beta-lactamase in Class B (Bicknell et al., 1985)

f. The use of DNA probing in the Ambler classification method.

Probing experiments facilitate the classification of beta-lactamases in this scheme as they infer the degree of nucleotide homology between beta-lactamase genes. Today DNA probing is widely used to distinguish different beta-lactamases and with the introduction of non-radioactive beta-lactamase probes (Carter et al., 1987) the utilization of such procedures will increase. However, discrepancies have already occurred concerning the hybridization of a TEM-1 probe with OXA-2 (Cooksey et al., 1985). This hybridization was later discounted when no significant homology could be detected between the amino acid sequences of TEM-1 and OXA-2. Ouellette et al. (1987) proposed that the cross hybridisation recorded by Cooksey et al. (1985) may have been caused by either the plasmid encoding OXA-2 containing a silent copy of TEM-1, or because the TEM-1 probe contained more than 200 base pairs outside the TEM-1 structural gene. Consequently, to some extent the efficacy of probing depends on the nature and length of the probe (Bush, 1988). Therefore, the

methodology and the type of probes used to detect specific beta-lactamase genes must be standardized.

Table 4 illustrates some of the relationships between different beta-lactamases which have been demonstrated by the use of DNA probes.

g. Advantages and future use of the Ambler classification.

The main advantage in the classification of beta-lactamases by their amino acid sequence is that such data are not susceptible to the huge variations seen with biochemical calculations. Ultimately to categorize a particular beta-lactamase into a specific Class, the amino acid sequence of the enzyme has to be determined. Although the determination of amino acid sequence data requires extensive experimentation, in time it will probably be recognized as the only efficient method of concluding the novelty of a beta-lactamase, and this in turn will complement the Ambler system of classifying beta-lactamases.

1.8.3. Chromosomal beta-lactamases which confer cephalosporin resistance in Gram-negative bacteria.

Matthew and Harris (1976) reported that virtually all Gram-negative bacteria produced chromosomally mediated beta-lactamases. Depending on the host strain, these beta-lactamases are either expressed basally or they are inducible. For example, Escherichia coli and Shigella and Proteus mirabilis species produce basal levels of cephalosporinase regardless of

Table 4. DNA-homology of beta-lactamase genes.

Beta-lactamase.	Homology.	Reference.
TEM-1	TEM-2, TLE-1	Levesque et al. (1987) Huovinen et al. (1988b)
TEM-1	TEM-2, OXA-2(*)	Cooksey et al. (1985)
TEM-1	TEM-3, 4, 5, 6, and 7	Sougakoff et al. (1988)
OXA-1	(OXA-2)	Ouellette and Roy (1986)
OXA-1	OXA-4	Huovinen et al. (1988b) Levesque et al. (1987) Ouellette et al. (1988).
OXA-2	(OXA-3)	Boissinot et al. (1987) Houvinen et al. (1988b)
PSE-1	PSE-4, CARB-3	Levesque et al. (1987) Houvinen et al. (1988b)
PSE-2	OXA-6, (OXA-5)	Huovinen et al. (1988b)
ROB-1	None	Levesque et al. (1987)
SHV-1	(LCR-1), (OXA-2), (CEP-1)	Bisessar and James (1988)
SHV-1	SHV-2	Huovinen et al. (1988b)
OHIO-1	None(@)	Shales et al. (1986)

(*) = Explained in section 1.8.2f.
(@) = There is strong immunological evidence that OHIO-1 is SHV derived (Vedel et al. 1989).
Beta-lactamases in parentheses hybridised weakly.

the presence of a beta-lactam antibiotic. Such beta-lactamases are unimportant in conferring cephalosporin resistance (Livermore, 1987).

a. Cephalosporin resistance caused by inducible chromosomal cephalosporinases.

Species such as Pseudomonas aeruginosa, Enterobacter cloacae, Enterobacter aerogenes produce inducible beta-lactamases (Sykes and Smith, 1979). These beta-lactamases were responsible for the resistance to many of the early cephalosporins. Rosselet and Zimmerman (1973) proved that the induction of these beta-lactamases was the mechanism of cephalosporin resistance as they showed that mutants which produce only basal levels of these enzymes remain sensitive to cephaloridine.

Most Gram-negative bacteria, which produce inducible cephalosporinases, are sensitive to 3GCs but are resistant to the first and second generation cephalosporins. This does not result from an ability of the 3GCs to resist hydrolysis by such cephalosporinases, rather that 3GCs are much poorer inducers of these beta-lactamases (Curtis et al., 1986; Livermore and Yang, 1987). Indeed, if secondary inducers are added to the media resistance to 3GCs increases (Bryan et al., 1984). However, this response is not seen with mutants which are capable of producing only basal amounts of the enzyme (Bryan et al., 1984).

b. Cephalosporin resistance caused by the selection of SDM.

Bacterial species, which produce inducible cephalosporinase, can produce spontaneous stably derepressed mutants (SDM) at a frequency of between 10^{-5} and 10^{-10} according to the species, strain and selection conditions (Philippon, 1987; Wiedemann, 1986). These mutants have the same broad spectrum of resistance as strains which produce high levels of cephalosporinase after induction and consequently, confer resistance to all generations of cephalosporins (Curtis et al., 1986). However, because the high enzyme activity of SDM confer resistance to the challenge of the newer cephalosporins the selective advantage of the SDM may allow them to out grow the susceptible bacteria (Livermore, 1987). This phenomenon has also been demonstrated to be the cause of emergence of resistance during the therapeutic use of the third generation cephalosporins.

c. Emergence of cephalosporin resistance in therapy.

The emergence of such resistance during therapy was rare prior to the use of third generation cephalosporins (Sugarman and Pesanti, 1980). However, the following examples illustrate the selection of SDM during therapy. King et al. (1983) demonstrated a reduced sensitivity to ceftazidime during treatment of P.aeruginosa; Paull and Morgan, (1986) showed the emergence of ceftriaxone resistance whilst treating P.aeruginosa in cystic fibrosis patients; Dworzack et al. (1987) showed that six out of 49 strains, from 44 patients on broad spectrum cephalosporin therapy had

become resistant as a result of the selection of SDM; Follath et al. (1987) showed that in 18 patients resistant strains of initially sensitive Gram-negative bacteria emerged during therapy with a third generation cephalosporin.

In addition to these studies Hopkins et al. (1987) demonstrated that it may not only be the selection of derepressed mutants which cause resistance to third generation cephalosporins, but other factors may also be involved. Hopkins and Towner (1990) later demonstrated that changes in outer membrane proteins were responsible for the enhanced cefotaxime resistance in certain strains of Enterobacter aerogenes.

d. Biochemical characterisation of cephalosporinases.

The beta-lactamases produced by SDM in P.aeruginosa, and Ent.cloacae confer high level resistances to the later cephalosporins. Further studies with Ent.cloacae revealed that this species produced at least three different types of chromosomal cephalosporinases (Seeberg et al., 1983; Minami et al., 1980a) which conferred resistance to all generations of cephalosporins. These enzymes hydrolysed the first generation cephalosporin but had no detectable hydrolytic activity against the later generation cephalosporins. Then and Angehrn (1982) showed that these "unhydrolysable cephalosporins" inhibited the enzyme's hydrolysis of the chromogenic cephalosporin nitrocefin. It was consequently hypothesized that these cephalosporinases were trapping and inactivating the non-hydrolysable cephalosporins (Then and Angehrn, 1982; Gutmann and

Williamson, 1983). However, Vu and Nikaido (1985) concluded that this mechanism could not explain the high level of resistance conferred by strains overproducing these cephalosporinases. They proposed that conventional hydrolysis assays were performed with beta-lactam concentrations which were too high to be a realistic reflection of the physiological situation within the bacterial cell. Subsequently, they performed assays at much lower beta-lactam concentrations and revealed that these "non-hydrolysable" substrates were slowly hydrolysed. However, Sanders and Sanders (1986) suggested that trapping and hydrolysis were not mutually exclusive mechanisms for beta-lactamase mediated resistance. It was hypothesised that if a substrate was slowly hydrolysed it would be found primarily in biological inactive complexes (Murakami and Yoshida, 1985; Sanders and Sanders, 1986). This mechanism prevents the build up of active drug in the cell. In addition, the poor penetration of cephalosporins into Gram-negative cells promotes the protection afforded by the enzyme, so that slow hydrolysis of poor substrate drugs can also be a very efficient mechanism of resistance (Sanders, 1989).

These cephalosporin specific beta-lactamases are also resistant to clavulanic acid (Bush, 1988). Consequently, if the genes encoding these enzymes become incorporated into plasmid DNA this would create a huge threat to the future therapeutic use of beta-lactam antibiotics.

e. Chromosomally mediated cefuroximases.

Inducible chromosomal cefuroximases are produced by Proteus vulgaris

(Aspiotis et al., 1986; Matsubara et al., 1981) and Pseudomonas cepacia (Hirai et al., 1980), whereas Pseudomonas pseudomallei produces constitutively low levels of the enzyme (Livermore et al., 1987). These enzymes are sensitive to clavulanic acid inhibition and have high activity against cefuroxime and cefotaxime.

f. Broad spectrum chromosomal beta-lactamases.

Metalloenzymes.

Xanthomonas maltophilia produces an unusual chromosomal beta-lactamase called L-1 which is Zn II dependent and hydrolyses most penicillins and cephalosporins as well as imipenem. This enzyme is acknowledged as being responsible for the wide spread resistances associated with X.maltophilia (Saino et al., 1982). X.maltophilia has been reported to produce one other type of chromosomal beta-lactamase called L-2, but this enzyme does not confer such a broad spectrum of resistances and is not thought to be a metallo-enzyme (Saino et al., 1984).

Several other imipenem hydrolysing beta-lactamases have been described and because of their dependence on Zn II and their susceptibility to EDTA have been suggested to be chromosomal metalloenzymes. These include those beta-lactamases produced by Flavobacterium odoratum (Sato et al., 1985) Legionella gormanii (Fujii et al., 1986) and Bacteroides fragilis (Cuchural et al., 1986; Yotsuji et al., 1983). Each of these enzymes have a very broad spectrum of activity and hydrolyse almost all cephalosporins, and they are resistant to inhibition by clavulanic acid (Sanders, 1989).

Klebsiella enzymes.

The broad spectrum chromosomal enzymes of Klebsiella spp. show slightly higher rates of hydrolysis for penicillins than for cephalosporins. Many of these enzymes hydrolyse and confer resistance to cloxacillin, the oxyiminocephalosporins and aztreonam. They generally have no activity against cephamycins, ceftazidime or imipenem and provide no protection against these beta-lactams. The klebsiella chromosomal enzymes are also sensitive to inhibition by clavulanic acid (Sanders, 1989). Klebsiella spp. chromosomal beta-lactamases have strong similarities with the SHV-1 and TEM-1 plasmid mediated beta-lactamases. Amino acid homology studies based on the sequences of TEM-1, SHV-1, and LEN-1 (a chromosomal beta-lactamase of Klebsiella pneumoniae) have inferred that the rather K.pneumoniae confined SHV-1 beta-lactamase could have evolved from the K.pneumoniae chromosomal beta-lactamase. Also the 68% amino acid sequence homology between SHV-1 and TEM-1 implies that the ubiquitous TEM beta-lactamase may have evolved from the SHV-1 beta-lactamase (Barthelemy et al., 1988a).

1.9. Cephalosporin resistance conferred by plasmid mediated beta-lactamases.

Beta-lactamases encoded by transmissible plasmids were first reported by Datta and Kontomichalou (1965). The genes encoding these enzymes provide a greater threat to beta-lactam therapy because they can transfer from one

bacterial cell to another, and often into different species of bacteria. This spread of resistance is enhanced if the gene is encoded by a transposon. These freely transferable DNA fragments can move easily between different plasmids or between the plasmid and chromosome (Hedges and Jacob, 1974). Medeiros (1989) listed 13 beta-lactamases which were encoded by transposons.

The number of plasmid mediated beta-lactamases has increased rapidly since 1979 when Matthew et al. (1979) described 11 different types. Payne described 28 different types in 1986 and today approximately 60 different plasmid encoded beta-lactamases have been characterised.

1.9.1. Cephalosporin resistance conferred by plasmid encoded beta-lactamases (pre-1983).

Most of the earlier plasmid mediated beta-lactamases such as TEM-1/2, OXA-1, 2, 3, and PSE-1, 2, 3, 4, hydrolyse cephaloridine, cephalothin, cefamandole and cefazolin (Glaxo, 1980). From studies with these enzymes in the same host strain (Escherichia coli J53-2), these beta-lactamases provide protection from each of the cephalosporins described above. However, none of the enzymes conferred resistance to ceftazidime or cefotaxime (Glaxo, 1980). Consequently, until 1983, the only beta-lactamases conferring third generation cephalosporin resistance were chromosomally encoded beta-lactamases.

1.9.2. Cephalosporin resistance conferred by plasmid encoded beta-lactamases (post-1983).

Before 1983 the only beta-lactamase conferred resistance to broad spectrum cephalosporins had been reported to be because of chromosomally mediated beta-lactamases. In 1983 Shah and Stille reported a number of Escherichia coil and Klebsiella pneumoniae strains which were more sensitive to cefoxitin than 3GCs. This had previously only been reported by Hart and Percival (1982). Such strains justified further investigation and Knothe et al. (1983) concluded that these strains carried plasmids which encoded transferable resistance to 3GCs, although the resistance mechanism was not determined.

a. Identification of SHV-2.

Kliebe et al. (1985) performed further studies on the strains reported by Knothe et al. (1983) and identified that SHV-2 was responsible for the transferable cefotaxime resistance. Consequently, SHV-2 was the first reported example of a plasmid encoded beta-lactamase which conferred transferable resistance to a 3GC. This beta-lactamase had the same isoelectric point as SHV-1 (pI 7.7) and heteroduplex analysis revealed extensive homology between SHV-1 and SHV-2. The hypothesis that SHV-2 had derived from SHV-1 was substantiated by the isolation of a cefotaxime resistant mutant which produced an enzyme identical to SHV-2 (Kliebe et al.,

1985). Comparison of the amino acid sequence of SHV-1 (Barthelemy et al., 1988a) and SHV-2 (Barthelemy et al. 1988b) revealed that these two enzymes only differed by a single amino acid substitution. This substitution allowed SHV-2 to hydrolyse broad spectrum cephalosporins due to an increased affinity for such compounds (Kliebe et al., 1985).

b. Identification of TEM-3 (CTX-1).

In July 1984 a multiresistant K.pneumoniae strain was isolated from the wound of a patient hospitalized for five months in an intensive care unit at Clermont-Ferrand, France. Its complex resistance phenotype to beta-lactam antibiotics was found to be attributable to the production of a beta-lactamase of pI 6.3. This enzyme was called CTX-1 (Sirot et al., 1987), and later renamed TEM-3, when DNA probing studies showed it was a TEM derived enzyme (Sougakoff et al., 1988). During 1985 10% (89 isolates) of K.pneumoniae strains isolated from patients in intensive care units in Clermont-Ferrand were suspected to produce the same enzyme. This was verified in 50 of the strains and the incident was reported as "an epidemic of antibiotic multiply-resistant strains of K.pneumoniae producing a new beta-lactamase" (Sirot et al., 1987).

More than 300 miles away in Paris, a similar outbreak of 3GC resistant K.pneumoniae was occurring at the same time. The outbreak included three different intensive care units and spread from one unit to another and

then to four wards. The resistance to 3GCs also resulted from a transferable beta-lactamase of pI 6.3 with identical properties to TEM-3 (Brun-Buisson et al., 1987). Consequently, TEM-3 was the first reported example of a TEM derived enzyme which caused transferable resistance to a 3GC. However, it is possible that such enzymes have been present in bacterial populations long before this initial discovery.

c. Amino acid sequence of TEM-3.

When Sougakoff et al. (1988) determined the amino acid sequence of TEM-3, they found the beta-lactamase differed in two amino acids from that of TEM-2. At residue 104 (Ambler numbering system, 1980) glutamic acid (TEM-1) was substituted for lysine (TEM-3), and at residue 238 glycine (TEM-2) was substituted by a serine (TEM-3).

The ability to change the catalytic activity of TEM-1, by altering key amino acid residues was first illustrated by Hall and Knowles (1976), although these substitutions were not the same.

d. Other TEM derived enzymes.

During 1986 and 1987, TEM-4 (Paul et al., 1989), TEM-5 (Petit et al., 1988), TEM-6 (Bauernfeind and Horl, 1987) and TEM-7 (Gutmann et al., 1988) were identifed. The genes encoding each of these beta-lactamases

hybridised with a TEM probe, thus verifying that they exhibited homologous nucleotide sequences with TEM-1/2 (Sougakoff et al., 1988). The first reported example of transferable resistance in the UK was identified at the Royal Hallamshire Hospital by Spencer et al. (1987). This enzyme was called RHH-1 (later renamed TEM-9) and although the resistance was transferable no plasmid could be extracted and visualized. All these beta-lactamases are detailed in table 53.

e. Selection of mutant enzymes of TEM-1 which confer resistance to broad spectrum cephalosporins.

Further work revealed that it was possible to obtain 3GC hydrolysing beta-lactamases spontaneously from TEM-1/2 producing organisms. Sougakoff et al. (1988b) and Gutmann et al. (1988) both obtained a beta-lactamase which resembled TEM-7 from a TEM-2 producing strain. These mutant beta-lactamases are detailed in table 51.

1.9.3. Plasmid mediated beta-lactamases - epidemiological studies.

Table 5 summarises nine beta-lactam resistance surveys which have been conducted at different centres around the world. The majority of the surveys have investigated the beta-lactamases produced by ampicillin resistant strains, and the table only shows the percentages of E.coli and Klebsiella spp. which produce TEM-1, TEM-2, or SHV-1 "potentially" plasmid mediated beta-lactamases.

Table 5. Summary of Ampicillin resistance surveys illustrating the ubiquitous occurrence of TEM-1 and SHV-1.

Strain	Number of resistant organisms.	Chromo only (%).	Plasmid beta-lactamases. TEM-1 (%)	TEM-2 (%)	SHV-1 (%)	Reference, country.
E.coli	374	12	77	-	2	Medeiros, (1984), Germany, Brazil, USA, France, Indonesia Thailand, S.Africa
E.coli	110	16	67	0	1	Simpson et al.
K.pneumoniae	63	18	8	8	78	(1980), England.
E.coli	94	0	94	1	0	Roy et al.(1883),
Klebsiella spp.	46	9	44	4	61	Spain.
E.coli	130	13	78	4	13	Simpson et al.
Klebsiella spp.	44	61	0	0	39	(1986), Germany Mexico, Amman Colombia.
E.coli	90	-	72	-	-	Carter et al. (1987), England.
E.coli	47	-	85	-	2	Huovinen et al.
K.pneumoniae	9	-	67	-	33	(1988b), USA.
E.coli	104	24	73	2	0	*Reid et al.
Klebsiella spp.	23	70	22	4	9	(1988), Scotland
E.coli	96	-	85	1	-	Jouvenot et al.
Klebsiella spp.	41	-	17	0	-	(1987), France.
E.coli	27	-	74 ("TEM")		4	Wiedemann et al.
Klebsiella spp.	78	-	23 ("TEM")		67	(1989), Germany.

- = not mentioned in survey. *Study of cephaloridine resistant organisms. "TEM" = both TEM-1 and TEM-2.

A very small proportion of these strains produce both SHV-1 and a TEM type of beta-lactamase. To maintain the simplicity of the table such strains are registered more than once.

The surveys illustrate that TEM-1 is responsible for the vast majority of ampicillin resistance in E.coli, and it confers a significant amount of resistance in Klebsiella spp.. SHV-1 is far more ubiquitous in Klebsiella spp. than E.coli which confirms the view that SHV-1 was derived from the klebsiella chromosomal beta-lactamases. TEM-2 is found in very few strains of ampicillin resistant klebsiella or E.coli. TEM-1 and TEM-2 have almost identical biochemical characteristics and only differ by one amino acid residue. However, the TEM-2 gene has not even approached the success achieved by the TEM-1 gene.

Consequently, TEM-1 appears to have a selective advantage over other plasmid mediated beta-lactamases including TEM-2. This success could be attributed to its efficient hydrolysis of penicillins and cephalosporins along with it being encoded by particularly promiscuous transposons and plasmids. However, it is possible that in the physiological environment of the bacterial cell TEM-1 may exhibit an advantage over other plasmid mediated beta-lactamases which we cannot detect (Amyes, 1989).

1.10. Clinical implications of transferable resistance to broad spectrum cephalosporins.

The following section forecasts the potential threats which these enzymes may present to the future therapeutic use of third generation cephalosporins.

1.10.1. Treatment of Klebsiella infections.

Klebsiella spp. are a renowned source of transferable antibiotic resistance (Casewell and Phillips, 1981), and several outbreaks caused by multi-resistant Klebsiella pneumoniae have been reported in the 1970s (Thomas et al., 1977; Petrocheilou, et al., 1977; Rennie and Duncan, 1977; Courtney et al. 1980). In the 1980s such outbreaks have probably been reduced because of the introduction of third generation cephalosporins.

However, table 5 shows that most of the TEM or SHV derived enzymes occur more frequently in Klebsiella spp. than any other species. Consequently, these beta-lactamases could seriously jeopardize the future success of the later cephalosporins, such as cefotaxime, ceftazidime or aztreonam, in treating Klebsiella spp. infections.

1.10.2. Spontaneous occurrence of TEM and SHV derived beta-lactamases.

It has been discussed that 3GC hydrolysing beta-lactamases could occur spontaneously in SHV-1, TEM-1 or TEM-2 producing organisms. As TEM-1/2 and SHV-1 have been illustrated to be the most wide spread plasmid mediated beta-lactamases there is a high proportion of strains which are potential producers of 3GC hydrolysing beta-lactamases. Consequently, it would be fair to expect high levels of these 3GC hydrolysing enzymes in bacterial populations.

1.10.3. The occurrence of TEM and SHV derived beta-lactamases related to third generation cephalosporin utilization.

The use of broad spectrum cephalosporins in the UK is very low, and a study of 14 000 Enterobacteriaceae strains isolated in the UK between 1985 and 1987 yielded only one plasmid mediated 3GC hydrolysing beta-lactamase (Spencer et al., 1987). However, the amount of the 3GCs used in France is much higher than in the UK. In particular, the isolated examples detailing outbreaks of these enzymes have occurred in areas of intense 3GC usage. Therefore, the more extensive the use of 3GCs the higher the probability of 3GC hydrolysing enzymes evolving. Thus, as general resistance to the earlier cephalosporins increases, more broad spectrum cephalosporins will be used. In conclusion, this predicament could yield many more examples of transferable resistance and novel enzymes in the future.

1.10.4. Occurrence of different types of TEM or SHV derived beta-lactamases.

Seven different plasmid mediated 3GC hydrolysing beta-lactamases have been discussed in this Introduction, these were all identified between 1983 and 1987. All of these beta-lactamases probably differed from TEM-1/2 or SHV-1 by only a few amino acid residues. Therefore, for the reasons stated earlier, it is fair to assume that over the next few years, many more of these enzymes will evolve. These new enzymes will all have different

specificities and may confer even broader resistance profiles to cephalosporins. In the future the transferable 3GC hydrolysing beta-lactamases have the potential to create huge problems for the future use of 3GCs.

1.11. Aims of this Thesis.

1. To establish the origin of 3GC hydrolysing beta-lactamases.

2. To characterise any novel 3GC hydrolysing beta-lactamases identified in clinical isolates.

3. To establish how long these beta-lactamases have been in bacterial populations.

4. To create a method for classifying the 3GC hydrolysing beta-lactamases.

5. To devise quicker and more convenient methods for the separation and purification of beta-lactamases.

6. Investigation of beta-lactamase satellite bands.

2.0. METHODS

2.1. Bacterial strains.

The standard bacterial stains used in this thesis are shown in table 6 and the standard bacterial plasmids are detailed in table 7. Details of the strains producing standard beta-lactamases are shown in table 8. All strains were maintained at -70°C in nutrient broth with 10% glycerol v/v. The API 20E system (API System, S.A., France) was used for the identification of bacterial strains.

2.2. Antimicrobial agents.

The antimicrobial agents used in this Thesis, along with their suppliers are listed in table 9.

Table 6. Standard bacterial strains.

Bacterial strain.	Markers.	Reference.
E.coli K12 J62-2 (DP55)	pro^- his^- trp^- $lac^+ r_f^R$	Bachmann (1972)
E.coli K12 J53-2 (DP53)	pro^- met^- $lac^+ r_f^R$	Bachmann (1972)
E.coli K12 SA 10 (DP257)	met^-	S. G. B. Amyes

Table 7. Standard bacterial plasmids.

Plasmid designation.	Markers.	Size (Kb).	Reference.
R1 (DP 6)	Ap Cm Km Sm Su incFII	89	Meynell and Datta (1966)
RP4 (DP 42)	Ap Km Tc incP-I	52	Datta et al. (1971)
R6K (DP 43)	Ap Sm inc X	38	Kontamichalou et al. (1970)

DP Numbers denotes D.Payne culture collection.

Table 8. Beta-lactamase producing strains.

Bacterial strain and Number.	B-lactamase produced.	Plasmid.	Original reference for B-lactamase or source.
E.coli J53-2 DP43, 2136E	TEM-1	R6K	Hedges et al. (1974)
E.coli J53-2 DP43, 2137E	TEM-2	RP4	Hedges et al. (1974)
E.coli J62-1 DP189	TEM-3	pCF04	Sirot et al. (1987) Brun-Buisson et al. (1987)
E.coli J53-2 DP193	TEM-4	pUD16	Paul et al. (1989)
E.coli J53-2 DP70	TEM-5	pCFF14	Petit et al. (1988)
E.coli J53-2 DP314, 3491E	TEM-6		Bauernfeind et al. (1987)
E.coli J62-1 DP187	TEM-7		Gutmann et al. (1988)
E.coli J53-2 DP41, 2639E	TEM-9		Spencer et al. (1987)
E.coli J53-2 DP211	TEM-10	pJRQ100	Quinn et al. (1989)
E.coli J53-2 DP37, 2651E	TLE-1	pMG204b	Medeiros et al. (1985)
E.coli J53-2 DP38, 2141E	SHV-1	R1010	Petrocheilou et al. (1977)
E.coli J53-2 DP192	SHV-2	pUD17	Kliebe et al. (1985)
E.coli J53-2 DP191	SHV-3	pUD18	Jarlier et al. (1988)
E.coli J53-2 DP324	SHV-5	pAFF-1	Gutmann et al. (1989)
E.coli J53-2 DP44, 2505E	TEM-1,TLE-1		Glaxo culture collection
E.coli J53-2 SA319	OXA-2	R46	Dale and Smith (1974)
P.aeruginosa PU21 AR34	PSE-1		Matthew and Sykes (1977)
P.aeruginosa PU21 AR35	PSE-2		Matthew (1978)
E.coli J53-2 DP2	PSE-4	R1818	Furth (1975)
K.pneumoniae CF 504 DP68	TEM-1, TEM-5 SHV-1	pCFF14	Petit et al. (1988)
K.aerogenes DP36, 1976E	SHV-1		Glaxo culture collection
K.pneumoniae DP39, 1957E	TEM-1,SHV-1		Glaxo culture collection
K.pneumoniae DP40, 1082E	K-1		Marshall et al. (1972)
E.coli D31 AR39	CEP-1	R22Ka	Bobrowski et al. (1976)
Ent. cloacae P99	Type B (*)		Fleming et al. (1963)
Ent. cloacae	Type A (*)		Seeberg et al. (1983)

DP Numbers denote D.Payne culture collection.
E Numbers denote Glaxo Group Research culture collection.
AR Numbers denote A.Reid culture collection.
SA Numbers denote S.G.B. Amyes culture collection.
(*) Chromosomal beta-lactamases as classified by Seeberg et al. (1983).

Table 9. Antimicrobial agents

Compound.	Supplier.
Ampicillin	SmithKline Beecham Pharmaceuticals.
Aztreonam	E.R. Squib and Sons, Middlesex.
Carbenicillin	SmithKline Beecham Pharmaceuticals.
Cefotaxime	Roussel Laboratories Ltd., Middlesex.
Cefoxitin	MSD Ltd., Herts.
Ceftazidime	Glaxo Group Research Ltd. Greenford, Middlesex.
Ceftriaxone	Roche Products Ltd. Herts.
Cefuroxime	Glaxo Laboratories Ltd. Greenford, Middlesex.
Cephaloridine	Glaxo Group Research Ltd. Greenford, Middlesex.
Cephradine	E.R. Squibb and Sons, Middlesex.
Clavulanic acid	SmithKline Beecham Pharmaceuticals.
Kanamycin	Bristol Laboratories, Middlesex.
Nitrocefin	Glaxo Group Research Ltd. Greenford, Middlesex.
Rifampicin	Lepetite , Milan, Italy.

2.3. Media.

All growth media, both agar and broth, were sterilized by autoclaving at 15 pounds/square inch for 15 minutes.

2.3.1. Complex media.

The following complex media were used; Nutrient broth No.2 (CM67), Isosensitest Broth (CM473), Diagnostic Sensitivity Test Agar (CM261), all supplied by Oxoid, Basingstoke, Hants.

2.3.2. DM minimal medium agar plates (DM agar).

Double strength minimal salts medium was prepared as described by Davies and Mingioli (1950) (table 10). 50ml of double strength DM was then supplemented with the appropriate amount of the required amino acid stock solutions to achieve the final concentrations as shown in table 11. The appropriate amounts of antibiotics were then added. Finally, 2.5ml of a 20% glucose solution was added and the volume made up to 60ml with sterile distilled water. This solution was mixed and added to 40ml of molten Bacteriological Agar No1 (1g of agar/40ml distilled water) and, after gentle mixing, the plates were poured.

Table 10. Preparation of double strength Davis and Mingioli basal medium.

Ingredient	Quantity dissolved in 1L (g).
K_2HPO_4	14.0
KH_2PO_4	6.0
Tri-sodium citrate	1.0
$MgSO_4.7H_2O$	0.2
$(NH_4)_2SO_4$	2.0

Table 11. Amino acid solutions.

Solution	Strength of stock solution.	Final concentration in DM agar.
L-histidine (BDH)	5mg/ml	50mg/L
L-methionine (BDH)	5mg/ml	50mg/L
L-proline (BDH)	5mg/ml	50mg/L
L-tryptophan (Sigma)	2mg/ml	50mg/L

All amino acid solutions were sterilised by steaming for 30 minutes.

2.4. Antibacterial drug susceptibilities.

Susceptibility tests and minimum inhibitory concentrations (MICs) of antibacterial drugs were determined on solid media containing the appropriate concentrations of antimicrobial drugs. An inoculum of 10^5 colony forming units (cfu)/ml was used in each case. In some experiments the MICs of ampicillin, ceftazidime and cefotaxime were additionally measured in the presence of clavulanic acid (2mg/L) or sulbactam (2mg/L).

2.5. Conjugation experiments.

In all mating experiments 0.1ml of an overnight culture of the donor strain was mixed with 1ml of an overnight culture of the recipient strain in 4.5ml of nutrient broth, as described previously by Amyes & Gould (1984). This mixture was then incubated at 37°C for 6 hours and then harvested by centrifugation and resuspended in 5.6ml of DM media. E. coli J53-2 transconjugants of the clinical strains were selected on agar containing the appropriate amounts of rifampicin and beta-lactam antibiotic. Transfers from E.coli J62-2 to E.coli J53-2 were performed in the same way but the E.coli J53-2 transconjugants were selected on DM agar containing proline (50mg/L), methionine (50mg/L) and ceftazidime (4mg/L). Transconjugants resulting from transfers from E.coli J53-2 to E.coli K12

SA10 were selected on DM agar containing methionine (50mg/L) along with the appropriate beta-lactam antibiotic. E.coli J62-2 transconjugants produced from conjugation of E.coli K12 SA10 with E.coli J62-2 were selected on DM agar supplemented with histidine (50mg/L), tryptophan (50mg/L), proline (50mg/L) and the appropriate antibiotic. Transfer frequencies were calculated as follows;

$$\text{Transfer frequency} = \frac{\text{Number of transconjugant cells / ml}}{\text{Number of donor cells / ml}}$$

2.6. Mutation experiments.

In the spontaneous mutation experiments 10ml of nutrient broth was inoculated with a colony of the particular strain under investigation and either grown statically or on an orbital shaker at 37°C. The broth cultures were subjected to a range of growth conditions. The cells were then harvested by centrifugation at 4500rpm for 15 minutes (Heraeus Christ Bactifuge). The resultant pellet was resuspended in 1ml of sterile distilled water and 0.1ml aliquots of this suspension were inoculated onto nutrient agar containing varying concentrations of different selection agents. These plates were incubated for 24-48 hours and over this period all the resistant colonies were purified on identical drug containing nutrient agar plates. The integrity of these mutant colonies was checked by API 20E analysis, and as most of the parent strains were E.coli J53-2

or E.coli J62-2, the auxotrophic requirements of the mutant strains were also tested. Once the mutant strains had satisfied these two criteria their resistance profile was determined and their beta-lactamases examined by IEF. Those mutant strains producing a different beta-lactamase from the parent strain were characterised in more detail.

In those experiments studying the effects of methotrexate or metronidazole, the 10ml of nutrient broth was inoculated with 0.1ml of a 16 hour broth culture of the particular strain under investigation.

A full list of the different factors incorporated into each of the mutation experiments are shown in tables 13 and 19.

2.7. Preparation of crude beta-lactamase preparations.

One litre of nutrient broth, in a two litre conical flask, was inoculated with the particular strain producing the beta-lactamase under investigation. In the case of ceftazidime resistant strains, the bacteria were grown in the presence of ceftazidime (4mg/L). The culture was incubated for 16 hours at 37^{o}C on an orbital shaker. The culture was then harvested by centrifugation at 6000g for 15 minutes at 4^{o}C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments). The resultant bacterial pellet was resuspended in 10ml of 25mM sodium

phosphate buffer (pH 7.0) and centrifuged at 4500 rpm for 15 minutes (Heraeus Christ Bactifuge). The final cell pellet was resuspended in 1ml of 25mM sodium phosphate buffer (pH 7.0). This sample was suspended in an ice/water bath and subjected to two 30 second treatments of ultrasonication (8um), with a one minute cooling period between treatments (MSE Soniprep 150 MSE Instruments, Crawley). The resultant lysate was then cleared by centrifugation at 32 000g for 30 minutes at 4°C (Sorval). The supernatant was removed and could be stored successfully for up to 3 years.

2.8. Assessment of beta-lactamase activity of beta-lactamase preparations.

Thirty three ul of the beta-lactamase extract was mixed with 100ul of a nitrocefin solution (50mg/L) in a microtitre tray. The time taken in seconds for the mixture to change colour from yellow to red was taken as an indication of the beta-lactamase activity of the enzyme preparation.

2.9. Analytical isoelectric focusing of beta-lactamases.

Beta-lactamases were identified by analytical isoelectric focusing as

described by Matthews et al. (1975). The extracts were focused on a thin layer polyacrylamide gel containing carrier ampholines. The composition of the gel is shown in table 12. The beta-lactamase preparations were first examined by wide range IEF employing a broad range ampholine (3.5-10 pH). If the beta-lactamase of interest had an acidic pI it was subsequently re-examined on a gel containing a 1:1 mixture of 4-6 and 3.5-10 pH ampholines. Alternatively, if the beta-lactamase had an alkali pI it was re-examined on a gel containing a 1:1 ratio of 9-11 and 3.5-10 pH ampholines. In all cases novel beta-lactamases were focused along side beta-lactamases of known pI (table 8). Samples of the beta-lactamases were loaded near the anode on the surface of the gel. The amount of each extract applied depended on its activity; for each second of activity possessed by the extract (see section 2.8) 1ul of the sample was applied.

2.10. Determination of the molecular weight of beta-lactamases.

The method of gel filtration was employed to determine the molecular weights of the different beta-lactamases investigated in this study. This was achieved with a Sephadex G-75 column as described by Andrews (1964).

Table 12. Composition of IEF gel for the analysis of beta-lactamases.

Material.	Supplier.	Volume used (ml).	Final concentration.
5% tetramethyl-ethylenediamine (TEMED) in distilled water.	Sigma	0.2	0.25mg/L
40% ampholines w/v (various pH ranges).	LKB	2.0	2% w/v
Acrylamide (100g) plus methylene bisacrylamide (2.7g) in water (300ml).	BDH	9.0	acrylamide 75g/L bisacrylamide 2g/L
Distilled water.		25.0	
Riboflavin (20mg/L).	Sigma	4.0	2mg/L

2.10.1. Preparation of the Sephadex G-75 column.

Sephadex G-75 (Pharmacia) was swollen with the appropriate volume of 25mM sodium phosphate buffer (pH 7.0) at 100^{o}C for an hour. The slurry was allowed to cool and then poured carefully into an LKB gel filtration column (50 X 1.5cm^{2}). When the column was full, the top was connected and the flow started in a upward direction with a LKB peristaltic pump. After the initial pouring of the gel the column was left to equilibrate for 48 hours with 25mM sodium phosphate buffer (pH 7.0). Following this period a flow rate of 15ml/hour was maintained by adjusting the speed of the peristaltic pump.

2.10.2. Calibration of the Sephadex G-75 column.

Before the column was used to determine the unknown molecular weights of beta-lactamases it had to be calibrated with proteins of known molecular weight. Therefore, 10mg each of cytochrome C (12.38 Kd), chymotrypsinogen (25.5 Kd) ovalbumin (45.0 Kd) and were dissolved in 1ml of 25mM sodium phosphate buffer (pH 7.0) and applied to the column. Following the application of the standard proteins 100 fractions were collected by an Ultrorack fraction collector. Each fraction contained 2ml of the elutate. The absorbance of each fraction at 280nm was measured to give a rough estimate of the amount of protein in each fraction. The absorbance reading was then plotted against fraction number. This gave three well separated peaks and the fraction which corresponded to the pinnacle of each peak

represented the elution position of that particular standard molecular weight protein. Therefore, the molecular weight of each of the standard proteins was plotted against its representative fraction number to give a standard curve. This was used to determine the unknown molecular weights of the different beta-lactamases.

2.10.3. Determination of the molecular weight of beta-lactamases.

One ml of beta-lactamase sample, prepared as described in section 2.7, was applied to the Sephadex G-75 column. The settings of the peristaltic pump and fraction collector were maintained at exactly the same parameters as those used in the calibration procedure. Once again 100 2ml fractions were eluted from the column. A nitrocefin spot test assay was performed on each of the fractions to ascertain which of the tubes contained the beta-lactamase. The hydrolytic activities of those fractions exhibiting beta-lactamase activity were then assayed spectrophotometrically by measuring the rate of hydrolysis of nitrocefin (10^{-4}M). The activity of each fraction was plotted against its fraction number, and the molecular weight of the enzyme was determined from the standard curve. This was achieved by calculating the molecular weight which corresponded to the fraction exhibiting the greatest beta-lactamase activity. Once the molecular weight had been determined, all the fractions containing significant beta-lactamase activity were pooled and used for the spectrophotometric assays described in section 2.11.

2.11. Kinetic studies.

For all spectrophotometric assays either a Pye Unicam SP1800 uv/vis Spectrophotometer or a Perkin Elmer Lambda 2 Spectrophotometer were used. Both these machines had thermostatically controlled cell carriers and an automatic cell change facility, all measurements were performed at 37^{o}C. Kinetic measurements were performed only on beta-lactamases which had been partially purified by gel filtration and which had originated from E.coli transconjugants.

2.11.1. Determination of Vmax and Km.

The rates of hydrolysis of cephalosporins were measured at concentrations between 0.01mM and 0.1mM, whereas the rates of hydrolysis of penicillins were measured at concentrations between 0.1mM and 1mM. The reciprocals of the rate of hydrolysis values obtained in these assays were plotted against the reciprocal of the substrate concentration. The Km and Vmax values for the hydrolysis of a particular beta-lactam by a particular beta-lactamase were then obtained by the Lineweaver-Burk (1934) method. The Vmax values were normalised with respect to ampicillin as proposed by Bush and Sykes (1986). The efficiency of hydrolysis of a particular substrate was determined from the ratio of Vmax/Km and the relative efficiency of hydrolysis of the enzyme was calculated as outlined by Sykes et al. (1981), as follows;

$$\text{Relative efficiency.} = \frac{\text{Efficiency of hydrolysis of substrate}}{\text{Efficiency of hydrolysis of ampicillin}} \times 100$$

2.11.2. Measuring beta-lactamase inhibition.

The ID_{50} value is defined as the amount of inhibitor required to reduce the hydrolytic activity of an enzyme by 50%. The ID_{50} values for the beta-lactamase enzymes were determined by spectrophotometric assay. Firstly, the rate of hydrolysis of nitrocefin (10^{-4}M) by the beta-lactamase was measured. This procedure was then performed in the presence of 10^{-8}M of the potential inhibitor and repeated with increasing concentrations of the inhibitor until inhibition approached 100%.

Percentage inhibition was then plotted against log concentration of inhibitor and the concentration of inhibitor which gave 50% inhibition was calculated from the graph.

2.11.3. Specific activities.

Protein concentrations were determined by the method of Waddell (1956) and specific activities were expressed as nmoles substrate hydrolysed per minute per mg of protein (nmoles/min/mg).

2.12. Isolation of plasmid DNA.

Overnight broth cultures (4.5ml) were harvested by centrifugation (4000rpm, 15 minutes, Christ Bactifuge). The method of Takahashi and Nagano (1984) was then followed to extract and visualise plasmid DNA.

2.12.2. Restriction of plasmid DNA by restriction endonuclease enzymes.

The method of Takahashi and Nagano (1984) was used to prepare plasmid DNA for restriction by endonucleases. For each restriction digest, 4.6ul of 10X concentrated restriction buffer (Boehringer Mannheim, SuRE/Cut buffers) was added to 40ul of the plasmid preparation followed by 5-10 units of the restricting enzyme (Boehringer Mannheim). The reactions were left for between 1 and 18 hours at 37^{o}C.
The amount of restricted plasmid sample electrophoresed depended on the size of the agarose gel used.

2.12.3. Agarose electrophoresis of plasmid DNA.

Horizontal slab gel electrophoresis was performed by the method of Meyers et al. (1976). Both large (14 by 25 X 0.5cm) and small (Bio-Rad Mini Sub Cell) horizontal slab gels were used for the electrophoresis of plasmid preparations. The gels consisted of between 0.5% and 1% agarose (Sigma) in Tris-acetate buffer (40mM Tris-acetate, 2mM disodium EDTA,

pH 7.9). Electrophoresis was performed with the gels submerged in Tris-acetate buffer at 70V overnight for the large gels and at 100V for 2 hours with the mini-gels. The gels were stained in a 0.75mg/L solution of ethidium bromide for 15 minutes and then briefly washed in water. The DNA was visualized over a long-wave ultra-violet light source (Ultra-violet Products Inc., Cambridge).

Before the analysis of each DNA sample, loading buffer was added to each preparation to create a ratio of loading buffer to sample of 1:4 respectively.

2.12.4. Sizing of DNA bands on agarose gels.

The sizes of restricted fragments were determined by running standard molecular weight markers in parallel with the samples. Lambda DNA restricted with Hind III (Gibco BRL) and ϕ X 174 RF DNA (Gibco BRL) restricted with Hae III were used for this purpose. The sizes of intact plasmids were determined by running plasmids of known sizes (table 7) along side the clinical plasmids of unknown size.

The distance travelled by DNA through the gel is inversely proportional to the logarithm of its molecular size. Consequently, the unknown sizes could be calculated from a graph of log molecular size against distance migrated by the plasmid.

3.0. RESULTS

3.1 Spontaneous selection of beta-lactamases which confer resistance to 3GCs.

The genes responsible for the transferable 3GC resistance discussed in the Introduction may have resulted from simple mutation of the common TEM beta-lactamase genes. In order to test this hypothesis, mutation experiments were performed. The mutation procedure (section 2.6) was used to investigate which of the strains in table 13 would yield 3GC hydrolysing beta-lactamases when subjected to the selective pressure of ceftazidime, and in some cases, cefotaxime. Both anaerobic and aerobic cultures were examined. In some experiments the broth cultures were grown in the presence of very low levels of ceftazidime, for long incubation periods, in an attempt to mimic the environment faced by a bacterium infecting a patient on ceftazidime therapy.

Table 13. Bacterial strains and growth conditions used to obtain mutant beta-lactamases (conditions for experiments 1-9 are listed on the following page).

Strain.	Beta-lactamase produced.	Experiment number (see following page)								
		1	2	3	4	5	6	7	8	9
E.coli J53-2 2136E	TEM-1	+	+	+	+a	+	-	-	+	+
E.coli J53-2 2137E	TEM-2	+	+	+	+a	+	+	-	+	-
E.coli J53-2 2141E	SHV-1	+	+	+	-	+	-	-	-	-
E.coli J53-2 2651E	TLE-1	+	+	+	-	-	-	-	-	-
E.coli J53-2 AR273	PSE-4	-	-	-	-	-	-	+a	+	-
E.coli J53-2 Nc	TEM-1	-	-	-	-	+a	-	-	-	-
E.coli 2505E	TEM-1 TLE-1	+	+b	+	-	+	-	-	-	-
K.pneumoniae 1957E	TEM-1 SHV-1	+	+	+	-	+	-	-	-	-
K.aerogenes 1976E	SHV-1	+	+	+	-	+	-	-	-	-

a = Denotes an experiment which yielded mutant beta-lactamases.

b = Culture grown in the presence of a range of ceftazidime concentrations (7.5×10^{-2} - 2.5×10^{-4} mg/L).

N.B. some of these experiments may have been performed more than once.

+ denotes that the strain was tested.
- denotes that the strain was not tested.

Experimental conditions discussed in table 13.

1. Anaerobic, 80 hours incubation, static, cultures grown in the presence of 0.001mg/L of ceftazidime. Mutants selected on ceftazidime (4mg/L).

2. Aerobic, 80 hours incubation, shaken, cultures grown in the presence of 0.001mg/L of ceftazidime. Mutants selected on ceftazidime (4mg/L).

3. Anaerobic, 70 hours incubation, static. Mutants selected on ceftazidime (8mg/L).

4. Aerobic, 24 hours incubation, shaken. Mutants selected on ceftazidime (2, 4 or 10mg/L).

5. Aerobic, 20 hours incubation, shaken. Mutants selected on ceftazidime (0.8, 4, 10mg/L).

6. Aerobic, 20 hours incubation, shaken. Mutants selected on ceftazidime (2mg/L).

7. Aerobic, 8 hours incubation, shaken. Mutants selected on ceftazidime (1mg/L).

8. Aerobic, 22 hours incubation, shaken. Mutants selected on cefotaxime (1mg/L).

9. Aerobic, 21 hours incubation, shaken. Mutants selected on cefotaxime (1 or 2mg/L) or cefuroxime (20 or 40mg/L)

As the vast majority of the beta-lactamases which conferred 3GC resistance had occurred in Klebsiella spp. both Klebsiella spp. and Escherichia coli strains, which produced the TEM-1 beta-lactamase, were examined for their ability to produce mutants. Many of the strains which produced a TEM derived 3GC hydrolysing beta-lactamase also manufactured SHV-1. Therefore, strain 1957E was investigated in order to identify whether SHV-1 exhibited any synergistic effect on the mutation capabilities of the TEM-1 beta-lactamase to a 3GC hydrolysing enzyme. Both TEM-1 and TEM-2 beta-lactamase producing strains were investigated because the origin of the new TEM-like enzymes was not clear. A strain of E.coli producing TLE-1 was also studied, as TLE-1 has been shown to be closely related to the TEM resistance gene (Levesque et al.,1987). The strains producing SHV-1 were investigated to ascertain whether other 3GC hydrolysing SHV-derived beta-lactamases could be obtained. The E.coli producing PSE-4 was examined to establish whether the selective pressure of 3GCs could produce spontaneous changes in beta-lactamases other than the TEM or SHV-1 related beta-lactamases.

3.1.1 Mutant beta-lactamases obtained spontaneously from the TEM-1 produced by E.coli J62-2 Nc.

The particular growth conditions which gave rise to these mutants are shown in table 13.

a. Rationale for studying E.coli J62-2 Nc.

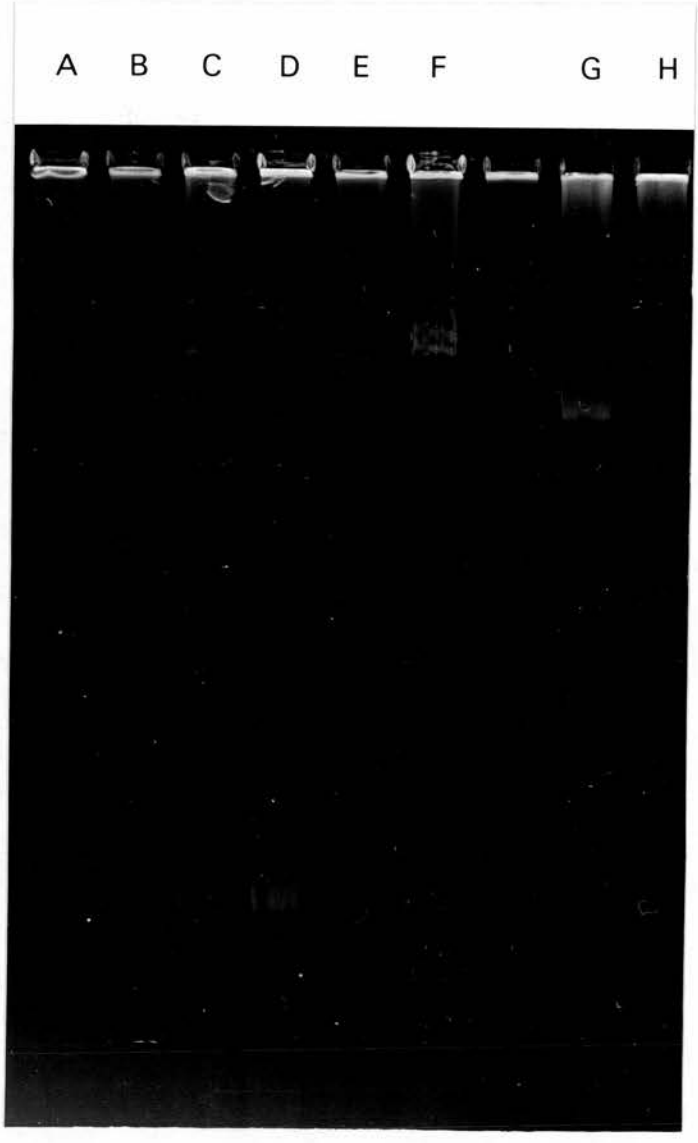
Klebsiella pneumoniae CF504 was a clinical strain isolated in

Clermont-Ferrand, France. It was resistant to ceftazidime and cefotaxime and the gene encoding this resistant determinant was located on a 150kb plasmid (pCFF14) (Petit et al, 1988). IEF studies showed that this strain also produced the TEM-1 beta-lactamase as well as the TEM-5 enzyme. This strain was conjugated with the rifampicin-resistant E.coli J62-2 and the transconjugants were selected on agar plates containing rifampicin and ceftazidime. The transconjugants were purified and their integrity checked. Analysis of the plasmid DNA revealed that the E.coli J62-2 transconjugant of Klebsiella pneumoniae CF504 had lost the 150Kb plasmid (pCFF14), which has been shown to encode the TEM-5 beta-lactamase (Petit et al, 1988), and possessed only a 100Kb plasmid along with two smaller plasmids (fig. 5). This transconjugant reverted to the normal characteristics of a TEM-1 producing E.coli J62-2 strain. This strain (denoted as E.coli Nc) was then used as the parent in the spontaneous mutation experiments. The rationale for this was that there should be a higher chance of obtaining a 3GC hydrolysing mutant enzyme from a plasmid encoded TEM-1 beta-lactamase gene found in a strain which was host to a TEM-like 3GC-hydrolysing beta-lactamase, rather than a standard laboratory strain hosting a TEM-1 beta-lactamase gene.

b. Selection of Mutants.

Overnight broth cultures were plated onto ceftazidime-containing plates. The ceftazidime resistant mutants grew on plates containing 0.8 and 4mg/L of ceftazidime. These mutants appeared at a frequency of 1 in 10^9, and following purification the strains were examined for any change in

Fig. 5. Plasmid profiles of strains producing Mutant beta-lactamases A or B along with their E.coli J53-2 transconjugants and the original transconjugants of K.pneumoniae CF504 (0.7% agarose) .



A. E.coli J53-2 transconjugant producing Mutant beta-lactamase B; B. E.coli J53-2 transconjugant producing Mutant beta-lactamase A; C. Original mutant E.coli J62-2 producing Mutant beta-lactamase A; D. Original mutant E.coli J62-2 producing Mutant beta-lactamase B; E. E.coli J62-2 Nc, transconjugant of K.pneumoniae CF504 (ceftazidime sensitive, wild type strain used in mutation experiments); F. E.coli J62-2 Nb, transconjugant of K.pneumoniae CF504 (ceftazidime resistant); G. R6K; H. R1818.

beta-lactamase profile. In all the colonies studied, ceftazidime resistance was associated with the mutation of the TEM-1 beta-lactamase gene to produce one of two different beta-lactamases (Mutant beta-lactamase A and Mutant beta-lactamase B). Twenty mutant colonies were examined and the beta-lactamases which they produced were characterised. The ratio of the occurrence of Mutant beta-lactamase A to Mutant beta-lactamase B was 4:1. The genes encoding the mutant beta-lactamases were transferred by conjugation into E.coli J53-2 with selection of the transconjugants on ceftazidime containing agar plates (table 14). The acquisition of the plasmids elevated the MIC of ceftazidime for the E.coli J53-2 recipient more than 60 fold from 0.13 to 8-16mg/L (tables 22 and 27). The transferability of the resistance determinant confirmed that the mutation had occurred in the plasmid encoded genes and not in the chromosome. Both the original mutants and their E.coli J53-2 transconjugants possessed the 100Kb plasmid, but the two smaller plasmid bands were seen only in the original mutant strains (fig. 5).

c. Isoelectric Focusing.

Mutant beta-lactamase A focused as a doublet band at pI 5.3 with an additional weak TEM-1 enzyme band. Mutant beta-lactamase B had a pI marginally above the TEM-1 enzyme but below the TEM-7 beta-lactamase (fig. 10).

Table 14. Transferability of mutant beta-lactamases A and B to E.coli J53-2.

Wild type strain.	Mutant B-lactamase.	pI	Mutation frequency.	Transfer frequency per donor[+].
E.coli Nc	A	5.30[*]	$1.0X10^{-9}$	$1.6X10^{-7}$
E.coli Nc	B	5.41	$1.0X10^{-9}$	$5.4X10^{-8}$

[+]Ceftazidime (4mg/L) used as the selecting agent in minimal media agar containing proline and methionine.

[*]Doublet band

d. Minimum Inhibitory Concentrations.

E.coli J53-2 transconjugants expressing the mutant enzymes were all resistant to ampicillin, carbenicillin, cephaloridine, ceftazidime, and aztreonam. However, they were sensitive to all other cephalosporins tested including cefotaxime (tables 22 and 27). Clavulanic acid and sulbactam both overcame the resistance to a similar extent when ceftazidime was used as the principal antibiotic. However, clavulanic acid was seen to be the slightly more efficient beta-lactamase inhibitor when ampicillin was the principle antibiotic (tables 22 and 27).

e. Biochemical Profiles of the Mutant beta-lactamases A and B.

The two mutant enzymes had molecular weights similar to the TEM-1 beta-lactamase (table 25). The substrate profiles of Mutant beta-lactamases A and B are compared to TEM-1 in table 23. The profiles were similar to TEM-1 for ampicillin, carbenicillin and cephaloridine. However, both mutant enzymes showed a low, but significant, rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed no hydrolysis of either of these substrates. The relative Vmax and the Km values for these enzymes against a number of beta-lactams were determined. These values were combined to give the relative efficiency of hydrolysis values (rel Vmax/Km, ampicillin = 100%) which takes into account

both the binding and the hydrolysis capabilities of the enzyme with a particular beta-lactam. Mutant beta-lactamase B had similar efficiency of hydrolysis values for ceftazidime and cefotaxime and Mutant beta-lactamase A hydrolysed cefotaxime more efficiently than ceftazidime (table 24). This is a paradox because both enzymes confer a far greater resistance to ceftazidime. Both enzymes were inhibited by clavulanic acid with ID_{50}s similar to TEM-1 (table 26).

3.1.2. Mutant beta-lactamase selected from the TEM-1 produced by E.coli 2136E.

Only one ceftazidime resistant colony was obtained from the experiments conducted with this strain. The conditions which yielded the strain which produced this mutant beta-lactamase are shown in table 13. The strain was selected on a plate containing 2mg/L of ceftazidime (mutation frequency 1in 10^9). The MIC of ceftazidime for the sensitive strain used in these tests was 0.06mg/L, the increase in MIC of the mutant strain to 4mg/L was demonstrated to be caused by the production of Mutant beta-lactamase B. No other mutant beta-lactamases were obtained from organisms producing TEM-1.

3.1.3. Mutant beta-lactamases selected from E.coli 2137E.

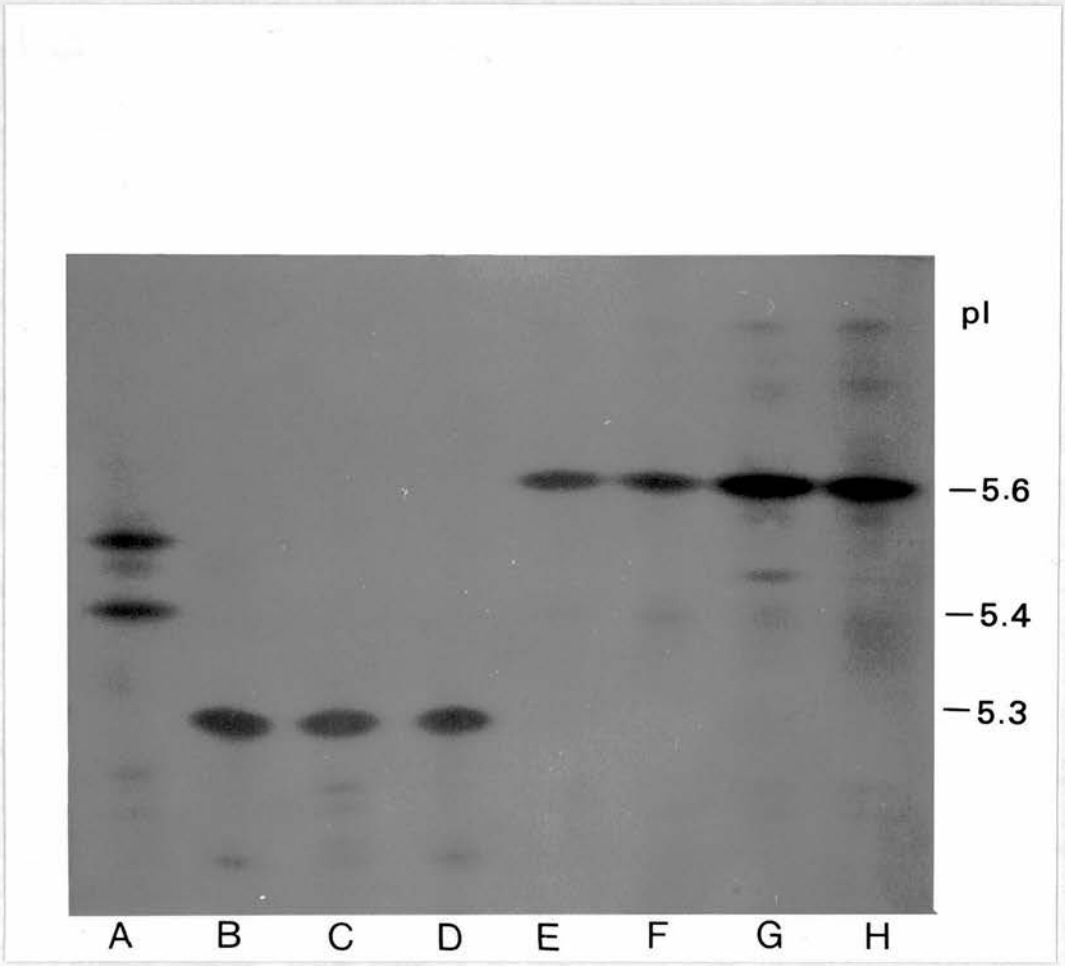
The strains producing these mutant beta-lactamases were identified on plates containing 4 or 2 mg/L of ceftazidime. The conditions which gave rise to these mutant beta-lactamases are shown in table 13. In these experiments two types of spontaneous mutants were identified. The first type was produced at a mutation frequency of 1 in 5×10^8 and produced

Mutant beta-lactamase C which had the same pI as TEM-2 (fig. 6). The strains producing Mutant beta-lactamase C were approximately 60 times more resistant to ceftazidime than the original sensitive strain and more than 8 times more resistant to aztreonam. This mutant strain became sensitive to ceftazidime in the presence of clavulanic acid (2mg/L) or sulbactam (2mg/L) (table 15) which strongly suggested that the ceftazidime resistance was conferred by Mutant beta-lactamase C.

Attempts to transfer this ceftazidime resistance were performed with kanamycin or ceftazidime as the selecting agents. Transferable resistance to kanamycin and ceftazidime could be demonstrated when ceftazidime was used as the selecting agent. However, the ceftazidime resistance could not be transferred to an E.coli J62-2 recipient when kanamycin was the selecting agent (table 16).

The second type of mutant beta-lactamase selected from the TEM-2 produced by E.coli 2137E was Mutant beta-lactamase D. Isoelectric focusing of this enzyme revealed that Mutant beta-lactamase D focused marginally above TEM-2 (fig. 19). The genes encoding for this beta-lactamase could be transferred to an E.coli K12 (SA 10, methionine deficient) recipient strain with a concomitant transfer of resistance to ceftazidime. The E.coli K12 (SA10) transconjugants selected on kanamycin or ceftazidime were all resistant to ceftazidime and produced Mutant beta-lactamase D (fig. 19). However, the frequency of transfer of ceftazidime resistance was 600 times greater with kanamycin as the selecting agent than when the selection was performed with ceftazidime (table 16). This phenomenon was studied in greater detail.

Fig. 6. IEF of Mutant beta-lactamase C and Mutant beta-lactamase E.



A. E.coli J53-2 producing TEM-1; B. Original ceftazidime resistant E.coli J53-2 mutant producing Mutant beta-lactamase E; C. Ceftazidime resistant E.coli J62-2 transconjugant of original E.coli J53-2 mutant selected on kanamycin; D. E.coli J53-2 (AR273) producing PSE-4, used as the sensitive strain in mutation experiments; E. E.coli J53-2 (2137E) producing TEM-2, used as the sensitive strain in mutation experiments; F. Original ceftazidime resistant E.coli J53-2 mutant producing Mutant beta-lactamase C; G. E.coli J62-2 transconjugant of original E.coli J53-2 mutant selected on kanamycin (ceftazidime sensitive); H. E.coli J62-2 transconjugant of original E.coli J53-2 mutant selected on ceftazidime.

Table 15. MICs conferred by Mutant beta-lactamase C and the mutant beta-lactamases produced from K.pneumoniae 1957E (10^{-5} inoculum).

	Minimum Inhibitory Concentrations (mg/L)				
Beta-lactam antibiotic.	E.coli J53-2 (TEM-2)	E.coli J53-2 producing β-lactamase C	Klebsiella 1957E	Klebsiella 1957E Mutant 3a	Klebsiella 1957E Mutant 5a
Ampicillin	>250	>250	>250	>250	>250
A +clav	2	4	ND	ND	ND
B +sulb	>250	>250	ND	ND	ND
Carbenicillin	>250	>250	>250	>250	>250
Cephaloridine	16	32	64	250	32
Cephalexin	1	8	8	16	8
Cefoxitin	0.5	4	4	4	2
Cefuroxime	1	2	4	8	4
Ceftazidime	<0.06	4	0.25	4	8
A +clav	<0.06	0.13	ND	ND	ND
B +sulb	<0.06	0.5	ND	ND	ND
Cefotaxime	<0.06	<0.06	<0.06	0.13	0.13
Ceftriaxone	<0.06	<0.06	<0.06	0.13	0.13
Aztreonam	<0.06	0.5	<0.06	0.25	0.25
imipenem	0.13	<0.06	0.13	<0.06	0.13

A +clav = in combination with 2mg/L clavulanic acid.

B +sulb = in combination with 2mg/L sulbactam. ND = Not done.

Table 16. Transferability of Mutant beta-lactamases.

Wild type strain.	Mutant Beta-lactamase.	pI	Mutation frequency.	Transfer frequencies to E.coli recipient*.	
				CAZ-1 Rf-25	Km-40 Rf-25
E.coli J53-2 2137E (TEM-2).	C	5.60	$5X10^{-8}$	$8.0X10^{-9}$	$^{+}1.5x10^{-4}$
E.coli J53-2 2137E (TEM-2)	D	5.61	$1X10^{-9}$	$5.2X10^{-9}$	$3.5X10^{-6}$
E.coli J53-2 AR273 (PSE-4).	E	5.30	$1X10^{-7}$	$1.1X10^{-7}$	$2.2X10^{-3}$

*no transconjugants were detected when kanamycin and ceftazidime were used in combination as the selecting agents.

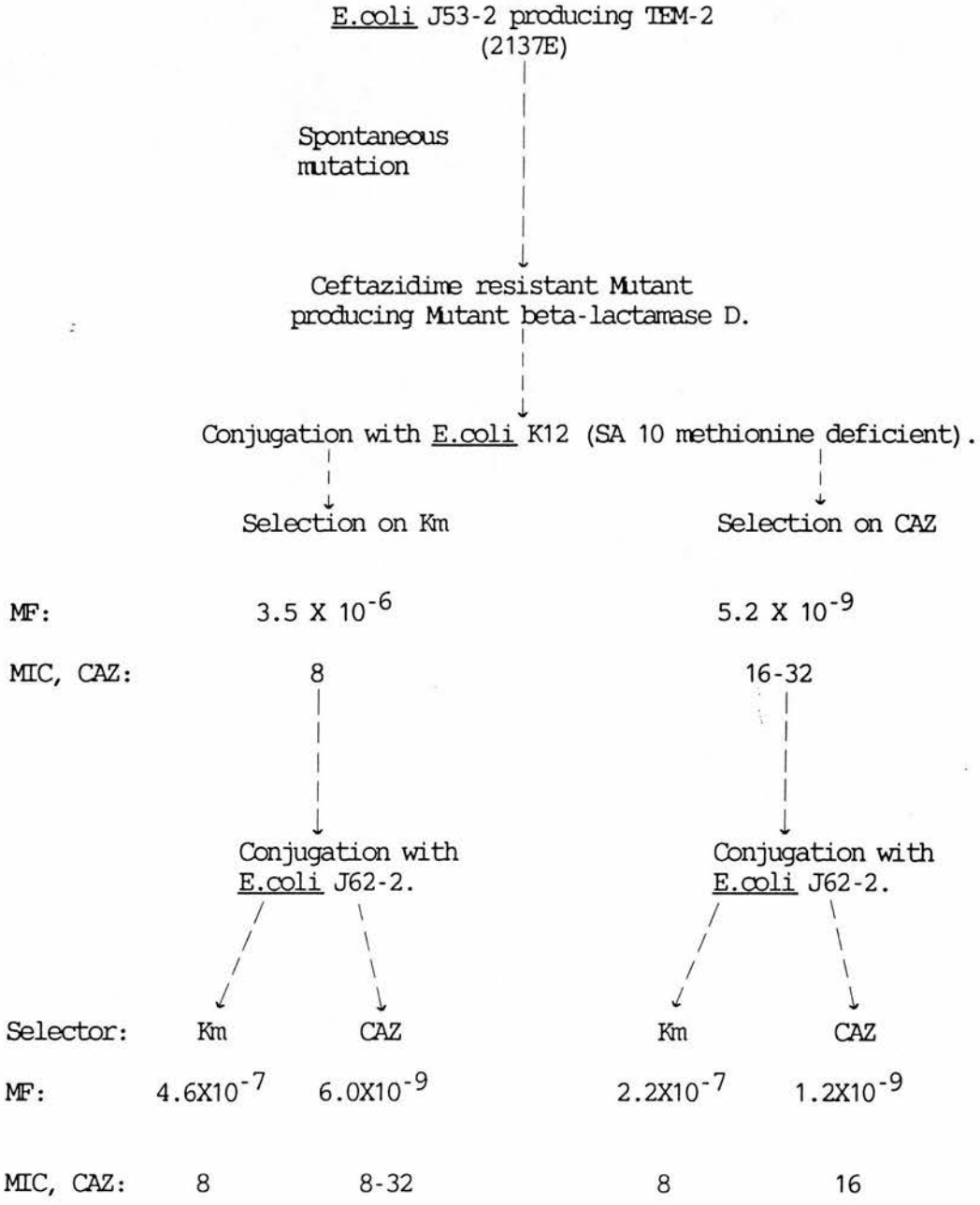
+transconjugants were sensitive to ceftazidime.

CAZ-1 = 1mg/L of ceftazidime added to the nutrient agar to select for transconjugants.

Km-40 = 40mg/L of kanamycin added to the nutrient agar to select for transconjugants.

Rf-25 = 25mg/L rifampicin added to the nutrient agar to select for transconjugants.

Fig. 7. Conjugation experiments involving the transfer of the Mutant beta-lactamase D gene.



Km = Kanamycin
CAZ = Ceftazidime
MF = Mating frequency.
MIC, CAZ = Minimum inhibitory concentration of ceftazidime in mg/L (10^5 inoculum).

The E.coli K12 (SA10) transconjugants selected on kanamycin were then conjugated with E.coli J62-2 and the transconjugants were selected on agar containing the appropriate nutrients and either ceftazidime or kanamycin. Once again much lower mating frequencies were recorded when the E.coli J62-2 transconjugants were selected on ceftazidime. This procedure was repeated for the E.coli K12 (SA10) transconjugants selected on ceftazidime, and again the number of transconjugants selected on kanamycin was far greater than the number selected on ceftazidime (fig. 7).

a. Resistances conferred by Mutant beta-lactamase D.

An E.coli J53-2 strain producing Mutant beta-lactamase D was sensitive to cefotaxime and aztreonam but resistant to ceftazidime. This strain was sensitive to ceftazidime in the presence of clavulanic acid (2mg/L) demonstrating that the ceftazidime resistance was mediated by Mutant beta-lactamase D (table 33).

b. Biochemical characterisation of Mutant beta-lactamase D.

Mutant beta-lactamase D hydrolysed cefotaxime and ceftazidime at low, but significant rates (table 34). However, Mutant beta-lactamase D had much higher affinity for cefotaxime than ceftazidime (table 35). Table 36 shows that Mutant beta-lactamase D hydrolyses cefotaxime more efficiently than ceftazidime, although the enzyme conferred far greater resistance to ceftazidime. The molecular weight of Mutant beta-lactamase D was estimated

to be similar to that of TEM-1 (table 25).

c. Plasmid analysis.

Mutant beta-lactamase D was encoded by a plasmid of 52Kb, which was identical to the size of the RP4 plasmid which encoded TEM-2 in the original strain (fig. 18). EcoR I digests of each of these plasmids yielded a linear restriction fragment of 52Kb (fig. 21), and demonstrated that they were indeed the same plasmid.

3.1.4. Mutant beta-lactamase obtained from E.coli J53-2 DP2 (PSE-4).

The conditions which led to the production of these mutants are illustrated in table 13. The spontaneous ceftazidime resistant mutants were obtained at a frequency of 1 in 10^8. The ceftazidime resistance, exhibited by these strains, was transferable to an E.coli J62-2 recipient, and ceftazidime resistant transconjugants could be selected with either kanamycin or ceftazidime. However, the frequency of transfer was far greater when kanamycin was the adopted selection agent (table 16). Both the original mutant strain and the E.coli J62-2 transconjugant produced Mutant beta-lactamase E which aligned directly with the PSE-4 enzyme produced by the original strain (fig. 6).

a. Resistances conferred by Mutant beta-lactamase E.

The E.coli J53-2 and the E.coli J62-2 transconjugant strain, which both produced Mutant beta-lactamase E, were approximately 60 times more resistant to ceftazidime than the sensitive strain. These strains were sensitive to ceftazidime and ampicillin in the presence of clavulanic acid (2mg/L) or sulbactam (2mg/L), illustrating that the ceftazidime resistance was beta-lactamase mediated (table 17).

b. Biochemical characterisation of Mutant beta-lactamase E.

The hydrolytic activities of Mutant beta-lactamase E and PSE-4 were determined for a range of beta-lactam substrates. The Vmax, Km and Vmax/Km values for these substrates were similar for both the PSE-4 and Mutant beta-lactamase E. Neither beta-lactamase had any measurable hydrolytic activity against cephradine, cefuroxime, cefotaxime, or ceftazidime (table 18). Their ID_{50} values for clavulanic acid were also similar (table 26) and high concentrations of ceftazidime (10^{-3} M) had no inhibitory effect on the ability of either beta-lactamase to hydrolyse nitrocefin. Table 25 shows that there was no significant difference between the molecular weights of Mutant beta-lactamase E and PSE-4.

Table 17. MICs conferred by Mutant beta-lactamase E (10^{-5} inoculum).

Beta-lactam antibiotic.	Minimum Inhibitory Concentrations (mg/L)			
	E.coli J53-2 (AR273).	*E.coli* J53-2 producing β-lactamase E. (a)	*E.coli* J62-2 transconjugant of (a).	*E.coli* J62-2
Ampicillin	>250	>250	>250	4
A +clav	8	8	ND	2
B +sulb	16	32	ND	4
Carbenicillin	>250	>250	>250	4
Cephaloridine	4	4	4	2
Cephalexin	4	4	8	2
Cefoxitin	2	4	8	2
Cefuroxime	4	4	8	2
Ceftazidime	0.13	8	8	<0.06
A +clav	<0.06	0.5	ND	0.13
B +sulb	<0.06	<0.06	ND	<0.06
Cefotaxime	<0.06	<0.06	<0.06	<0.06
Ceftriaxone	<0.06	<0.06	0.13	<0.06
Aztreonam	<0.06	0.13	0.25	<0.06
Imipenem	0.06	0.25	0.25	0.13

A +clav = in combination with 2mg/L clavulanic acid

B +sulb = in combination with 2mg/L sulbactam

ND = Not done.

Table 18. Kinetic parameters of PSE-4 and Mutant beta-lactamase E.

Beta-lactamase.	Beta-lactam substrate AMP	PEN	CARB	CER	CED	CXM	CAZ	CTX
*Vmax								
PSE-4	100	106	124	27	UM	UM	UM	UM
Enzyme E	100	111	186	28	UM	UM	UM	UM
#Km								
PSE-4	200	166	263	400	UM	UM	UM	UM
Enzyme E	167	167	313	333	UM	UM	UM	UM
*Rel Vmax/Km								
PSE-4	100	128	94	14	UM	UM	UM	UM
Enzyme E	100	111	99	14	UM	UM	UM	UM

*Values expressed as a percentage of the value for ampicillin.

#Km Values in uM.

UM = Unmeasurable because hydrolysis of substrate too low.

AMP = ampicillin; PEN = penicillin; CARB = carbenicillin; CER = cephaloridine; CED = cephradine; CXM = cefuroxime; CAZ = ceftazidime CTX = cefotaxime.

3.2. Investigation of the effects of methotrexate or metronidazole on the production of mutant beta-lactamases.

The strain expressing TEM-9 had been isolated from a patient undergoing anti-leukaemic and metronidazole therapy. Consequently, the ability of methotrexate and metronidazole to mutate beta-lactamases to enzymes which confer resistance to ceftazidime was investigated.

Metronidazole is only activated in the absence of oxygen, therefore, both anaerobic and aerobic cultures were examined. Nevertheless, only one of the experiments shown in table 19 produced stable ceftazidime resistant mutants.

3.2.1. Mutant beta-lactamases produced by K.pneumoniae 1957E.

When K.pneumoniae 1957E was grown anaerobically in the presence of metronidazole (table 19), ceftazidime resistant mutants were selected which possessed slightly different beta-lactamase profiles from the original strain (fig. 8). As similar numbers of mutants were also selected from control cultures, containing no metronidazole, it was assumed that metronidazole did not have any significant effect on the mutation of the beta-lactamases produced by strain 1957E.

Table 19. Culture conditions implemented to study the effects of methotrexate and metronidazole on the selection of mutant beta-lactamases with altered substrate profiles.

Potential mutagen used.	Concentrations of potential mutagen tested (mg/L).	Incubation conditions.	Concentration of ceftazidime used to select mutants. (mg/L).	Strains tested.
Metronidazole (a)	0, 64, 125, 250, 1000	Anaerobic, 24 and 43 hours.	2 or 4	2136E (TEM-1)
Metronidazole	0, 64, 125, 250, 1000	Anaerobic, 24 hours.	2 or 4	1957E (SHV-1, TEM-1) 1976E (SHV-1) 2137E (TEM-2)
Metronidazole	0, 0.1, 0.5, 1, 10	Anaerobic, 45 hours.	10	(c)1957E (SHV-1, TEM-1) 2137E (TEM-2) 2505E (TEM-1, TLE-1) 175 (SHV-1,TLE-2, TEM-1)
Metronidazole	0, 64, 125, 500, 1000 (b)	Anaerobic, 68 hours.	4	1957E (SHV-1, TEM-1) 2505E (TEM-1, TLE-1)
Metronidazole (a)	0, 64, 125, 500, 1000	Aerobic, 24 hours.	8	2136E (TEM-1)
Methotrexate (a)	0, 64, 125, 500, 1000	Anaerobic 24 and 43 hours.	8 or 4	2136E (TEM-1)
Methotrexate (a)	0, 64, 125, 500, 1000	Aerobic 24 hours.	8	2136E (TEM-1)

See following page for (a), (b) and (c) foot notes.

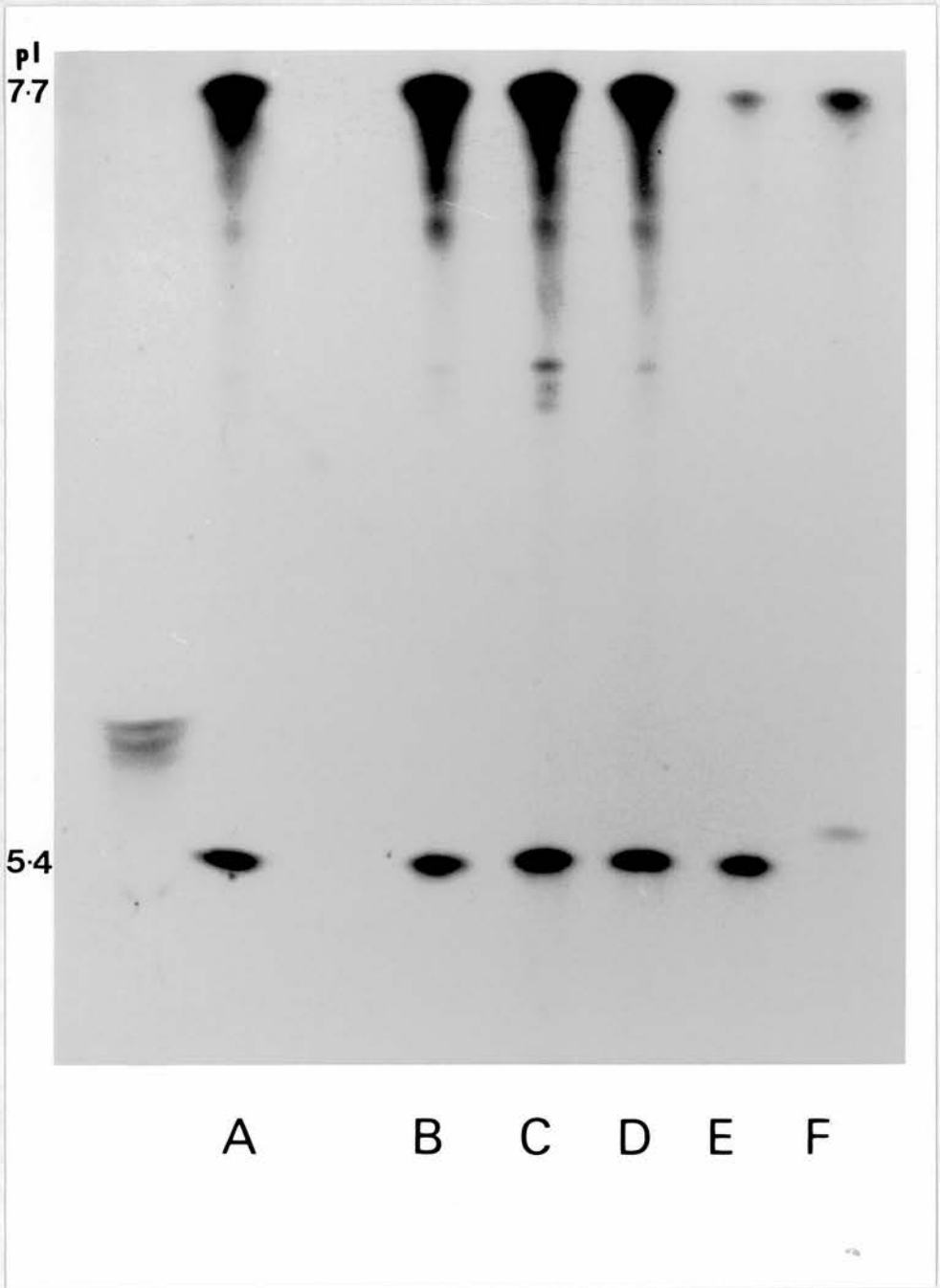
From table 19 (previous page).

(a) These experiments were first conducted by adding the potential mutagen at the same time as the bacterial inoculum. They were then repeated adding the potential mutagen two hours after the bacterial inoculum (in all other experiments the potential mutagen was added at the same time as the bacterial inoculum).

(b) All the cultures investigated in these experiments also contained 25mg/L methotrexate.

(c) Strains which yielded mutant beta-lactamases under these conditions (see text for details).

Fig. 8. Isoelectric focusing of mutant beta-lactamases obtained from K.pneumoniae 1957E.



A.; B.; C.; D.; Mutant beta-lactamases produced by ceftazidime resistant type 3a mutants obtained spontaneously from K.pneumoniae 1957E; E. Beta-lactamases produced by K.pneumoniae 1957E, original strain used in mutation experiments; F. Mutant beta-lactamases produced by ceftazidime resistant 5a mutant obtained spontaneously from K.pneumoniae 1957E.

K.pneumoniae mutant 5a was characteristic of one of the two types of mutants obtained in this experiment (mutation frequency 1 in 10^9). It was 32 times more resistant to ceftazidime than the original K.pneumoniae 1957E (table 15), but transfer of this resistance could not be achieved. The 5a mutant strains produced a beta-lactamase of similar pI to Mutant beta-lactamase B (fig. 8).

The other type of ceftazidime resistant mutant, K.pneumoniae mutant 3a, was obtained at approximately the same frequency. This mutant was 16 times more resistant to ceftazidime than the original 1957E strain (table 15), and the ceftazidime resistance could not be transferred. When the beta-lactamase profiles of the sensitive strain and mutant 3a were compared by isoelectric focusing, it was apparent that both strains produced a TEM-1 band of equal intensity. However, the mutant 3a strain produced a far larger SHV-1 band. This might be explained by the induction effect of ceftazidime on the production of SHV-1.

3.3. Selection of enhanced 3GC resistance from beta-lactamases already conferring 3GC resistance.

Experiments were performed to examine whether it was possible to spontaneously select for a beta-lactamase conferring a broad spectrum of 3GC resistance from an enzyme already mediating a narrow or low degree of 3GC resistance.

3.3.1. Selection of enhanced ceftazidime resistance from Mutant beta-lactamase E.

Mutant beta-lactamase E confers only low level resistance to ceftazidime, therefore, these experiments attempted to mutate the Mutant beta-lactamase E gene to produce an enzyme which would confer greater ceftazidime resistance. As described previously, aliquots of an overnight culture of E.coli J53-2 producing Mutant beta-lactamase E were plated onto nutrient agar plates containing ceftazidime (64 or 125mg/L). However, no ceftazidime resistant colonies were detected.

3.3.2. Selection of cefotaxime resistance from TEM-E1.

TEM-E1 confers resistance to ceftazidime but not to cefotaxime (see section 3.4.1). Therefore, aliquots of an overnight culture of E.coli J53-2 producing TEM-E1 were plated onto agar containing cefotaxime (8mg/L). However, no cefotaxime resistant colonies were detected.

3.4. Plasmid mediated beta-lactamases identified in clinical isolates which confer resistance to 3GCs.

3.4.1. Beta-lactamase TEM-E1.

This beta-lactamase was produced by the ceftazidime resistant E.coli 7891 which was isolated in Belgium in 1987 (table 20). This enzyme was designated TEM-E1, because it had TEM-like qualities and it was characterised in Edinburgh.

a. Conjugation experiments.

The beta-lactam resistances expressed by E.coli 7891 were transferred to an E.coli J53-2 recipient with ceftazidime as the selecting agent (table 21). Both the original clinical isolate and the transconjugant possessed two plasmid DNA bands (sizes 31Kb and 3Kb) and TEM-E1 was believed to be located on the 31Kb plasmid which was designated pUK720.

b. Antibiotic sensitivities.

The MIC data (table 22) shows that both the clinical strain and the E.coli J53-2 transconjugant were ceftazidime resistant. The strains expressing the novel enzyme were seen to be sensitive to ceftazidime in the presence of clavulanic acid (2mg/L) or sulbactam (2mg/L). Clavulanic acid was the more efficient inhibitor when used in combination with ampicillin, but the

Table 20. Isolation details on the original clinical isolates expressing beta-lactamases which confer transferable resistance to 3GCs.

Beta-lactamase.	pI.	Clinical isolate.	Site of isolation.	Country of isolation.	Year of isolation.	Drug therapy of patient.	Plasmid encoding β-lactamase.
TEM-E1	5.41	E.coli 7891	Urine	Belgium	1987	No ceftazidime	pUK720
TEM-E2	5.30	K.oxytoca	Blood and CSF.	UK	1982	NS	pUK721
TEM-E3 (NMH)	5.55	E.coli 8001	Urine and Stool.	UK	1987	Ampicillin Cephalexin Cefuroxime Gentamicin Metronidazole	pUK722
TEM-E3 (RMH)	5.55	Ent. cloacae 7923	NS	UK	1987	NS	pUK723
TEM-E4	5.61	S.marcescens 7919	NS	Belgium	1987	NS	pUK724
DJP-1	7.90	K.pneumoniae 8825	Lymphoma patient	India	1989	NS	pUK725
BIL-1	8.80	E.coli	Raw swabs, tissue biopsy.	Pakistan	1989	Amikacin Cefotaxime	No name designated.

NS = not specified
NMH = North Middlesex Hospital
RMH = Royal Marsden Hospital

Table 21. Transfer of clinically derived plasmid mediated beta-lactamases into E.coli J53-2.

Donor strain.	Beta-lactamase.	pI.	Selectors (mg/L).	Transfer frequency into E.coli J53-2.
E.coli 7891	TEM-E1	5.41	Rf-50, CAZ-4	$3.9X10^{-6}$
K.pneumoniae 5445	TEM-E2	5.30	Rf-200, CAZ-4	$5.0X10^{-6}$
			Rf-200, AMP-20	$3.8X10^{-6}$
			Rf-200, AMP-20, CAZ-4	$6.3X10^{-7}$
E.coli 8001	TEM-E3(NMH)	5.55	Rf-50, CAZ-1	$7.4X10^{-7}$
			Rf-50, CARB-100	$4.0X10^{-7}$
Ent. cloacae 7923	TEM-E3(RMH)	5.55	Rf-25, CAZ-4	$9.2X10^{-4}$
S.marcescens 7919	TEM-E4	5.61	Rf-25, CAZ-4	$1.3X10^{-4}$

Rf = Rifampicin.
CAZ = Ceftazidime.
AMP = Ampicillin.
CARB = Carbenicillin.

Table 22. MICs of E.coli strains harbouring Mutant beta-lactamase B and beta-lactamase TEM-E1

β-lactam (mg/L) antibiotic	Minimum Inhibitory Concentrations				
	Mutant B			Controls	
	J62-2 mutant	J53 trans.	*7891 xJ53	J62-2	J53-2
Ampicillin	>250	>250	>250	4	4
[1] + clav	ND	2	2	ND	2
[2] + sulb	ND	4	8	ND	4
Carbenicillin	>250	>250	>250	4	8
Cephaloridine	32	16	32	2	4
Cephalexin	4	4	8	4	4
Cefoxitin	1	1	1	2	2
Cefuroxime	2	1	2	2	2
Ceftazidime	4	8	32	<0.06	<0.13
[1] + clav	ND	0.06	0.25	ND	0.13
[2] + sulb	ND	0.13	0.25	ND	<0.06
Cefotaxime	<0.06	<0.06	0.13	<0.06	<0.06
[1] + clav	ND	<0.06	<0.06	ND	0.06
[2] + sulb	ND	<0.06	<0.06	ND	<0.06
Ceftriaxone	<0.06	<0.06	0.13	<0.06	<0.06
Aztreonam	0.13	0.5	0.5	<0.06	0.13
Imipenem	0.13	0.5	0.5	0.13	0.25
Augmentin	16	16	16	8	8

1 + clav = in combination with 2mg/L clavulanic acid

2 + sulb = in combination with 2mg/L sulbactam

* = produces TEM-E1. ND = not done

two inhibitors seemed to be equally effective when 3GCs were used as substrates. A comparison of the resistance profiles conferred by TEM-E1 and Mutant beta-lactamase B revealed distinct similarities (table 22).

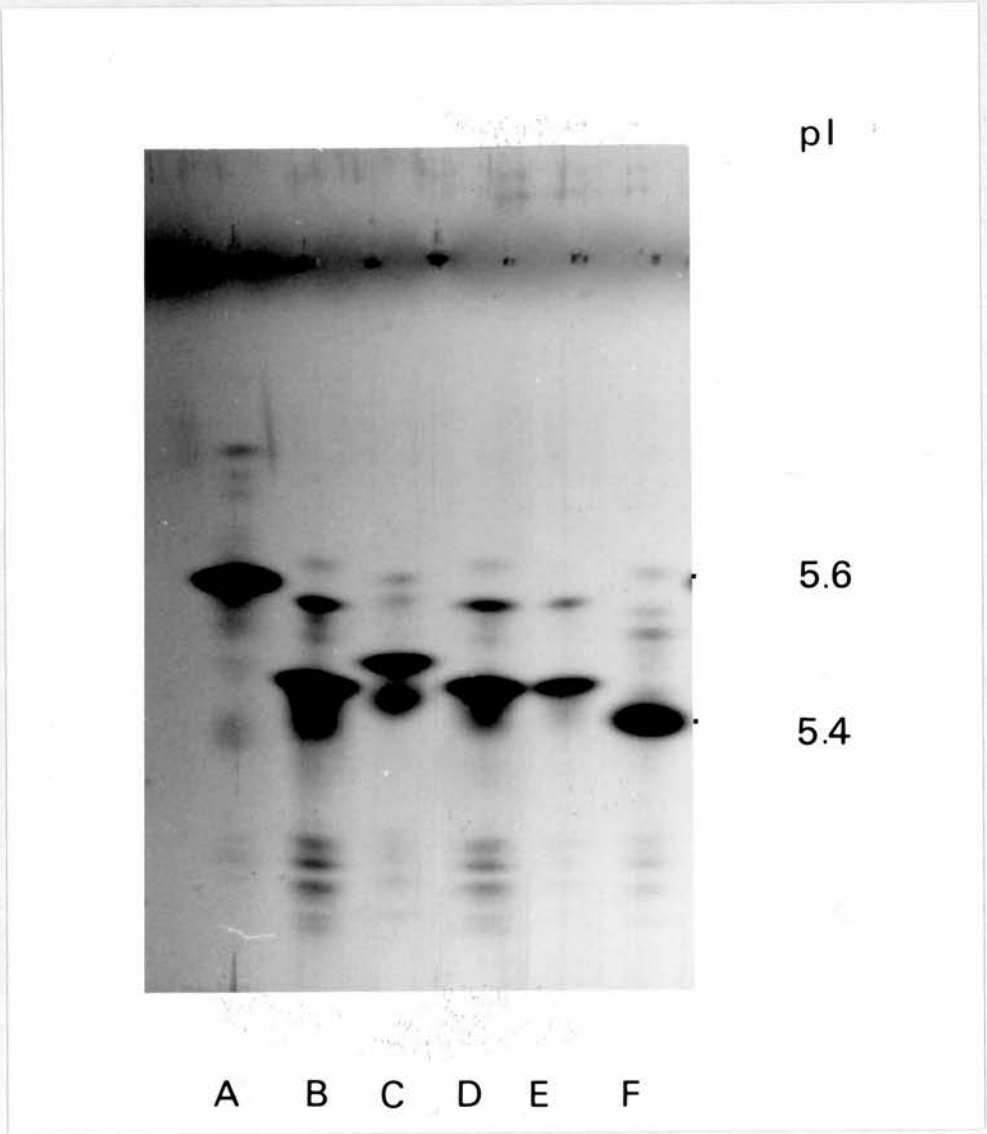
c. Isoelectric focusing.

Isoelectric focusing revealed both the E.coli J53-2 transconjugant and E.coli 7891 produced a beta-lactamase which focused between TEM-7 (pI 5.41) and TEM-1 (pI 5.4) (fig. 9). Further isoelectric focusing analysis revealed that TEM-E1 aligned directly with Mutant beta-lactamase B (fig. 10).

d. Biochemical characterisation.

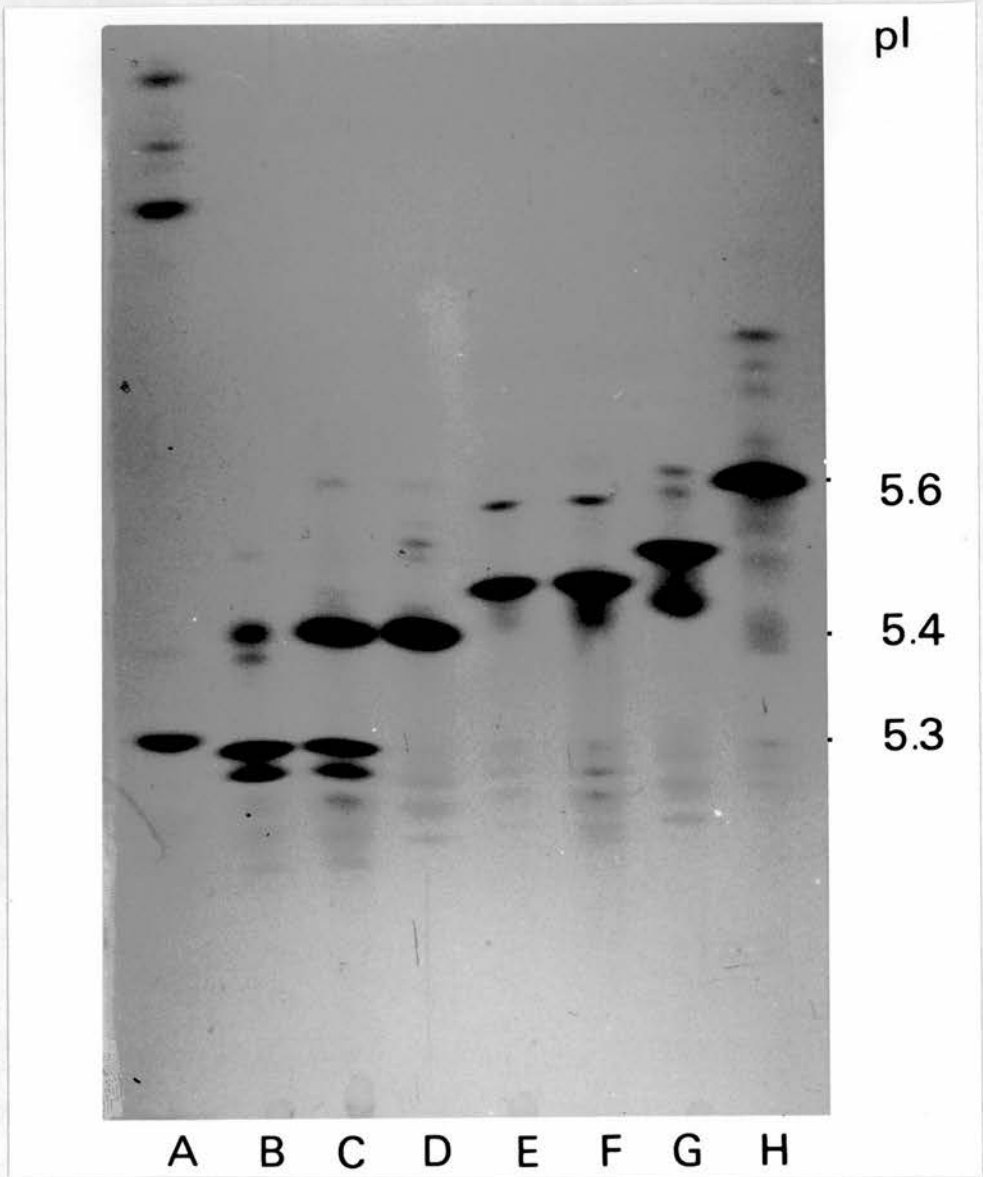
The substrate profiles of the TEM-1, TEM-E1, and Mutant beta-lactamase B are shown in table 23. The profiles are similar for carbenicillin, ampicillin, and cefuroxime. However, the TEM-E1 and Mutant beta-lactamase B showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed only a minute hydrolysis of either of these substrates. The relative efficiency of hydrolysis (Vmax/Km, ampicillin = 100%) values for TEM-1, TEM-E1 and Mutant beta-lactamase B for five beta-lactam substrates are shown in table 24. TEM-E1 hydrolysed ceftazidime more efficiently than cefotaxime, and Mutant beta-lactamase B had similar efficiency of hydrolysis values for both of these substrates. However, both these enzymes conferred a far greater resistance to ceftazidime than to cefotaxime.

Fig. 9. Isoelectric focusing pattern of the plasmid encoded beta-lactamase derived from strain 7891, compared with the enzymes TEM-1, TEM-2 and TEM-7 over a narrow pH range.



A. E.coli J53-2 producing TEM-2; B. E.coli J53-2 transconjugant of E.coli 7891 producing TEM-E1; C. E.coli BM694 producing TEM-7; D. as B; E. E.coli 7891 producing TEM-E1; F. E.coli J53-2 producing TEM-1.

Fig. 10. Isoelectric focusing patterns of mutant enzymes and beta-lactamases from clinical isolates over a narrow pH range.



A. E.coli J53-2 producing PSE-4; B. E.coli J53-2 producing Mutant beta-lactamase A; C. E.coli J53-2 producing TEM-E2 and TEM-1; D. E.coli J53-2 producing TEM-1; E. E.coli J53-2 producing Mutant beta-lactamase B; F. E.coli J53-2 producing TEM-E1; G. E.coli J53-2 producing TEM-7; H. E.coli J53-2 producing TEM-2.

Table 23. *Relative rates of hydrolysis of beta-lactamases TEM-E1, TEM-E2 and the Mutant enzymes A and B.

	Beta-lactamases.				
Beta-lactam antibiotic.	TEM-E1	Enzyme B	TEM-E2	Enzyme A	TEM-1
Ampicillin	100.0	100.0	100.0	100.0	100.0
Carbenicillin	29.8	17.4	19.3	26.3	10.9
Cephaloridine	42.1	68.0	41.6	38.2	18.0
Cefuroxime	0.25	0.55	0.22	0.52	0.014
Ceftazidime	0.31	1.11	0.23	0.92	0.017
Cefotaxime	1.48	1.36	0.58	2.27	0.014

*Values expressed as a percentage of the value for ampicillin.

Table 24 *Relative efficiency values for TEM-E1, TEM-E2, and the Mutant enzymes A and B.

Beta-lactam antibiotic.	Beta-lactamases.				
	TEM-E1	Enzyme B	TEM-E2	Enzyme A	TEM-1
Ampicillin	100.0	100.0	100.0	100.0	100.0
Carbenicillin	18.0	7.0	9.0	16.0	18.0
Cephaloridine	134.0	76.0	78.0	81.0	110.0
Ceftazidime	1.1	2.1	0.69	3.3	UM
Cefotaxime	2.5	0.9	1.5	6.1	0.16

*Values expressed as a percentage of the value for ampicillin.

UM = Unmeasurable because of insufficient hydrolysis of substrate.

The molecular weight of TEM-E1 was indistinguishable from Mutant beta-lactamase B and TEM-1 (table 25).

e. Inhibition studies

When assayed for the hydrolysis of nitrocefin, both TEM-E1 and Mutant beta-lactamase B were sensitive to inhibition by clavulanic acid (table 26).

These data revealed that Mutant beta-lactamase B and TEM-E1 were essentially similar. Consequently, it was concluded that they were the same enzyme.

3.4.2. Beta-lactamase TEM-E2.

The ceftazidime resistant isolate of Klebsiella oxytoca 5445 was isolated in 1982 from the blood and cerebrospinal fluid of a baby in the Neonatal Intensive Care Unit of Liverpool Hospital (table 20). Previous to the isolation of this strain, the intensive care unit had a number of fatalities in neonates owing to a gentamicin resistant K.oxytoca 5446. This strain was ceftazidime sensitive and produced TEM-1. K.oxytoca 5446 was eradicated for some months and then K.oxytoca 5445 appeared, which was resistant to both gentamicin and ceftazidime. The clinicians suggested that the ceftazidime sensitive K.oxytoca 5446 may have remained in areas where it had been exposed to sub-MIC levels of ceftazidime and this had eventually yielded the ceftazidime resistant K.oxytoca 5445. On initial analysis of K.oxytoca 5445, the ceftazidime resistance was not thought to

Table 25. Molecular Weight of beta-lactamases determined by Sephadex gel filtration.

Beta-lactamase.	Molecular Weight (x1000)
TEM-1	22.0
TEM-E1	22.0
TEM-E2	23.5
TEM-E3 (NMH)	25.5
TEM-E3 (RMH)	23.5
TEM-E4	24.5
Mutant beta-lactamase A	23.5
Mutant beta-lactamase B	24.0
Mutant beta-lactamase D	23.5
TEM-3	24.0
TEM-4	25.5
TEM-5	24.0
TEM-6	23.0
TEM-7	25.5
TEM-9	23.5
TEM-10	22.0
PSE-4	27.5
Mutant beta-lactamase E	26.0
BIL-1	29.0

Table 26. Concentrations of clavulanic acid required to inhibit the beta-lactamase hydrolysis of nitrocefin by 50% (ID_{50}).

Beta-lactamase	ID_{50} value (uM)
TEM-1	0.5
TEM-E1	0.6
TEM-E2	0.8
TEM-E3 (NMH)	0.2
TEM-E3 (RMH)	0.3
TEM-E4	0.3
Mutant beta-lactamase A	0.7
Mutant beta-lactamase B	0.5
TEM-7	0.5
TEM-9	0.3
TEM-10	0.3
PSE-4	0.7
Mutant beta-lactamase E	0.6
BIL-1	1995.0

be mediated by a beta-lactamase, because only TEM-1 was detected. Five years later the research of this Thesis revealed that the strain produced TEM-1 along with an additional beta-lactamase which focused as a doublet band at pI 5.3.

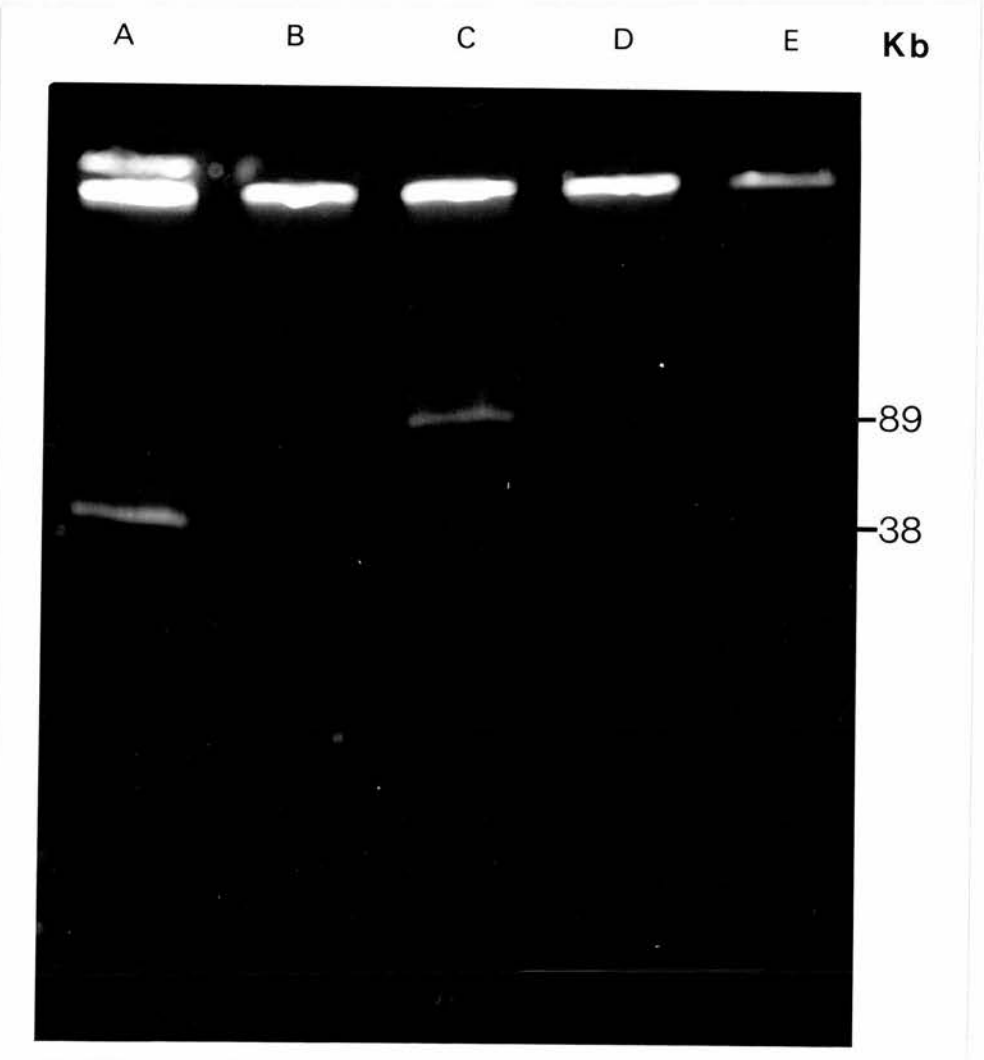
a. Conjugation experiments.

The genes encoding the TEM-1 and the doublet band beta-lactamases could be transferred by conjugation to a recipient strain of E.coli J53-2 with a concomitant transfer of resistance to ceftazidime. Similar transfer frequencies were obtained when ceftazidime (4mg/L), ampicillin (20mg/L) or ceftazidime (4mg/L) and ampicillin (20mg/l) were used as selectors (table 21). This illustrated that the TEM-1 and doublet band beta-lactamases were probably encoded by the same plasmid. The ceftazidime resistance was demonstrated to be encoded by a transferable plasmid designated pUK721. The size of plasmid pUK721 was originally calculated as 103Kb, but the correct size of pUK721 was found to be 141Kb (fig. 11, and fig. 12 for calculation).

b. Antibiotic sensitivities.

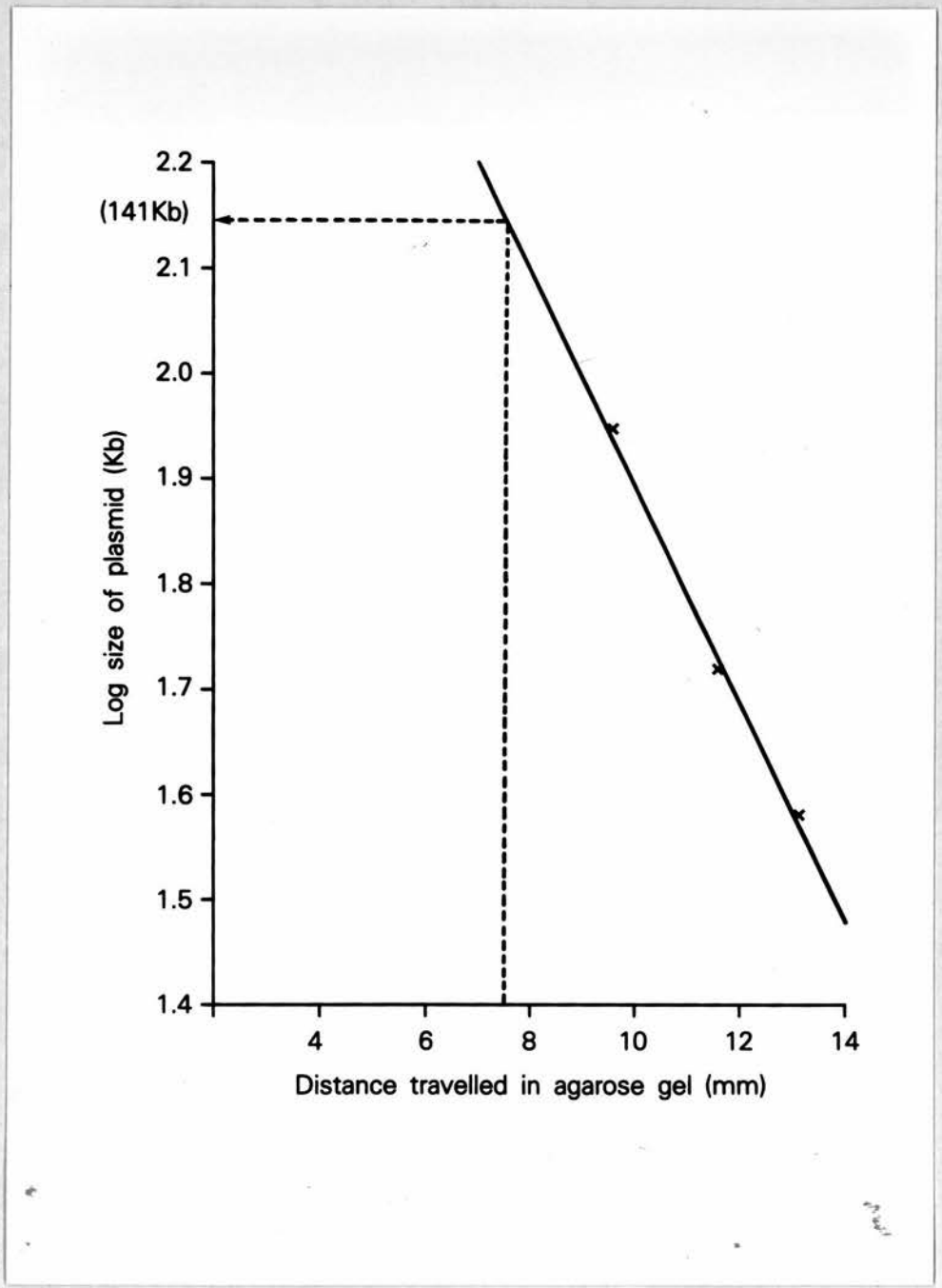
Both the original clinical isolate and the E. coli J53-2 transconjugant were resistant to ceftazidime but sensitive to cefotaxime and ceftriaxone. Both the transconjugants and the clinical strain became sensitive to ceftazidime in the presence of clavulanic acid (2mg/L) or sulbactam (2mg/L). However, clavulanic acid was the more efficient inhibitor when

Fig. 11. Agarose gel electrophoresis of pUK721 and standard plasmids (0.5% agarose).



A. R6K; B. RP4; C. R1; D. E.coli J53-2 transconjugant of K.oxytoca 5445; E. K.oxytoca 5445.

Fig. 12. Graph demonstrating the determination of the size of plasmid pUK721 encoding TEM-E2.



used in combination with ampicillin. The two inhibitors were similar in their activity when 3GCs were used as the principal antibiotic (table 27).

It was also observed that the E.coli J53-2 transconjugant of K.oxytoca 5445, which produced TEM-1 and the doublet beta-lactamase, exhibited a similar beta-lactam resistance profile to an E.coli J53-2 strain producing Mutant beta-lactamase A (table 27).

c. Isoelectric focusing.

The K.oxytoca 5445 isolate and the E. coli J53-2 transconjugant produced identical beta-lactamase bands. Both strains produced a band which co-focused with TEM-1 and a novel beta-lactamase which focused as a doublet band at pI 5.3. This novel beta-lactamase, TEM-E2, was clearly distinguishable from the other 3GC hydrolysing beta-lactamases (fig. 13) Further analysis of the TEM-E2 beta-lactamase demonstrated that it had an identical focusing position to Mutant beta-lactamase A (fig. 10).

d. Purification of the TEM-E2 beta-lactamase.

As it was assumed that the TEM-1 band of K.oxytoca 5445 had little bearing on the ceftazidime resistance, it was necessary to purify the TEM-E2 beta-lactamase (doublet band at pI 5.30) from TEM-1.

A method of electrodialysis was adopted to purify TEM-E2 from TEM-1. The protocol for this novel method of beta-lactamase purification is discussed

Table 27. MICs of E.coli strains harbouring Mutant beta-lactamase A and beta-lactamase TEM-E2.

Beta-lactam antibiotic.	Minimum Inhibitory Concentrations (mg/L).				
	Mutant A			Controls	
	J62-2 mutant	J53 trans	*5445 xJ53	J62-2	J53-2
Ampicillin	>250	>250	>250	4	4
[1] + clav	ND	2	8	ND	2
[2] + sulb	ND	4	250	ND	4
Carbenicillin	>250	>250	>250	4	8
Cephaloridine	8	16	64	2	4
Cephalexin	4	4	8	4	4
Cefoxitin	2	1	4	2	2
Cefuroxime	2	0.5	4	2	2
Ceftazidime	4	16	32	<0.06	<0.13
[1] + clav	ND	0.13	0.25	ND	0.13
[2] + sulb	ND	<0.06	0.5	ND	<0.06
Cefotaxime	0.06	0.06	0.25	<0.06	<0.06
[1] + clav	ND	<0.06	<0.06	ND	0.06
[2] + sulb	ND	<0.06	<0.06	ND	<0.06
Ceftriaxone	<0.06	0.13	0.13	<0.06	<0.06
Aztreonam	0.25	0.5	1	<0.06	0.13
Imipenem	0.13	0.5	0.25	0.13	0.25
Augmentin	8	16	32	8	8

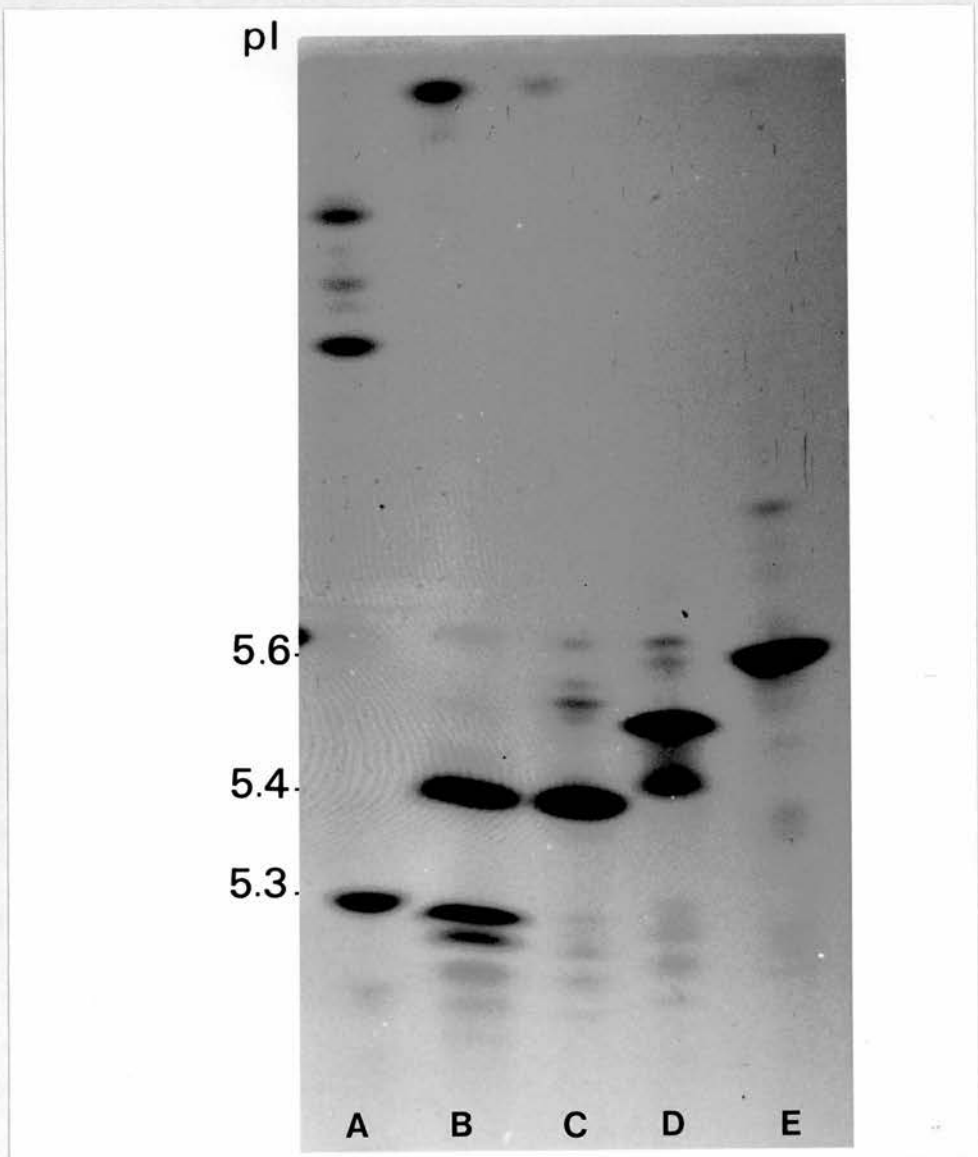
1 + clav = in combination with 2mg/L clavulanic acid

2 + sulb = in combination with 2mg/L sulbactam

* produces TEM-E2

ND not done

Fig. 13. Isoelectric focusing pattern of the plasmid encoded beta-lactamase derived from strain 5445 (TEM-E2), compared with enzymes PSE-4, TEM-1, TEM-2, and TEM-7, over a narrow pH range.

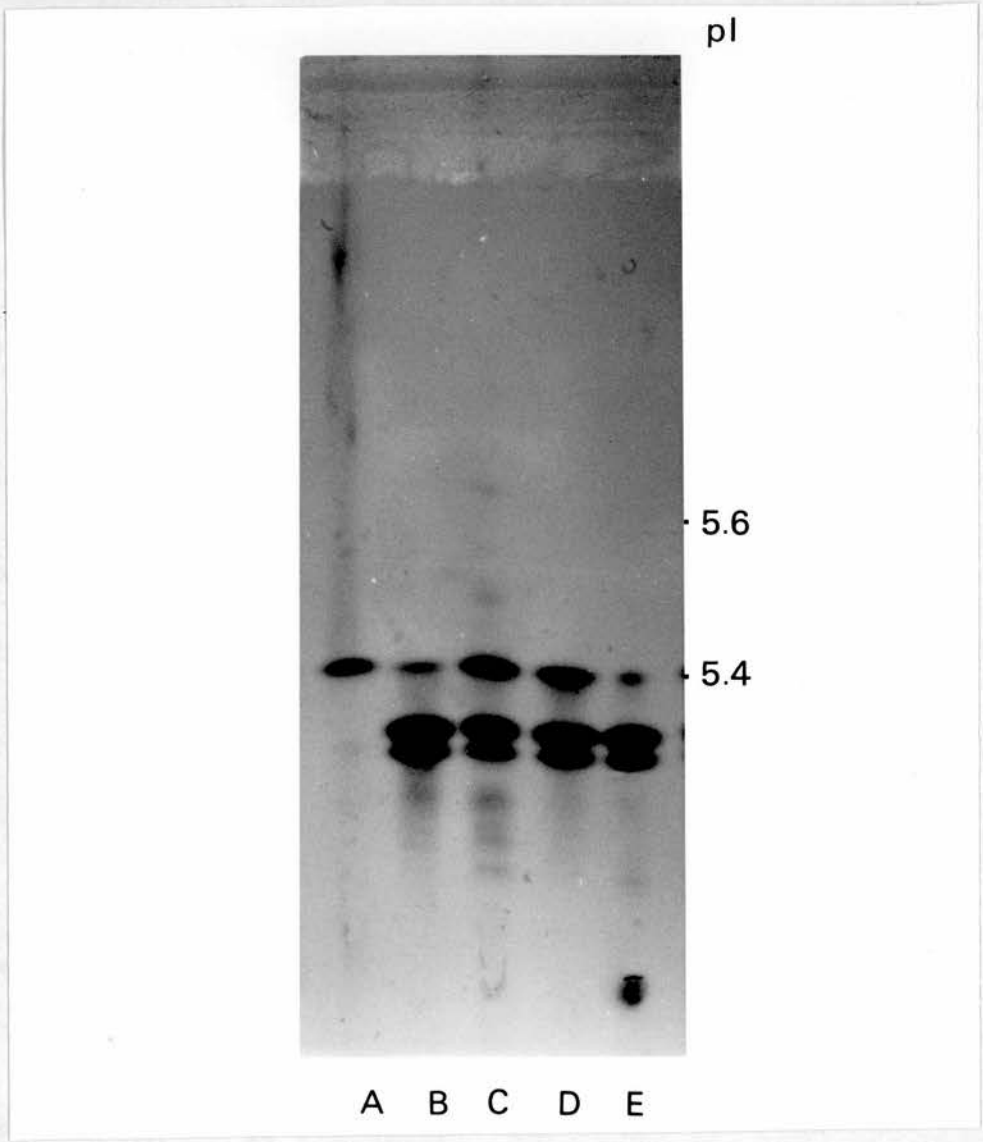


A. P.aeruginosa PAO8 producing PSE-4; B. E.coli J53-2 producing TEM-E2 and TEM-1; C. E.coli J53-2 producing TEM-1; D. E.coli BM694 producing TEM-7; E. E.coli J53-2 producing TEM-2.

below. Two ml of crude beta-lactamase solution were prepared from the E.coli J53-2 transconjugant of K.oxytoca 5445 (as described in section 2.7). This preparation was spread onto an analytical IEF polyacrylamide gel containing a 1:1.5 ratio of pH 6-8 and pH 4-6 ampholines. Care was taken not to cover the area of the gel where the beta-lactamase bands focused. Once the beta-lactamases had been focused a 1cm strip of filter paper, previously soaked in a 0.5mg/ml solution of nitrocefin, was placed from the cathode to the anode of the gel. This allowed the identification of the required plasmid beta-lactamase band so that it could be cut out of the gel. The portion of the gel extracted was then placed in dialysis tubing with a minimal amount of 25mM sodium phosphate buffer (pH 7.0). The dialysis sack was placed in the cathode reservoir of a Bio Rad Mini Sub Cell with 25mM sodium phosphate buffer (pH 7.0) as running buffer. A charge of 150 volts was applied for 10 minutes. The dialysis sack was then removed and the purity of the fraction was analysed by IEF.

The TEM-E2 preparation, purified by electrodialysis, is shown in fig. 14. It appears to have retained a small amount of TEM-1 activity, however, it is possible that this small TEM-1 band may be a satellite band of TEM-E2. Indeed, IEF of Mutant beta-lactamase A reveals a doublet band, which is identical to TEM-E2, along with a similar small TEM-1 beta-lactamase band (figs. 10 and 14). Therefore, this technique provided a quick and easy method for the separation and purification of beta-lactamases, particularly when the host strain produces more than one such enzyme with similar isoelectric focusing positions.

Fig. 14. IEF demonstrating the purification of TEM-E2 from TEM-1 by electrodialysis.



A. E.coli J53-2 producing TEM-1; B. TEM-E2 beta-lactamase purified from TEM-1 by electrodialysis; C. E.coli J53-2 transconjugant of K.oxytoca 5445 producing TEM-1 and TEM-E2; D. K.oxytoca 5445 producing TEM-1 and TEM-E2; E. E.coli J53-2 producing Mutant beta-lactamase A.

e. Biochemical characterisation of TEM-E2.

The rates of hydrolysis of a number of beta-lactam substrates by TEM-E2, Mutant beta-lactamase A, and TEM-1 are compared in table 23. None of the enzymes had any measurable activity against cefuroxime. TEM-1 had no activity against ceftazidime, but showed some affinity for cefotaxime. Both TEM-E2 and Mutant beta-lactamase A showed similar activity against both 3GC substrates, but had greater activity with cefotaxime. All three enzymes had similar activities for ampicillin, carbenicillin and cephaloridine. However, TEM-E2 and Mutant beta-lactamase A showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed negligible hydrolysis of either of these substrates (table 23). All three enzymes exhibited similar relative efficiency of hydrolysis values (Vmax/Km, ampicillin = 100%) for ampicillin, carbenicillin, and cephaloridine. Mutant beta-lactamase A and the TEM-E2 enzyme hydrolysed cefotaxime more efficiently than ceftazidime (table 24) although both enzymes conferred greater resistance to ceftazidime. When assayed for the hydrolysis of nitrocefin TEM-E2 and Mutant beta-lactamase A were sensitive to inhibition by clavulanic acid (table 26). The molecular weight of TEM-E2 (purified by electrodialysis from an analytical IEF gel) was 23,500. Under the same experimental conditions, the molecular weight of the TEM-1 enzyme was 22,000 (table 25). When the crude TEM-E2 enzyme preparation (i.e with the TEM-1 enzyme) was analysed by gel filtration, fractions were taken from the Sephadex G-75 column either side of the peak of beta-lactamase activity. Each of these fractions were examined by IEF to ascertain whether the TEM-1 and

the doublet band could be separated by virtue of their molecular weight. However, examination of each of these fractions showed that they all exhibited both the TEM-1 band and the doublet band. This confirms that the TEM-1 band and the doublet band had very similar molecular weights.

Biochemical characterisation of TEM-E2 and Mutant beta-lactamase A revealed that they were the same enzyme.

3.4.3. Beta-lactamase TEM-E3.

In 1987 a ceftazidime resistant strain of E.coli (serotype ONT H9) was isolated from two patients in the same ward at the North Middlesex Hospital, London (NMH) (table 20). This strain was later isolated on the ward mop and the bed pan washer. E.coli 8001 (E.coli ONT H9) was demonstrated to exhibit transferable resistance to ceftazidime by Drabu et al. (1989). The work of this Thesis showed that the transferable resistance resulted from a plasmid mediated beta-lactamase which was designated TEM-E3 (NMH).

A similar beta-lactamase was shown to be produced by Enterobacter cloacae 7923 which was isolated at the Royal Marsden Hospital, London (RMH) (table 20). This beta-lactamase was designated TEM-E3 (RMH).

a. Conjugation experiments.

The beta-lactam resistances expressed by E.coli 8001 were transferred to an E.coli J53-2 recipient strain with either carbenicillin or ceftazidime

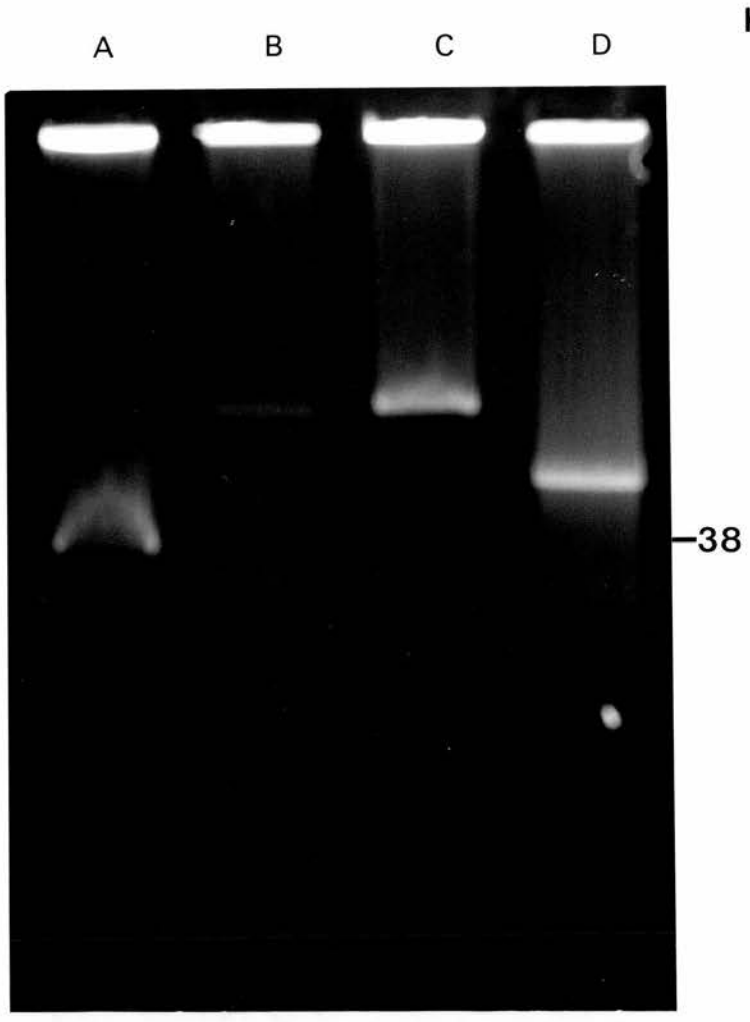
as the selecting agents. A similar frequency of transfer was observed for either selector (table 21). Both the original E.coli 8001 and its transconjugant possessed a 110Kb plasmid (pUK722) and it was assumed that this plasmid carried the ceftazidime resistance determinant.

The beta-lactam resistance expressed by Ent. cloacae 7923 was also successfully transferred to an E.coli J53-2 recipient strain. Both the transconjugant and the original clinical strain possessed a 100Kb plasmid (pUK723) and this was thought to encode for the beta-lactam resistance expressed by the transconjugant strain. Fig. 15 shows the plasmids encoding TEM-10, TEM-E3 (NMH), and TEM-E3 (RMH). The sizes of the TEM-E3 plasmids were determined separately by agarose gel electrophoresis in parallel with standard molecular size markers.

b. Antibiotic sensitivities.

Plasmids pUK722 and pUK723 conferred ceftazidime resistance in the E.coli J53-2 recipient strains. Both the transconjugants showed only a minor increase in resistance to cephalexin, cefuroxime and cefotaxime when compared to the E.coli J53-2 recipient strain. However, plasmids pUK722 and pUK723 conferred a significant increase in aztreonam and ceftazidime resistance (table 28). The ampicillin, ceftazidime and cefotaxime resistances conferred by the clinical isolates and transconjugants were considerably diminished in the presence of clavulanic acid (2mg/L) and sulbactam (2mg/L) (table 29). The beta-lactam resistance exhibited by the E.coli J53-2 transconjugants of Ent. cloacae 7923 and E.coli 8001 were compared with the MICs of E.coli J53-2 strains producing TEM-5, TEM-9, or

Fig. 15. Agarose gel electrophoresis of plasmid DNA from E.coli J53-2 transconjugants producing TEM-E3 or TEM-10 (0.6% agarose).



A. R6K plasmid; B. E.coli J53-2 transconjugant of Ent. cloacae 7923; C. E.coli J53-2 transconjugant of E.coli 8001; D. E.coli J53-2 transconjugant of the clinical isolate producing TEM-10.

Table 28. MICs (mg/L) of E.coli J53-2 transconjugants expressing TEM-E3, TEM-10, TEM-5 and TEM-9 (measured at 10^5 inoculum).

	Beta-lactamase					
Beta-lactam substrate.	TEM-5	TEM-E3 (NMH)	TEM-E3 (RMH)	TEM-10	TEM-9	E.coli J53-2 (recipient)
Ampicillin	>500	>500	>500	>500	>500	4
Cephalexin	8	16	8	8	16	4
Cefoxitin	4	8	4	4	2	2
Cefuroxime	64	8	8	8	8	2
Cefotaxime	1	1	0.5	0.5	2	<0.06
Ceftazidime	32	125	125	64	250	0.13
Aztreonam	2	32	64	32	64	<0.06
Imipenem	0.5	0.25	0.5	0.25	0.25	0.25

NMH = North Middlesex Hospital

RMH = Royal Marsden Hospital

Table 29. Effects of beta-lactamase inhibitors on strains producing TEM-E3.

Beta-lactam antibiotic.	Minimum Inhibitory Concentrations (mg/L)			
	E.coli 8001	*E.coli* 8001 X *E.coli* J53-2	*E.cloacae* 7923	*E.cloacae* 7923 X *E.coli* J53-2
Ampicillin	>250	>250	>250	>250
A +clav	4	8	16	8
B +sulb	4	4	64	16
Ceftazidime	64	64	250	125
A +clav	0.13	0.25	0.5	0.25
B +sulb	<0.06	0.5	2	0.5
Cefotaxime	1	1	2	1
A +clav	<0.06	<0.06	0.13	<0.06
B +sulb	<0.06	0.25	<0.06	<0.06

A +clav = in combination with 2mg/L clavulanic acid

B +sulb = in combination with 2mg/L sulbactam

TEM-10. This illustrated distinct similarities between the Ent. cloacae 7923 and E.coli 8001 transconjugants and the strain producing TEM-10. However, the E.coli J53-2 strain producing TEM-5 differed from the other transconjugants as it conferred a greater resistance to cefuroxime than any of the other transconjugants listed in table 28, also TEM-5 did not confer any significant resistance to aztreonam.

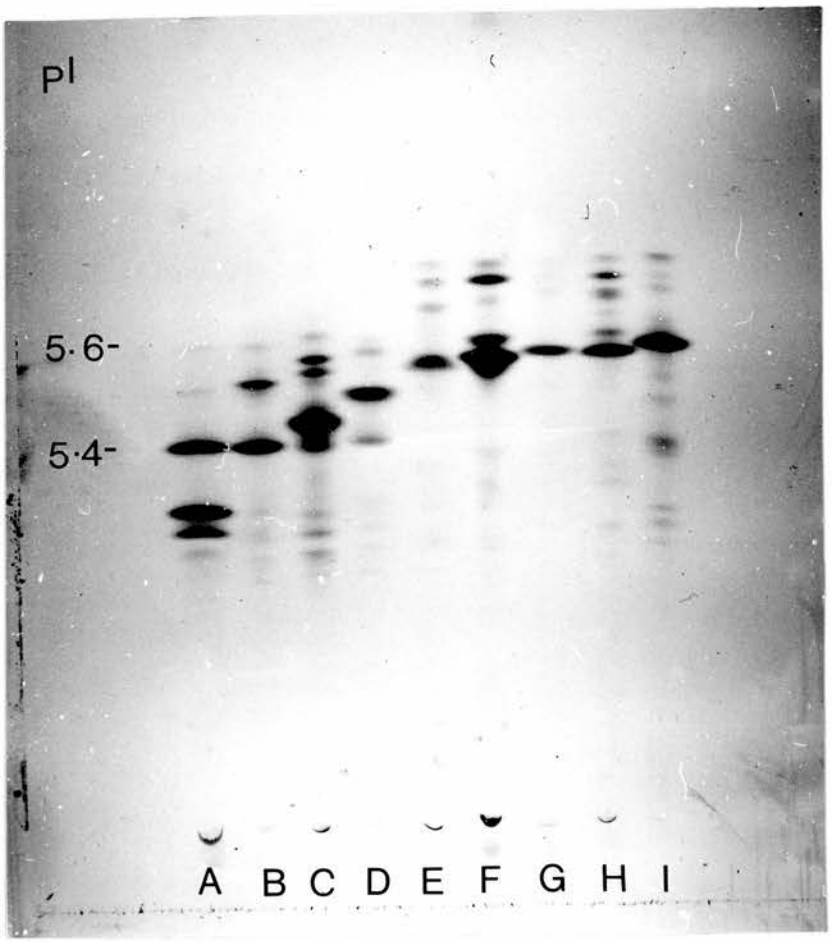
c. Isoelectric focusing.

Isoelectric focusing revealed that the E.coli J53-2 transconjugants of Ent. cloacae 7923 and E.coli 8001 produced beta-lactamases of identical pIs, and which focused marginally above TEM-5 and TEM-9 but below TEM-2 (fig. 16). These novel beta-lactamases were designated TEM-E3 (RMH) and TEM-E3 (NMH) respectively. Isoelectric focusing also illustrated that TEM-E3 aligned directly with TEM-10 (fig. 17). These six enzymes all focused over a range of 0.5 pI units. Therefore, further biochemical analysis was required to confirm these differences and similarities.

d. Biochemical characterisation of TEM-E3.

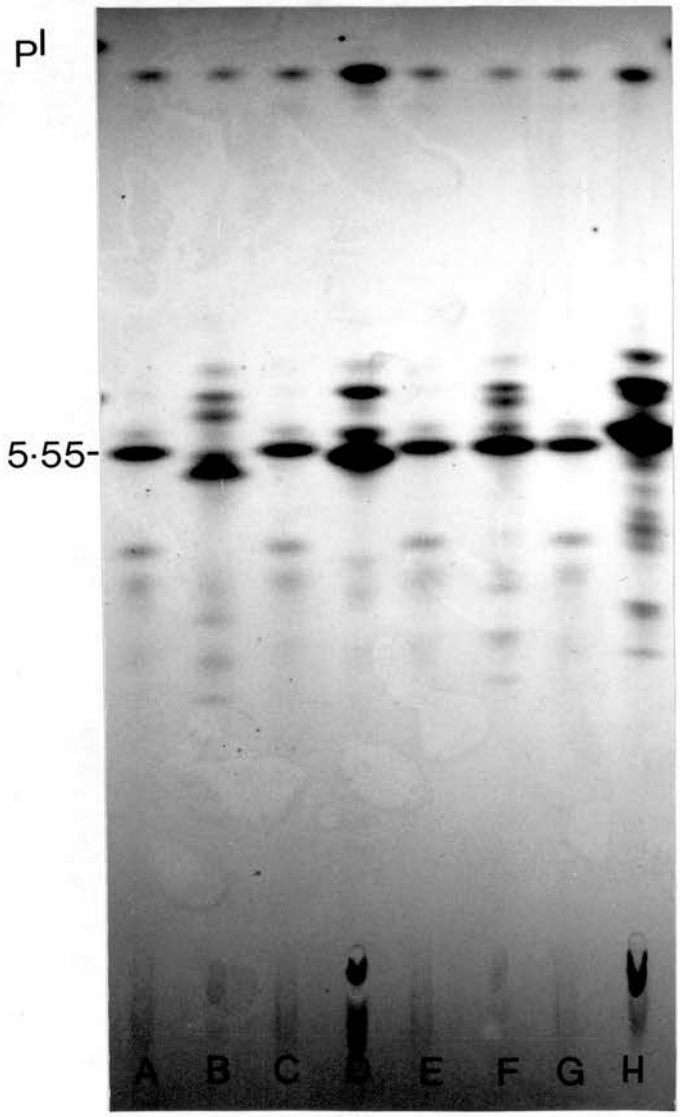
TEM-E3 (NMH), TEM-E3 (RMH) and TEM-10 had similar kinetic constants for all the beta-lactam drugs assayed. Their Vmax values, for the hydrolysis of ceftazidime, were more than 10 times greater than the Vmax values determined for cefuroxime or cefotaxime. However, these enzymes had much greater affinity for cefotaxime than any of the other beta-lactam substrates tested and, consequently, they hydrolysed this substrate fairly efficiently (tables 30-32).

Fig. 16. Isoelectric focusing of TEM-E3 (NMH) and TEM-E3 (RMH) over a narrow pH range.



A. E.coli J53-2 producing TEM-E2; B. E.coli J53-2 producing TEM-1; C. E.coli J53-2 producing TEM-E1; D. E.coli J53-2 producing TEM-7; E. E.coli J53-2 producing TEM-9; F. E.coli J53-2 producing TEM-5; G. E.coli J53-2 producing TEM-E3 (NMH); H. E.coli J53-2 producing TEM-E3 (RMH); I. E.coli J53-2 producing TEM-2.

Fig. 17. Isoelectric focusing of TEM-E3 compared to TEM-10, TEM-9 and TEM-5 over a narrow pH range.



A. E.coli J53-2 producing TEM-10; B. E.coli J53-2 producing TEM-9; C. same as A; D. E.coli J53-2 producing TEM-5; E. same as A; F. E.coli J53-2 producing TEM-E3 (RMH); G. same as A; H. E.coli J53-2 producing TEM-E4.

Table 30. *Relative Vmax values for TEM enzymes for a range of beta-lactams.

	Beta-lactam substrate					
Beta-lactamase.	AMP	CARB	CER	CXM	CAZ	CTX
TEM-5	100	51	410	102	135	26
TEM-E3 (RMH)	100	51	61	2.7	44	1.9
TEM-E3 (NMH)	100	30	63	5.1	43	3.2
TEM-10	100	48	58	2.0	30	2.2
TEM-9	100	46	73	1.8	115	6.3
TEM-1	100	11	23	UM	UM	0.06

Table 31. +Km values for TEM enzymes for a range of beta-lactams.

	Beta-lactam substrate					
Beta-lactamase.	AMP	CARB	CER	CXM	CAZ	CTX
TEM-5	69	38	143	250	330	21
TEM-E3 (RMH)	91	38	91	250	167	11
TEM-E3 (NMH)	76	33	125	286	167	21
TEM-10	111	39	100	167	100	15
TEM-9	128	43	200	167	500	40
TEM-1	167	100	167	UM	UM	286

+ Km measured in uM
*Values expressed as a percentage of the value with ampicillin.
UM = unmeasurable because hydrolysis of substrate was too low.

Table 32. *Relative efficiency of hydrolysis (Rel Vmax/Km) values for TEM enzymes.

	Beta-lactam substrate					
Beta-lactamase.	AMP	CARB	CER	CXM	CAZ	CTX
TEM-5	100	93	198	28	28	85
TEM-E3 (RMH)	100	122	61	1.0	24	16
TEM-E3 (NMH)	100	69	38	1.4	20	12
TEM-10	100	137	64	1.3	33	16
TEM-9	100	137	47	1.4	29	20
TEM-1	100	18	23	UM	UM	0.04

*Values expressed as a percentage of the value with ampicillin.
UM = Unmeasurable because hydrolysis of substrate was too low.

TEM-9 differed from the TEM-E3 and TEM-10 beta-lactamases by its much higher Vmax value for ceftazidime. However, this enzyme hydrolysed all the substrates tested with similar efficiencies to TEM-E3 (NMH), TEM-E3 (RMH) and TEM-10 (tables 30 and 32).

TEM-5 was distinguished from TEM-10 and the TEM-E3 beta-lactamases by its higher Vmax for the hydrolysis of cefuroxime, cefotaxime and ceftazidime. However, the affinities of TEM-5 for each of the substrates examined were similar to the other 3GC hydrolysing beta-lactamases. Lastly, TEM-5 hydrolysed cephaloridine, cefuroxime and cefotaxime far more efficiently than TEM-E3 (NMH), TEM-E3 (RMH), TEM-10, and TEM-9 (tables 30-32).

All the 3GC hydrolysing enzymes in tables 30-32 had similar molecular weights and ID_{50} values for clavulanic acid (tables 25 and 26 respectively).

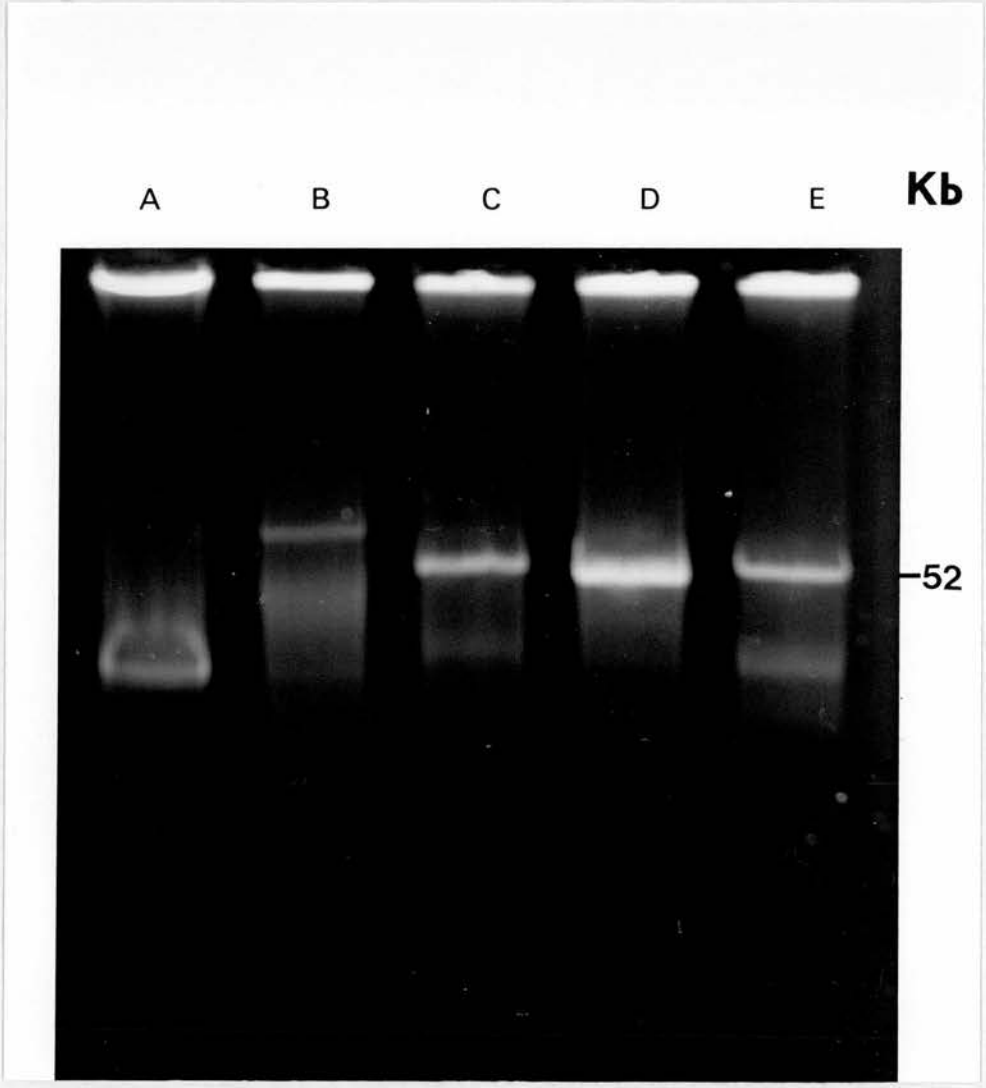
3.4.4. Beta-lactamase TEM-E4.

The isolation details of the strain producing TEM-E4 are shown in table 20.

a. Conjugation experiments.

The conjugation of the ceftazidime resistant S.marcescens 7919 and E.coli J53-2 gave rise to ceftazidime resistant E.coli transconjugants (table 21). Analysis of the plasmid DNA in these transconjugant strains showed a single plasmid band of 56Kb, this was designated pUK724 (fig. 18).

Fig. 18. Agarose gel electrophoresis of plasmid DNA of strains producing TEM-E4 or Mutant beta-lactamase D (0.5% agarose).



A. R6K plasmid; B. R1 plasmid; C. E.coli J53-2 producing TEM-2 mediated by plasmid RP4 (used as the wild type strain in mutation experiments; D. E.coli J53-2 transconjugant of S.marcescens 7919; E. E.coli J53-2 producing Mutant beta-lactamase D (derived from the TEM-2 gene in the RP4 plasmid).

This plasmid was also visualized in the S.marcescens strain.

b. Antimicrobial susceptibilities.

With the exception of imipenem, the S.marcescens 7919 strain was resistant to all the antibiotics tested in table 33. However, the E.coli transconjugant of this strain was resistant to ceftazidime but sensitive to all the other third and second generation cephalosporins tested. The ceftazidime and ampicillin resistances, expressed by the E.coli transconjugant, were diminished with the addition of clavulanic acid (2mg/L) to the media. This illustrated that the beta-lactam resistance of the E.coli J53-2 transconjugant of S.marcescens 7919 must be beta-lactamase mediated.

It was also noted that an E.coli J53-2 strain, producing Mutant beta-lactamase D, exhibited a similar profile of beta-lactam resistance to the E.coli transconjugant of S.marcescens 7919 (table 33).

c. Isoelectric focusing.

Both the original S.marcescens isolate and the E.coli transconjugant produced the TEM-E4 beta-lactamase which focused marginally above TEM-2 (pI 5.6) and aligned with Mutant beta-lactamase D (fig. 19). Further investigations demonstrated that TEM-E4 focused below TEM-6 (fig. 20) which illustrated the novelty of this beta-lactamase.

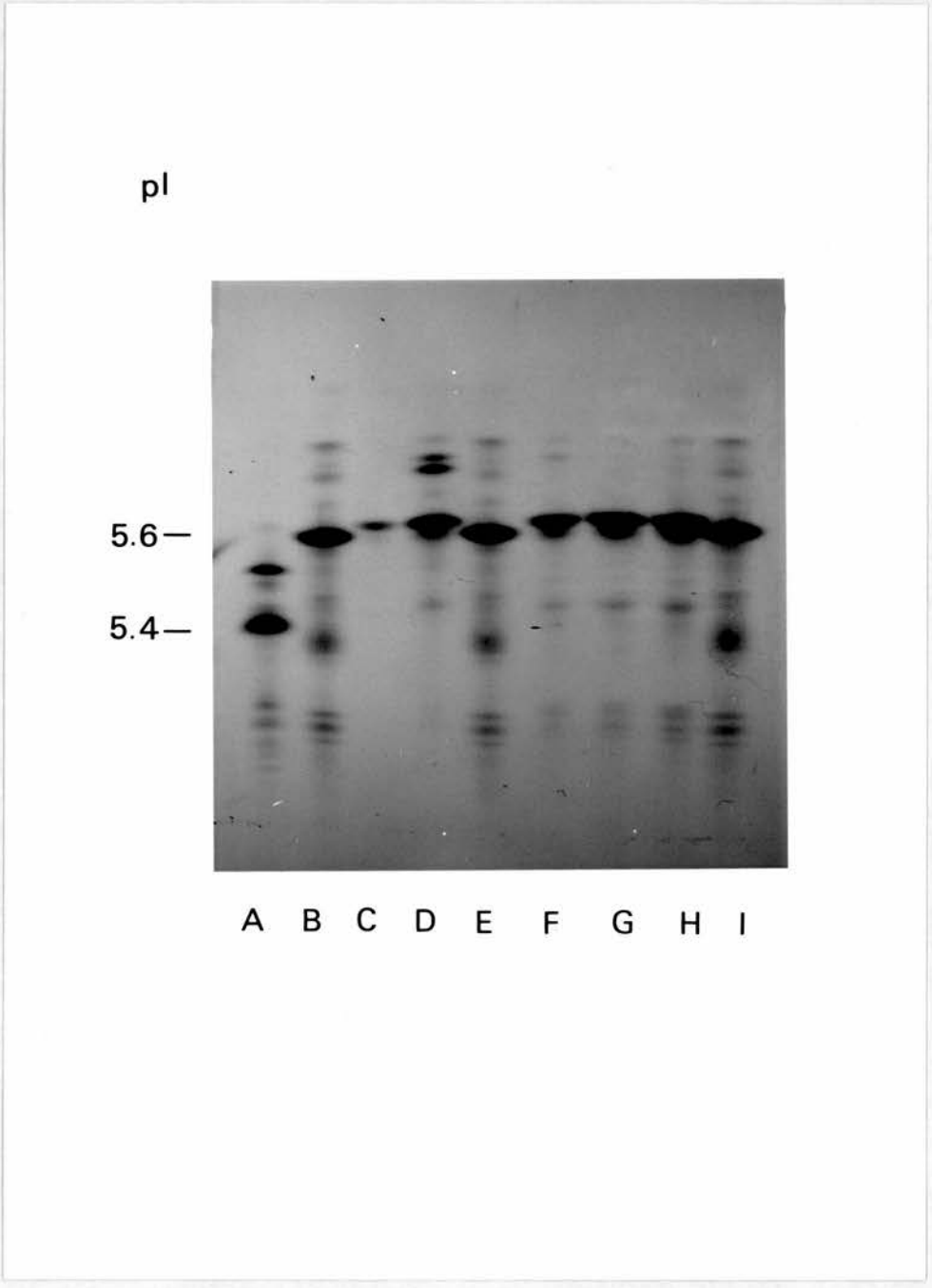
Table 33. MICs conferred by TEM-E4 and the Mutant beta-lactamase D (10^{-5} inoculum).

	Minimum Inhibitory Concentrations (mg/L)				
Beta-lactam antibiotic	*E.coli* J53-2 (TEM-2)	*E.coli* J53-2 producing β-lactamase D	*Serratia* 7919	*Serratia* 7919 X *E.coli* J53-2	*E.coli* J53-2
Ampicillin	>250	>250	>250	>250	4
A +clav	8	8	>32	8	4
Carbenicillin	>250	>250	>250	>250	8
Cephaloridine	32	16	>250	32	2
Cephalexin	4	8	>250	8	8
Cefoxitin	1	4	125	4	4
Cefuroxime	<1	4	>125	4	4
Ceftazidime	<1	8	32	16	<1
A +clav	<0.06	0.25	2	0.25	0.13
Cefotaxime	<1	<1	32	<1	<1
A +clav	<0.06	<0.06	>4	<0.06	<0.06
Ceftriaxone	<1	<1	16	<1	<1
Aztreonam	<1	<1	8	<1	0.25
Imipenem	<0.06	0.13	2	<0.25	<1

A +clav = in combination with 2mg/L clavulanic acid.

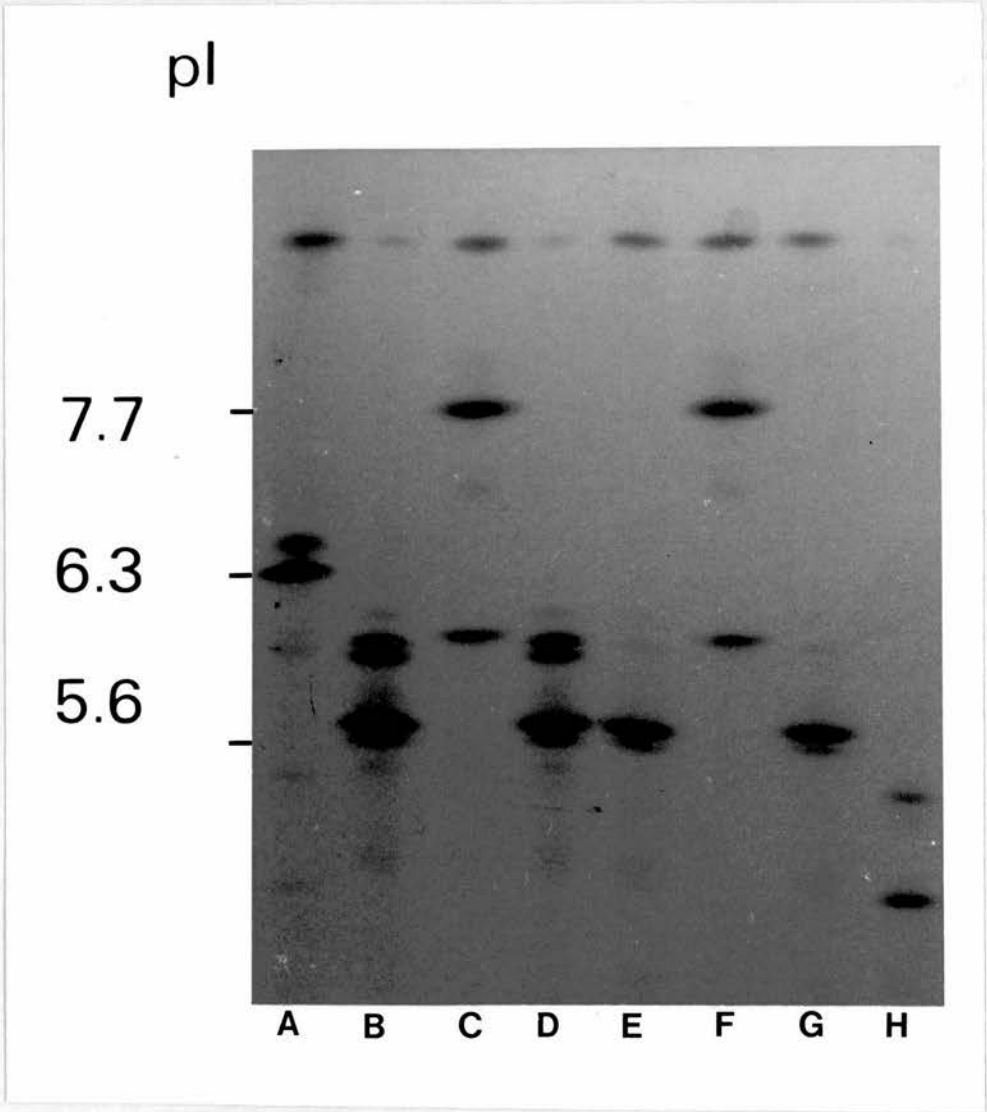
ND = Not done.

Fig. 19. IEF gel comparing the focusing positions of TEM-E4, Mutant beta-lactamase D and other TEM enzymes.



A. TEM-1; B. TEM-2; C. S.marcescens 7919; D. E.coli J53-2 transconjugant of S.marcescens 7919; E. TEM-2; F. E.coli J53-2 producing Mutant beta-lactamase D; G. E.coli K12 (SA 10) transconjugant producing Mutant beta-lactamase D (selected on 2mg/L ceftazidime); H. E.coli K12 (SA10) transconjugant producing Mutant beta-lactamase D (selected on 40mg/L Kanamycin); I. TEM-2.

Fig. 20. IEF gel demonstrating that TEM-E4 is different from TEM-6.



A. E.coli J53-2 producing CTX-1; B. E.coli J53-2 producing TEM-E4; C. E.coli J53-2 producing TEM-6 and SHV-1; D. same as B; E. E.coli J53-2 producing Mutant beta-lactamase D; F. same as C; G. same as E; H. E.coli J53-2 producing TEM-1.

d. Biochemical characterisation of TEM-E4.

Tables 34 and 35 show the Km and Vmax values determined for the hydrolysis of six different beta-lactam substrates by TEM-E4, Mutant beta-lactamase D and TEM-1. Mutant beta-lactamase D and TEM-E4 have similar kinetic constants for all the beta-lactams tested, they exhibit lower rates of hydrolysis for cefotaxime than ceftazidime (table 34), although they have a much higher affinity for cefotaxime (table 35). TEM-E4 and Mutant beta-lactamase D hydrolyse cefotaxime more efficiently than ceftazidime (table 36) which is paradoxical as these enzymes confer only a small degree of resistance to cefotaxime. Mutant beta-lactamase D and TEM-E4 had similar ID_{50} values for clavulanic acid (table 26) and similar molecular weights to TEM-1 (table 25).

e. Comparison of plasmids encoding TEM-E4 and Mutant beta-lactamase D.

Fig. 21 shows EcoR I digests of pUK724, RP4 encoding Mutant beta-lactamase D and RP4 encoding TEM-2. Plasmid pUK724 has at least three EcoR I restriction sites compared to the one EcoR I restriction site of RP4. Barth and Grinter (1977) showed that RP4 had only one EcoR I restriction site, therefore, these results confirm that the gene encoding Mutant beta-lactamase D was located on the RP4 plasmid.

Table 34. *Relative Vmax values Mutant beta-lactamase D and TEM-E4.

Beta-lactamase.	Beta-lactam substrate AMP	CARB	CER	CXM	CAZ	CTX
Mutant β-lactamase D	100	30	82	2.5	7.4	2.8
TEM-E4 (7919)	100	38	98	1.3	6.3	2.1
TEM-1	100	11	23	UM	UM	0.06

*Values expressed as a percentage of the value for ampicillin.

Table 35. +Km values for Mutant beta-lactamase D and TEM-E4.

Beta-lactamase.	Beta-lactam substrate AMP	CARB	CER	CXM	CAZ	CTX
Mutant β-lactamase D	37	139	100	250	1000	71
TEM-E4 (7919)	29	73	80	200	1500	77
TEM-1	167	100	167	UM	UM	286

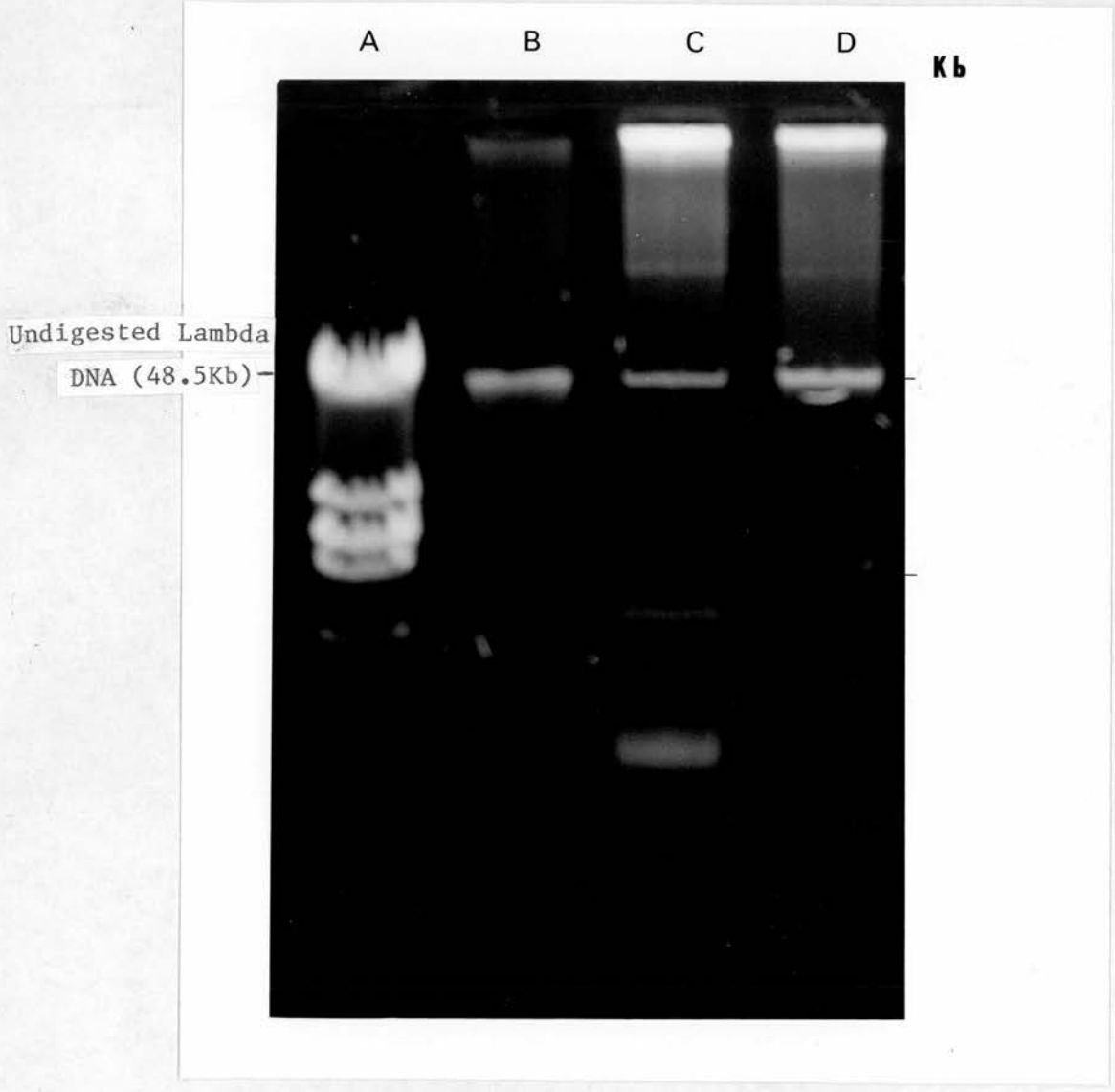
+Km values in uM.
UM=Unmeasurable because hydrolysis of substrate was too low.

Table 36. *Relative efficiency of hydrolysis (Rel Vmax/Km) values TEM-E4 and Mutant beta-lactamase D.

Beta-lactamase	Beta-lactam substrate AMP	CARB	CER	CXM	CAZ	CTX
Mutant β-lactamase D	100	8	30	0.37	0.27	1.50
TEM-E4	100	15	36	0.19	0.12	0.79
TEM-1	100	18	23	UM	UM	0.04

*Values expressed as a percentage of the value for ampicillin.
UM=Unmeasurable because hydrolysis of substrate was too low.

Fig. 21. Agarose gel electrophoresis of EcoR I digests of RP4 encoding Mutant beta-lactamase D or TEM-2 and pUK724 encoding TEM-E4 (1% agarose).



A. Lambda restricted with Hind III; EcoR I digests of: B. RP4 plasmid encoding TEM-2; C. Clinically derived plasmid encoding TEM-E4 (pUK724); D. RP4 plasmid encoding Mutant beta-lactamase D.

3.5. Manufacture of a radio-labelled TEM-E1 gene probe.

3.5.1. Manufacture of Polymerase Chain Reaction (PCR) primers.

In order to amplify a gene by PCR two primers must be synthesised which encode for a small piece of the DNA sequence either side of the gene. The 3' ends of each of these primers must point towards each other with the gene to be amplified in the middle. From previous experiments the reasonable assumption that the TEM-E1 gene was almost identical to TEM-1 was made. Therefore, analysis of the TEM-1 sequence suggested that the PCR primers 439E and 440E should be made. Their sequences are shown below.

Primer 439E.

5'- AAT TCT TGA AGA CGA AAG GGC CTC G -3'

Primer 440E.

5'- TTA CCA ATG CTT AAT CAG TGA GGC A -3'

Primer 439E (21 pmol/ul) and 440E (28.6 pmol/ul) were manufactured by the Oswell DNA Service, University of Edinburgh, Department of Chemistry.

Fig. 22 shows that primer 440E anneals to a section of the TEM-1 gene at the start of the structural gene and primer 439E anneals to a section in the promoter region of the gene. Therefore, the 3' ends of primers 439E and 440E point towards each other with the TEM gene in between.

3.5.2. Amplification of TEM-E1 gene by PCR.

Table 37 shows the ingredients of the PCR stock solution. This stock solution is sufficient for 15 PCR reactions. Aliquots of this stock mixture (89.7ul) were placed into 15 eppendorf tubes. One ul of a 1:10 diluted DNA preparation of plasmid pUK720, which contains the TEM-E1 gene, was added to each of the tubes. This was followed by 4.8ul of Primer 439E and 3.5ul of Primer 440E. These aliquots of primers provided 100pmols of each primer for each PCR reaction. Finally, 4.2U (1ul) of Taq Polymerase Type III (Cambio) was added to each of the reaction tubes. Sterile mineral oil (50ul) was then added to each of the reaction mixtures to prevent evaporation, and the bottom of each of the tubes was dipped in mineral oil to ensure maximum heat transfer from the heating block. The 15 tubes were then placed in a Cambio Intelligent Heating Block and the heating programme in table 38 was adopted.

Fig. 22. The TEM-1 gene.

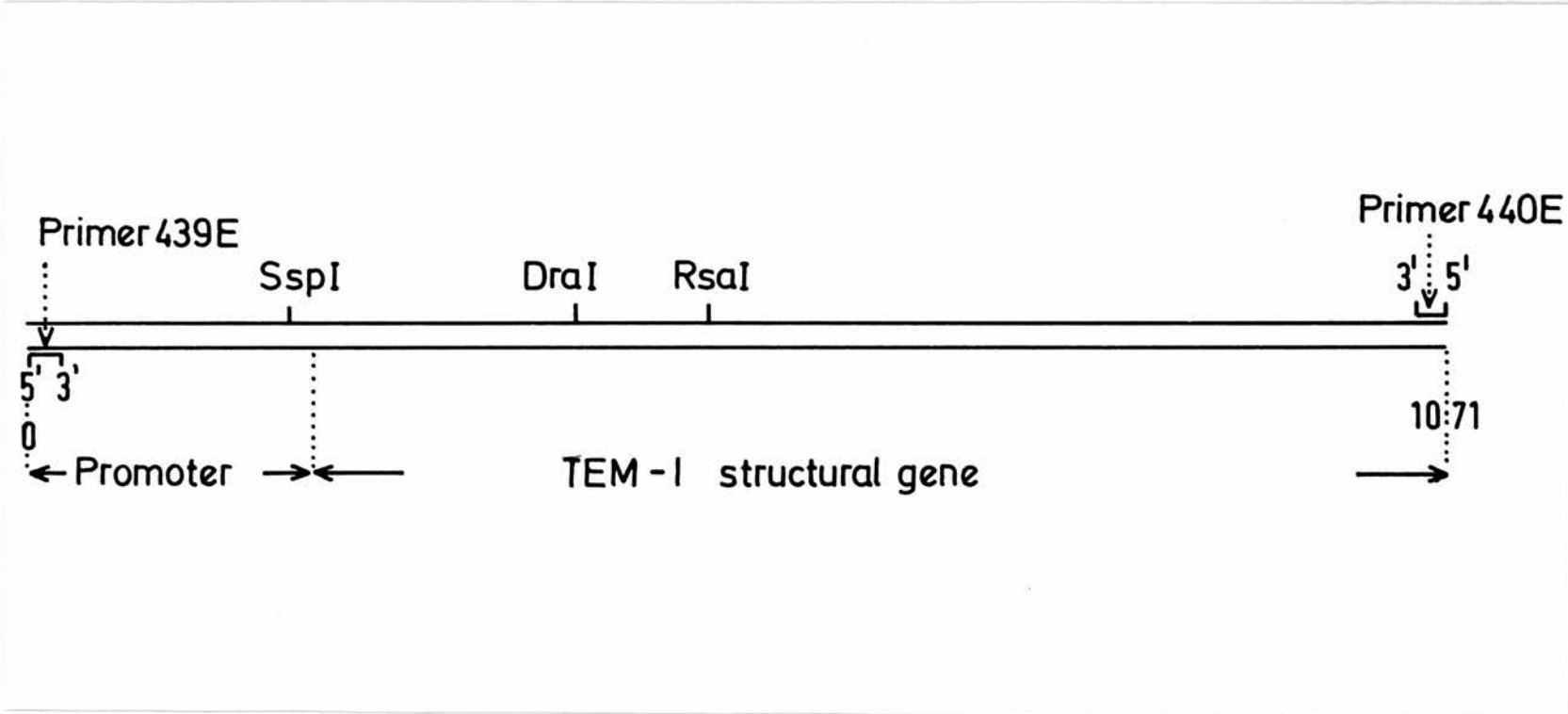


Table 37. Ingredients of the PCR stock solution.

Volume (ul).	Ingredient
805.5	Sterile water.
150.0	$MgCl_2$, 0.1M.
150.0	KCl, 0.5M.
150.0	Tris-HCl, 0.1M, pH8.4.
22.5	100mM 2'Deoxyadenosine 5'Triphosphate (dATP)
22.5	100mM 2'Deoxycytidine 5'Triphosphate (dCTP)
22.5	100mM 2'Deoxyguanosine 5'Triphosphate (dGTP)
22.5	100mM 2'Deoxythymidine 5'Triphosphate (dTTP)

Table 38. PCR protocol adopted for the amplification of the TEM-E1 gene.

	Temperature (^{o}C).	Purpose.	Time period (minutes).
1.	95	Denaturation.	10
	50	Annealing of primers.	5
2.	70	Extension of target DNA.	2
	95	Denaturation.	1
	50	Annealing of primers.	1
3.	70	Extension of all incompleted strands.	5

Step 2. was repeated 30 times.

On completion of the PCR reaction, 5ul was removed from each of the tubes and mixed with 2ul of loading buffer and loaded onto a 1% agarose gel with standard molecular weight markers.
Fig. 23 shows that 14 out of the 15 reactions produced a PCR fragment of approximately 1070bp. Analysis of the sequence of the TEM-1 beta-lactamase predicted that PCR primers 440E and 439E would produce a fragment of 1071bp. Therefore, these PCR cycles had yielded a DNA fragment of the correct size.

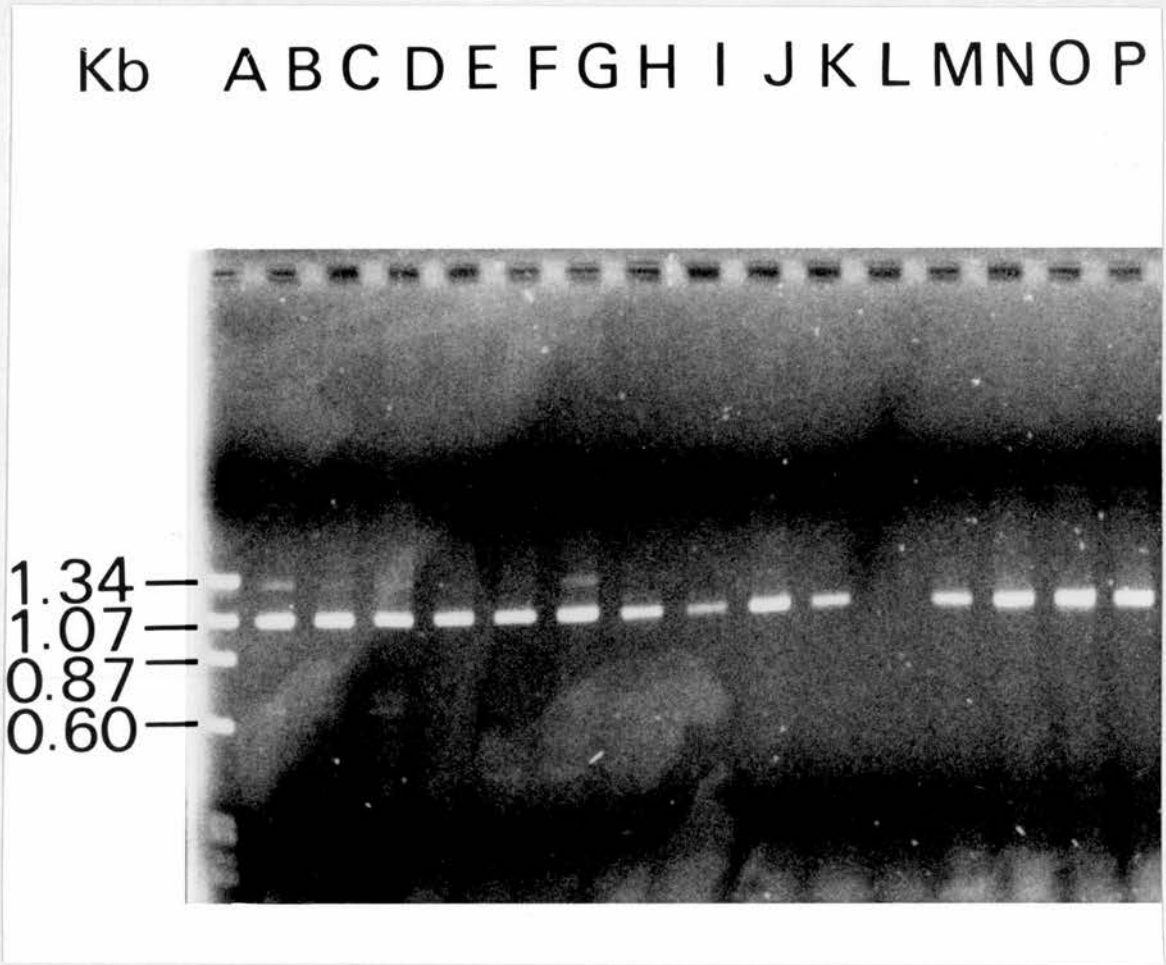
3.5.3. Extraction of mineral oil from PCR preparations.

After a PCR fragment of the correct size had been obtained 0.5ml of diethyl ether was added to each preparation. This mixture was vortexed and spun at high speed for 5 seconds. The diethyl ether fraction (top) was then removed and the procedure repeated twice. These tubes were then left for 15 minutes to allow any remaining diethyl ether to evaporate.

3.5.4. Checking the integrity of the PCR fragment.

In order to confirm that the 1071bp fragment actually contained the TEM-E1 gene, diagnostic restrictions with endonuclease enzymes were performed on three of the PCR preparations. It was assumed that TEM-E1 differed from TEM-1 by only a few point mutations. Therefore, from analysis of the TEM-1 nucleotide sequence, restriction endonuclease enzymes were selected that would cut the 1071bp into fragments which could be easily visualised on an agarose gel.

Fig. 23. Agarose gel electrophoresis of the amplified fragment obtained from PCR of pUK720.



A. ϕ X 174 RF DNA restricted with Hae III; (B.- P., aliquots of the 15 PCR preparations obtained from pUK 720); B. PCR preparation number 1; C. PCR prep. 2; D. PCR prep. 3; E. PCR prep. 4; F. PCR prep. 5; G. PCR prep. 6; H. PCR prep. 7; I. PCR prep. 8; J. PCR prep. 9; K. PCR prep. 10; L. PCR prep. 11; M. PCR prep. 12; N. PCR prep. 13; O. PCR prep. 14; P. PCR prep. 15.

It was deduced that if the 1071bp fragment contained a TEM derived beta-lactamase then Rsa I, Ssp I and Dra I should each have one restriction site (fig. 22). The sizes of the fragments which each of these restriction reactions should yield are shown in table 39.

The restriction with Rsa I gave two fragments of similar size and fig. 24 shows that there was a larger band of approximately 500bp suggesting that the two bands had not been resolved on this particular gel.

Fig. 24 shows that the Ssp I restriction endonuclease gave a large fragment of 878bp along with a small fragment of 193bp which is just visible.

Analysis of the TEM-1 structural gene suggested that Dra I should give fragments of 651bp and 420bp, these two fragments are clearly seen in fig. 24.

The diagnostic restrictions confirmed that the 1071bp fragment obtained from pUK720 contained a TEM-like gene which differed from TEM-1 by only a few amino acid residues.

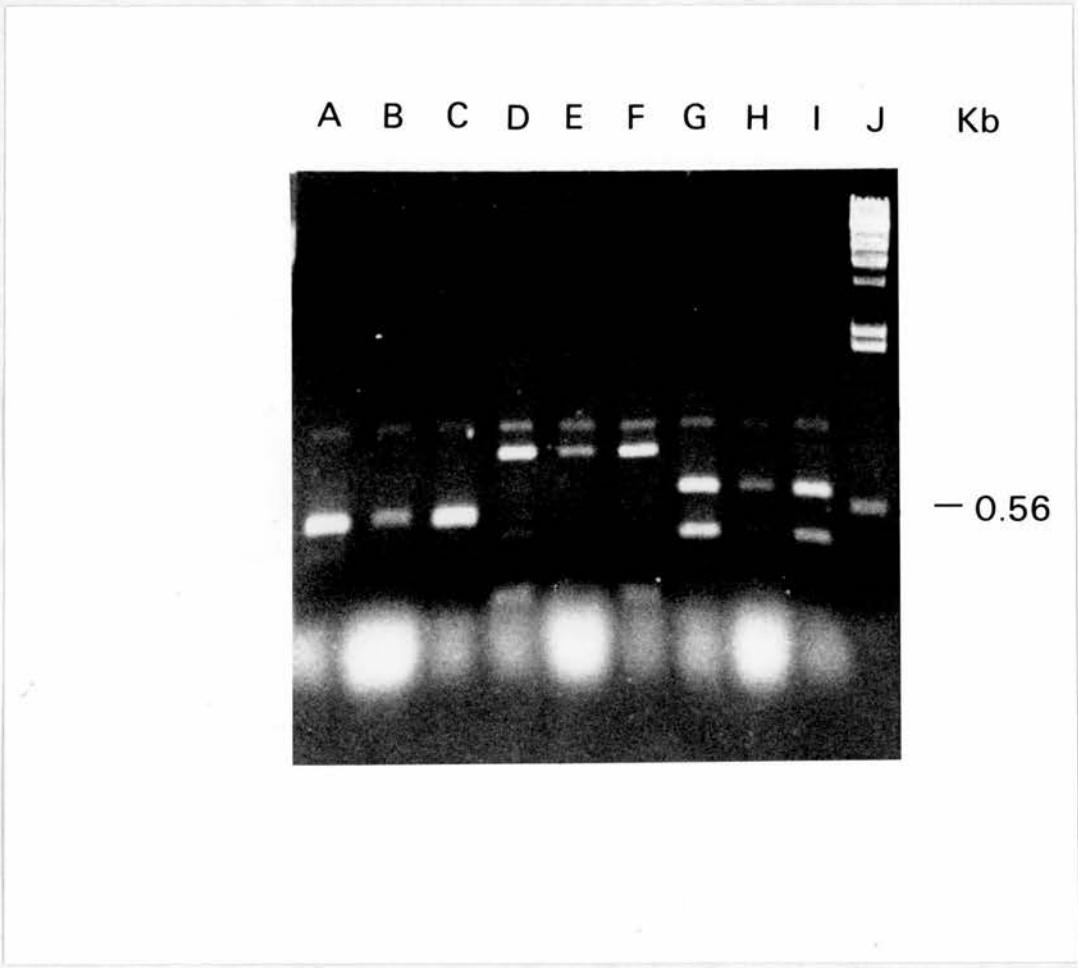
3.5.5. Purification of the PCR fragment.

The 14 PCR preparations were bulked together into three eppendorf tubes. These three samples were then completely dried in a Speed Vac Concentrator (Savant). Each of the samples were then resuspended in 25ul of TE buffer

Table 39. Diagnostic restrictions performed on PCR product.

Restriction endonuclease.	Fragments expected.
Rsa I	554, 517
Ssp I	878, 193
Dra I	651, 420

Fig. 24. Agarose gel electrophoresis of diagnostic restrictions of the PCR product (1% agarose).



A. PCR prep. 1 restricted with Rsa I; B. PCR prep. 5 restricted with Rsa I; C. PCR prep. 10 restriction with Rsa I; D. PCR prep. 1 restricted with Ssp I; E. PCR prep. 5 restricted with Ssp I; F. PCR prep. 10 restricted with Ssp I; G. PCR prep. 1 restricted with Dra I; H. PCR prep. 5 restricted with Dra I; I. PCR prep. restricted with Dra I; J. Lambda DNA restricted with Hind III.

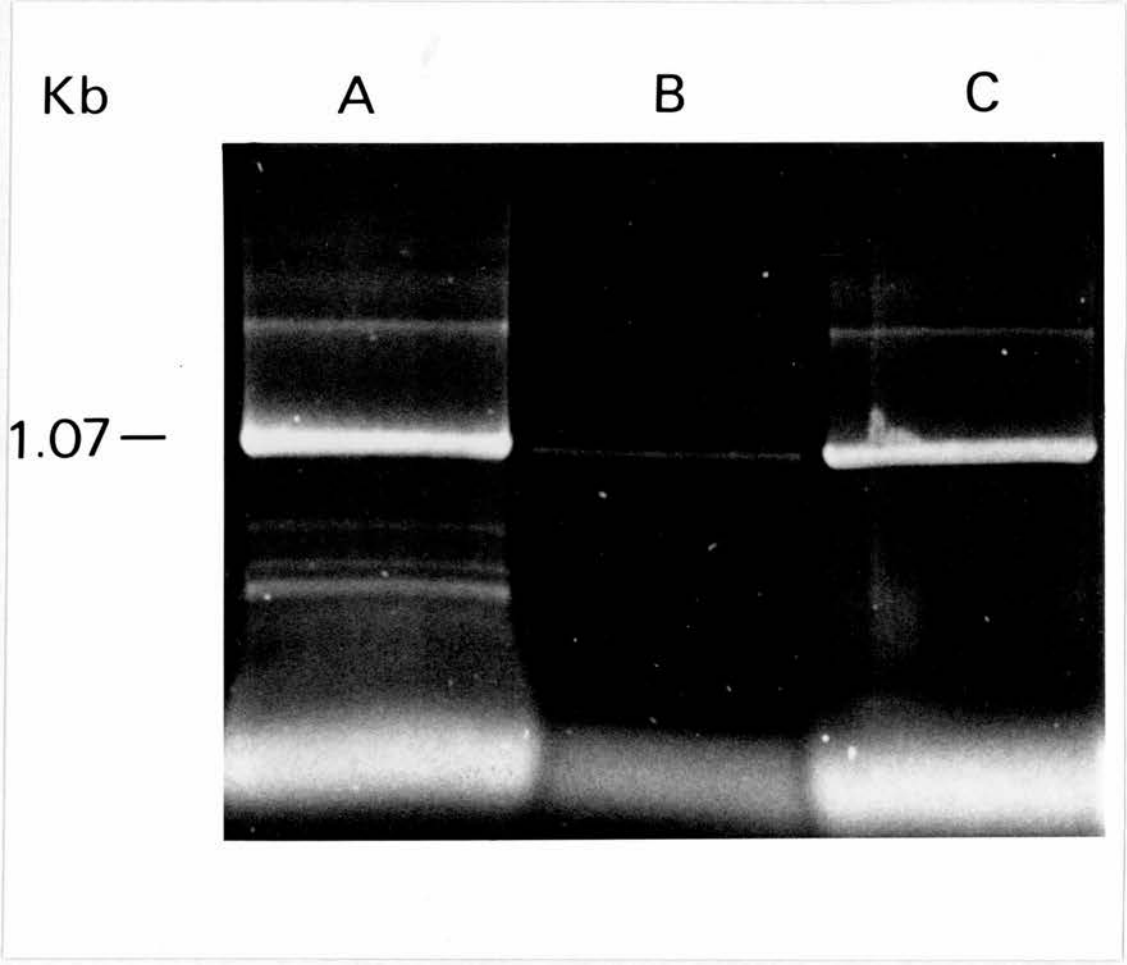
(10mM Tris acetate, 2mM disodium EDTA pH 8.0) and loaded onto a 1% Low Melting Point Agarose gel (fig. 25). The main PCR band from each preparation was cut out of the gel and resuspended in 1ml of elution buffer (500mM ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% SDS, pH 8.0) and left at 37°C overnight. The three preparations were then spun at high speed for five minutes to remove the agarose. In each case the supernatant was split into two 0.5ml aliquots 0.5ml of a 1:1 mixture of *phenol:chloroform/isoamyl alcohol (24 parts chloroform to one part isoamyl alcohol) was added to each preparation and mixed thoroughly. The mixture was then spun at high speed for two minutes and the top layer removed and placed in a clean eppendorf tube. This procedure was then repeated after which ethanol was added to give a final ratio of ethanol to DNA preparation of 3:1. The DNA was precipitated at -70°C for 20 minutes then harvested by spinning at high speed for 15 minutes, and the supernatant was discarded. The DNA was washed with 70% ethanol and spun at high speed for two minutes, the supernatant was removed and the pellet dried for five minutes in a Speed Vac Concentrator. The final pellets were combined and resuspended in a total of 50ul of TE buffer.

3.5.6. Restriction of the PCR product.

Fig. 22 shows that there is a Ssp I restriction site less than twenty base pairs away from the start of the TEM structural gene. Consequently, the purified PCR fragment was restricted with Ssp I to provide a fragment

*saturated with TE buffer.

Fig. 25. Low Melting Agarose Electrophoresis of combined PCR preparations obtained from pUK720.



A.; B.; C.; combined PCR preparations obtained from pUK720.

which contained predominantly the TEM structural gene. This would allow for the construction of a more discriminating TEM probe. The 50ul of purified PCR fragment was split into two 25ul aliquots and these were loaded onto a low melting agarose gel. Fig. 26 shows that the Ssp I restriction had yielded a fragment of 878bp and a fragment of 193bp. The 878bp fragment was extracted and purified as described previously. This preparation was finally resuspended in 30ul of TE buffer.

3.5.7. Radio labelling of the 878bp TEM-E1 fragment.

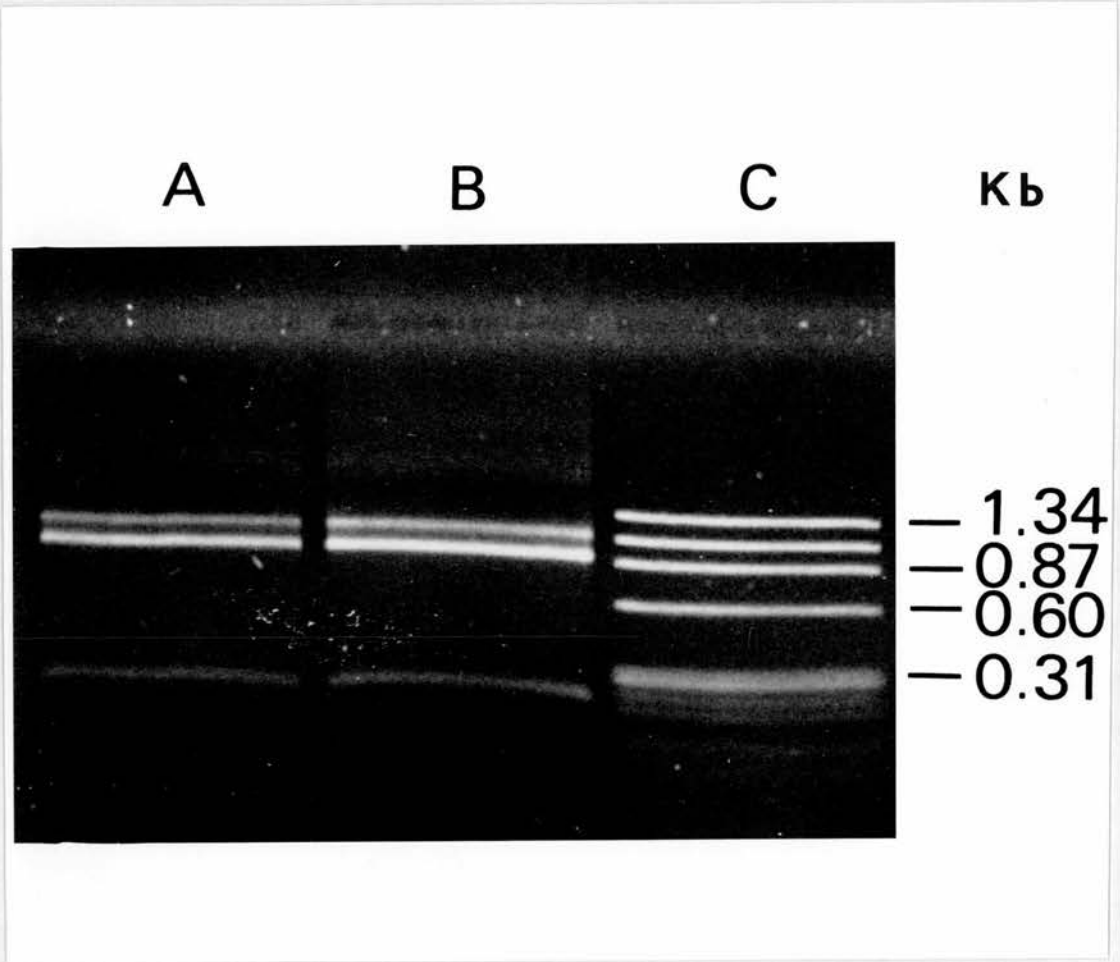
The Bio-Rad Random Labelling Primer DNA Labelling kit was used to radio label the TEM-E1 gene. Table 40 shows the ingredients of the labelling mix. These solutions were provided by the Bio-Rad kit and were mixed in a sterile eppendorf.

Five ul of the solution containing the TEM-E1 gene were mixed with 25ul of sterile water, boiled for 10 minutes and cooled rapidly in an ice/water bath. This mixture was then added to the labelling mixture and 1ul of Klenow Polymerase to give a final volume of 50ul. This solution was incubated for 4 hours at 37^{o}C. The reaction was terminated by the addition of 10ul of 10mM Tris pH 7.5, 100mM EDTA.

3.5.8. Blotting of agarose gel.

Following the electrophoresis of a range of TEM, and non-TEM beta-lactamase encoding plasmids the agarose gel was cut to the smallest possible size.

Fig. 26. Agarose electrophoresis of the PCR fragment restricted with Ssp I.



A. and B. PCR fragments restricted with Ssp I; C. ϕ X 174 RF DNA restricted with Hae III.

Table 40. The ingredients of labelling mix.

Ingredient.	Volume (ul)
5 X random primer buffer	10
10X BSA	5
dATP	1
dGTP	1
dTTP	1
[a ^{32}P]-dCTP	1

The gel was then incubated at room temperature in the following solutions.

1. 0.25M HCl for 15 minutes (depurination).
2. 0.4M NaOH, 0.6M NaCl for 2 X 15 minutes (denaturation).
3. 1.5M NaCl, 0.5M Tris, pH 7.5 for 30 minutes (neutralisation).

A piece of Genescreen Plus (Biotechnology Systems Dupont) was cut to the exact size of the agarose gel. Once the membrane was cut it curled up, and it is the concave side which must be laid on the agarose gel, this side was marked 'b'. The membrane was then submerged in distilled water and

transferred to 10X SSC (Salt Sodium Citrate, 1.5M sodium chloride; 0.15M sodium citrate) buffer for 15 minutes. The treated agarose gel was placed on a 3MM wick (Whatmann Chromatography Paper) soaked in 10X SSC. The membrane was then placed on top of the agarose gel (side 'b' in contact with the gel) and all air bubbles were removed. Short circuits were prevented by the addition of paraffin around the agarose gel. The following were then placed on top of the membrane;

1. 1 sheet of 3MM paper, this must be the exact size of the gel.
2. Approximately 7cm of absorbent tissues.
3. A 500g weight.

This was then blotted overnight, ensuring that there was plenty of 10X SSC in the reservoir.

On the following day the membrane was carefully removed from the gel and immersed in the following solutions;

1. 0.4M NaOH for 30 seconds.
2. 0.2M Tris, pH 7.5, 2X SSC for 30-60 seconds (neutralisation).

The filter was then left at room temperature until dry.

3.5.9. Hybridisation procedure.

The following pre-hybridisation mixture was prepared;

10% dextran sulphate

1% SDS

1.0M NaCl

Made up to 200ml with distilled water.

The ingredients were dissolved by heating to 90°C. Sufficient pre-hybridisation solution was added to cover the membrane and this was left at 65°C for five hours. Ten ul of a 10mg/ml salmon testes DNA were then added to the 50ul of the radio-labelled DNA probe made in section 3.5.7. This mixture was boiled for three minutes and cooled rapidly in an ice/water bath. The entire preparation was then added to the pre-hybridisation mixture and the hybridisation left overnight at 65°C. The following washes were then performed on the membrane;

(200ml per wash)

1. 2X SSC, 1% SDS, room temperature, 2 X 5 minutes.
2. 2X SSC, 1% SDS, 65°C, 2 X 30 minutes.
3. 0.1X SSC, 65°C, 1 X 30 minutes.

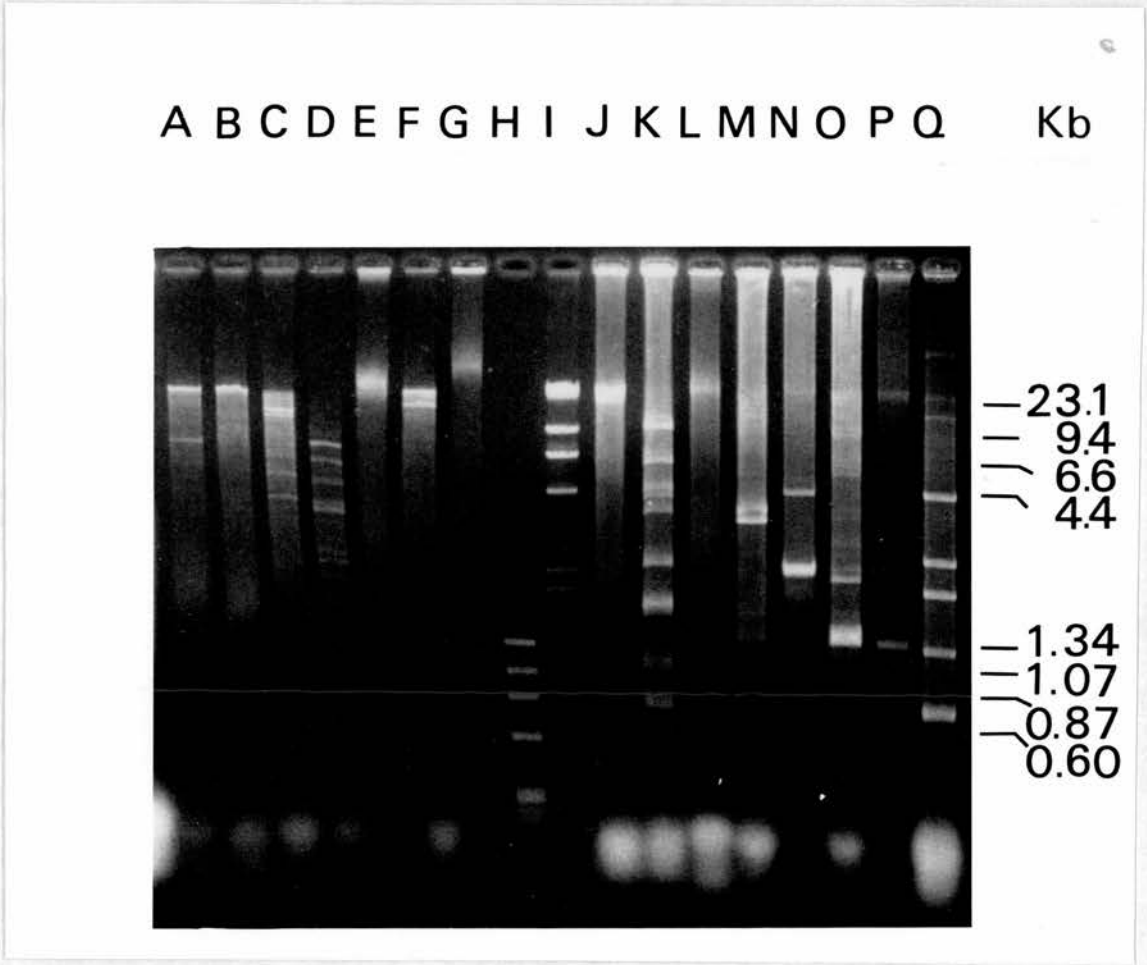
3.5.10. Probing of TEM-E1, TEM-E3, TEM-E4, TEM-1 and non-TEM beta-lactamases with a TEM-E1 probe.

Fig. 27 shows the agarose gel electrophoresis of plasmid DNA encoding TEM and non-TEM beta-lactamases. Fig. 28 shows the Southern blot of this gel probed with the TEM-E1 probe. The plasmid encoding TEM-E1 (pUK720) was restricted with Ssp I. In previous experiments employing this restriction, only a 4.3Kb DNA band hybridised with the TEM-E1 probe. However, in this case, although this band showed much stronger hybridisation than any other, other bands also showed some hybridisation but this was probably due to the incomplete restriction of the plasmid.

A non-restricted preparation of the TEM-E3 plasmid (pUK723) appeared to break up during the plasmid preparation. However, a faint band 19.1Kb did show some hybridisation. When the plasmid was restricted with EcoR I one fragment, (5.9Kb), showed strong hybridisation. Similarly, the non-restricted preparation of the TEM-E4 plasmid (pUK724) also appeared to have broken up during preparation but an EcoR I digest of this plasmid did produce fragments of 13.8Kb and 8.5Kb which strongly hybridised with the TEM-E1 probe.

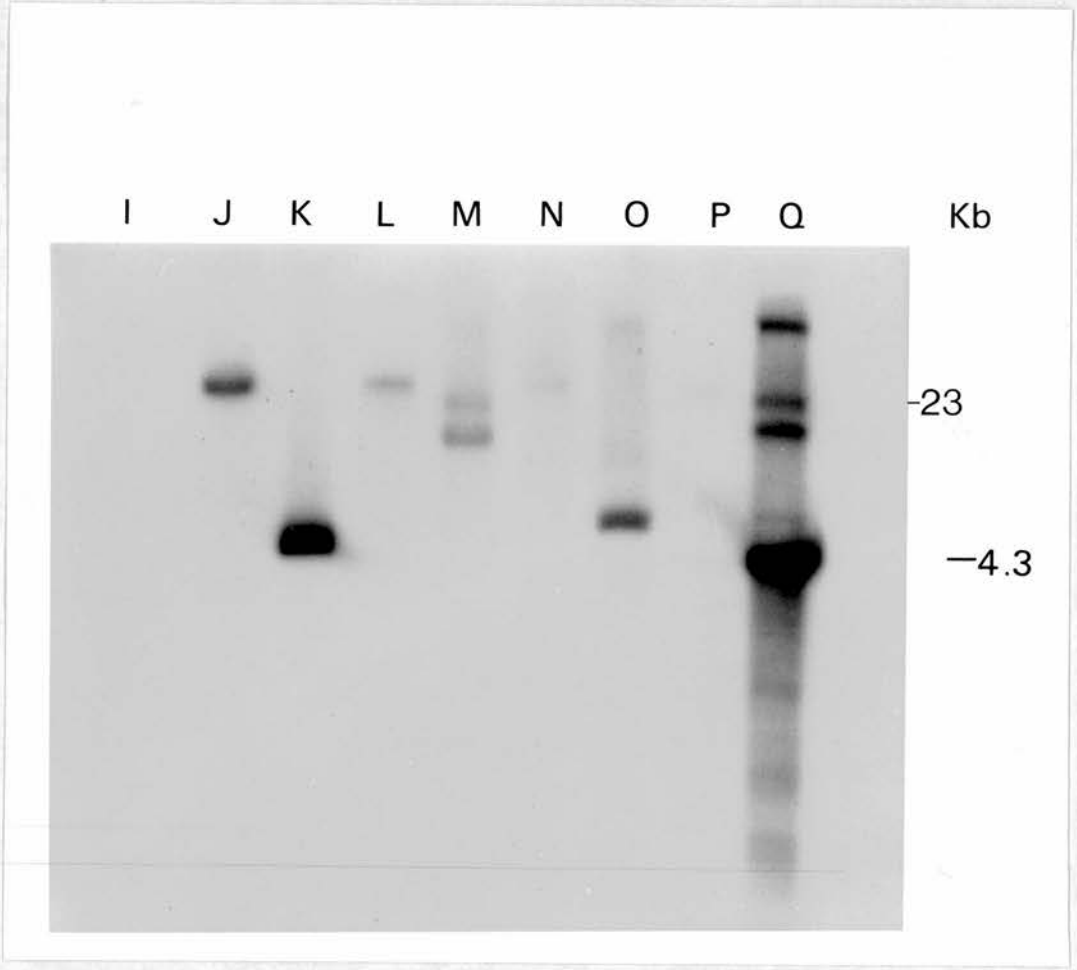
Ssp I and EcoR I restrictions of the R6K plasmid encoding TEM-1 yielded fragments of 19.1Kb and 4.9Kb respectively, which hybridised with the TEM-E1 probe.

Fig. 27. Agarose gel electrophoresis of plasmids encoding TEM-like or non-TEM beta-lactamases.



A. Plasmid R1010 (encodes for SHV-1) restricted with BamH I; B. R1010 restricted with Hind III; C. R1010 restricted with EcoR I; D. Plasmid R46 (encodes for OXA-2) restricted with Bgl I; E. R1010 non-restricted; F. R46 restricted with Sma I; G. R46 non-restricted; H. ϕ X RF DNA restricted with Hae III; I. Lambda DNA restricted with Hind III; J. Plasmid R6K (encodes for TEM-1) restricted with EcoR I; K. R6K restricted with Ssp I; L. R6K non-restricted; M. pUK724 (encodes TEM-E4) restricted with EcoR I; N. pUK724 non-restricted; O. pUK723 restricted with EcoR I; P. pUK723 non-restricted; Q. pUK720 (encoding TEM-E1) restricted with Ssp I.

Fig. 28. Southern blot of the agarose gel in fig. 27.



Tracks A-H were negative controls and showed no hybridisation with the TEM-E1 probe, tracks A - H not shown in figure.

A. Plasmid R1010 (encodes for SHV-1) restricted with BamH I; B. R1010 restricted with Hind III; C. R1010 restricted with EcoR I; D. Plasmid R46 (encodes for OXA-2) restricted with Bgl I; E. R1010 non-restricted; F. R46 restricted with Sma I; G. R46 non-restricted; H. ϕ X RF DNA restricted with Hae III; I. Lambda DNA restricted with Hind III; J. Plasmid R6K (encodes for TEM-1) restricted with EcoR I; K. R6K restricted with Ssp I; L. R6K non-restricted; M. pUK724 (encodes TEM-E4) restricted with EcoR I; N. pUK724 non-restricted; O. pUK723 restricted with EcoR I; P. pUK723 non-restricted; Q. pUK720 (encoding TEM-E1) restricted with Ssp I.

The R46 plasmid, encoding OXA-2 showed no hybridisation with the TEM-E1 probe. Restrictions of the OXA-2 encoding plasmid with Bgl I and Sma I also failed to yield fragments which hybridised with the TEM-E1 probe, even though these restrictions gave well defined bands on the original agarose gel. Similarly, the TEM-E1 probe did not hybridise with the R1010 plasmid which encodes for SHV-1 nor with any of the fragments produced by restrictions of this plasmid with EcoR I or Bgl I.

3.6. Ceftazidime resistant E.coli from Pakistan.

The work in the following three paragraphs was performed in conjunction with the Antibiotic Reference Laboratory, PHLS Colindale.

E.coli SHAFIQ Bilal (E.coli SB) was isolated from a patient at Queen Mary's University Hospital, London (table 20). The patient had previously been hospitalised in Pakistan. The strain was resistant to 3GC and it was found to possess three plasmids of approximate sizes, 80, 46, and 36Mdal. Mating experiments showed that the resistance to 3GCs was transferable to E.coli J62-1. This transconjugant possessed the 80Mdal plasmid along with a 28Mdal plasmid. The nature of the latter, which could not be demonstrated in the donor strain, was unclear, but may represent a deletion of a portion of one of the other parent strain plasmids.

Secondary matings were then conducted in an attempt to assess which of the two plasmids encoded the 3GC resistance determinant. The resultant E.coli J53-2 transconjugant, from this experiment, possessed only the 28Mdal plasmid. It was concluded that this plasmid encoded the transferable 3GC resistance. This E.coli J53-2 transconjugant was studied further to

characterise the factor responsible for transferable resistance to extended spectrum cephalosporins.

The 3GC resistance expressed by the E.coli J62-1 transconjugant (possessing the 80 and the 28Mdal plasmids) was shown to be transferable to recipient strains of Enterobacter cloacae 417, and Klebsiella oxytoca 478. These mating experiments yielded transconjugants with similar resistance profiles to E.coli SB. However, the resistance could not be transferred to either Proteus spp. or Serratia spp.

3.6.1. Conjugation experiments.

The E.coli J53-2 transconjugant (possessing only the 28Mdal plasmid) was mated with E.coli J62-2. Agar containing ampicillin (20mg/L) or ceftazidime (1mg/L), and the appropriate nutrients, was used to select for the transconjugants. However, these conjugation experiments were unsuccessful and no transconjugants were detected.

3.6.2. Antibiotic sensitivities.

The E.coli J53-2 transconjugant was resistant to all the first, second, and third generation cephalosporins tested (including cefoxitin) and, out of the 11 beta-lactam antibiotics studied, the strain was only sensitive to imipenem. The MICs of ceftazidime, cefotaxime, and ampicillin were not substantially reduced by the addition of clavulanic acid to the medium (table 41).

Table 41. MICs of E.coli SB and its E.coli J53-2 transconjugant.

	E.coli J53-2 recipient.	E.coli S.B.+	E.coli J53-2 transconjugant*.
Ampicillin	4	64	>250
Amp +clav	4	ND	>32
Carbenicillin	8	256	250
Cephaloridine	2	ND	125
Cephalexin	8	ND	>250
Cefoxitin	4	ND	64
Cefuroxime	4	>32	125
Ceftazidime	<1	>16	64
Caz +clav	0.13	ND	>4
Cefotaxime	<1	>16	16
Ctx +clav	<0.06	ND	>4
Ceftriaxone	<1	ND	32
Aztreonam	<1	ND	16
Imipenem	0.25	ND	0.25

+clav = in combination with clavulanic acid (2mg/L).

* Possesses only the 28Mdal plasmid.

+ MICs performed by the Antibiotic Reference Laboratory, PHLS Colindale.

ND = not done.

3.6.3. Isoelectric focusing.

Isoelectic focusing revealed that both the clinical isolate and the E.coli J53-2, E.cloacae 417, and K.oxytoca 478 transconjugants all produced a beta-lactamase which had a pI of 8.8 (fig. 29). This enzyme was given the preliminary designation of beta-lactamase BIL-1 after the patient it was isolated from.

3.6.4. Molecular weight.

The molecular weight of the beta-lactamase BIL-1 was determined as 29 000 by gel filtration with a Sephadex G-75 column. This was larger than any of the TEM- or SHV-derived 3GC hydrolysing beta-lactamases (table 25).

3.6.5. Hydrolysis of beta-lactam antibiotics by BIL-1.

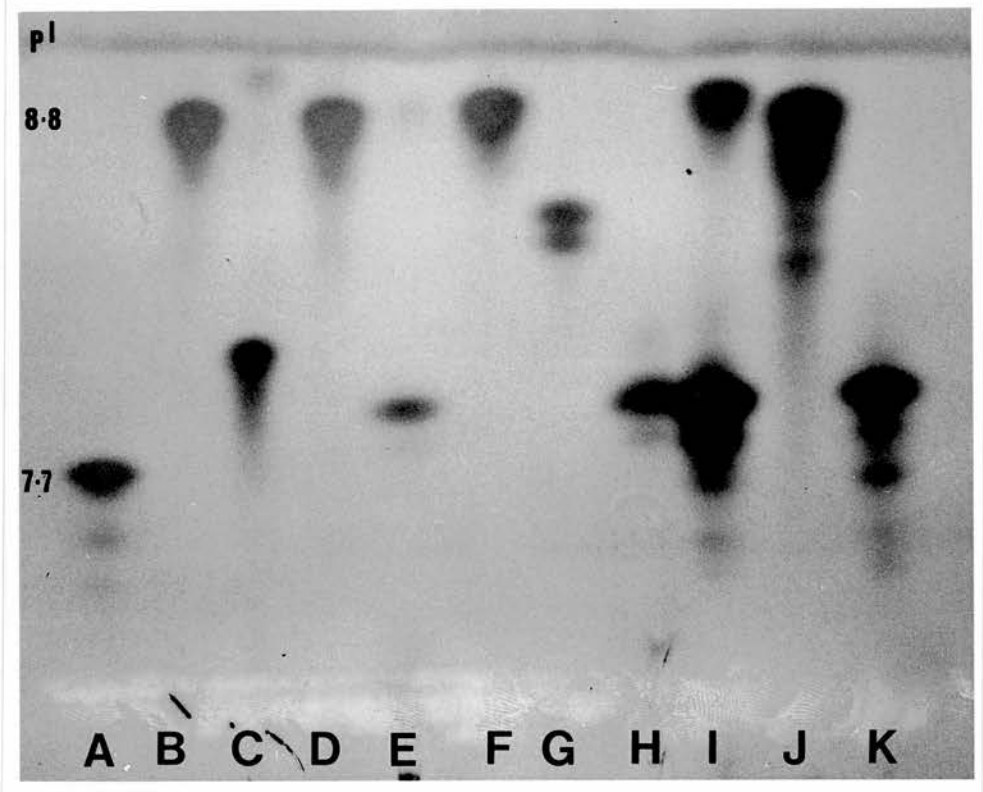
Enzyme BIL-1 had no detectable hydrolytic activity against cefotaxime, ceftazidime, cefuroxime, carbenicillin or ampicillin. However, hydrolysis of nitrocefin and cephaloridine was detectable (table 42).

BIL-1 hydrolysed nitrocefin and cephaloridine at similar rates but had a greater affinity for nitrocefin. BIL-1 and TEM-1 hydrolysed cephaloridine with similar efficiencies.

3.6.6. Inhibition of beta-lactamase BIL-1.

To test whether the novel enzyme was inhibited by the unhydrolysable

Fig. 29. IEF of novel enzyme and standard plasmid beta-lactamases of known pI.



A. E.coli J53-2 producing SHV-1; B. Original clinical isolate (E.coli SB) producing BIL-1; C. E.coli J62-1 producing SHV-5; D. E.coli J53-2 producing BIL-1; E. K.oxytoca 478; F. K.oxytoca 478 transconjugant producing BIL-1; G. CEP-1 (pI 8.2); H. Ent.cloacae 471; I. Ent.cloacae 471 transconjugant producing BIL-1; J. Ent.cloacae chromosomal beta-lactamase type A (pI 8.8); K. Ent.cloacaechromosomal beta-lactamase type B (pI 7.8).

Table 42. Kinetic constants for the hydrolysis of nitrocefin and cephaloridine by TEM-1 and BIL-1.

	Nitrocefin			Cephaloridine		
	Vmax	Km	Vmax/Km	Vmax	Km	Vmax/Km
TEM-1	2.44	77	100	2.00	166	38
Enzyme BIL-1	6.25	250	100	6.25	500	50

Efficiency of hydrolysis values (Vmax/Km) are expressed as a percentage of the value for nitrocefin.

Km value is measured in uM.

Vmax is measured in umoles/minute/ml of enzyme preparation.

Table 43. Concentration (uM) of beta-lactam antibiotics and clavulanic acid required to inhibit hydrolysis of nitrocefin by 50% (ID_{50} values).

Inhibitor.	TEM-1	Enzyme BIL-1
Ampicillin	224	4.20
Cefuroxime	>1000	0.04
Ceftazidime	>1000	8.90
Clavulanic acid	0.5	1995.00

substrates, the hydrolysis of nitrocefin was assayed in the presence of increasing concentrations of different beta-lactam compounds. The same assays were also performed on the TEM-1 beta-lactamase (table 43). These results show that ampicillin, ceftazidime, and cefuroxime are much more potent inhibitors of BIL-1 than TEM-1. Beta-lactamase BIL-1 was also almost 4000 times more resistant to inhibition by clavulanic acid than TEM-1.

3.6.7. Specific activity of BIL-1.

The specific activities of BIL-1 were compared with TEM-3 to TEM-10, TEM-E1 to TEM E4, and Mutant beta-lactamase A to E. The crude samples used in these assays all originated from E.coli J53-2 transconjugants of the original clinical isolates. These data revealed that the specific activity of BIL-1 was between 2 and 11 times greater than any of the other beta-lactamases investigated. This demonstrated that BIL-1 possessed the highest activity out of all the previously reported 3GC hydrolysing beta-lactamases.

3.7. Separation of plasmid mediated extended spectrum beta-lactamases by Fast Protein Liquid Chromatography System.

The 3GC resistant Klebsiella pneumoniae 8825 was isolated from a lymphoma patient at the Tate Memorial Hospital, Bombay, India (table 20). It was found to produce 4 beta-lactamases and the genes encoding 3 of these enzymes were transferred to an E.coli J53-2 recipient strain with a concomitant transfer of ceftazidime and cefotaxime resistance.

Extensive experimentation would normally be required to ascertain which of the 3 beta-lactamases was conferring transferable resistance to broad spectrum cephalosporins. Consequently, to investigate one of these enzymes it is imperative that it is separated from each of the other beta-lactamases. In the past these separation procedures were based on the enzymes ionic charge, because many of the beta-lactamases had similar molecular weights. For example, preparative IEF was used to separate TLE-2 from TEM-1 and SHV-1 (Reid et al., 1987) and as described previously electrodialysis has been used to separate TEM-E2 from TEM-1. This section discusses the development of a Fast Protein Liquid Chromatography System (FPLC System) technique for the rapid separation of three different beta-lactamases produced by the same strain. Previously, reverse phase High Performance Liquid Chromatography (HPLC) techniques have been used to

purify beta-lactamases such as SHV-1 (Barthelemy et al., 1988a), and FPLC System has been implemented to purify an inducible beta-lactamase produced by Proteus vulgaris (Cullmann and Seibert 1986), and a beta-lactamase from Clostridium butyricum (Kesado et al, 1989) but neither procedure has ever been used to separate three plasmid-encoded beta-lactamases produced by the same strain.

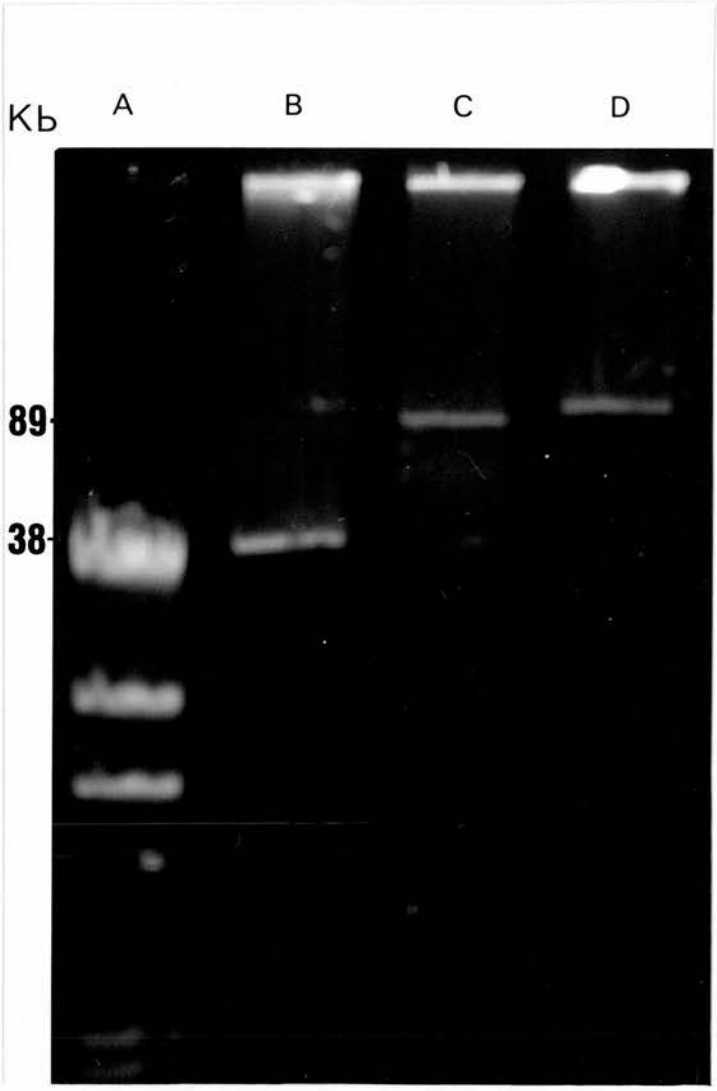
3.7.1. Conjugation experiments and plasmid analysis experiments.

E.coli J53-2 transconjugants of K.pneumoniae 8825 were detected on agar plates containing ceftazidime or carbenicillin. The frequency of transfer on the ceftazidime and rifampicin containing media was 1.18×10^{-6} per donor cell and the transfer frequency for the selection on carbenicillin and rifampicin was 3.53×10^{-6} per donor cell. Analysis of the plasmid DNA in the E. coli J53-2 transconjugant revealed plasmids of 100Kb and 2.5Kb (fig. 30). This suggested that the beta-lactamase genes conferring resistance to carbenicillin and ceftazidime were either one and the same or they were located on the same plasmid.

3.7.2. Antibiotic susceptibilities.

The K.pneumoniae 8825 strain was resistant to all the penicillins and to first, second and third generation cephalosporins tested, with the exception of cefoxitin. All these resistance determinants were transferred to the E.coli J53-2 in the conjugation experiments (table 44). In addition, the clinical strain and the transconjugant were both sensitive to imipenem. The K.pneumoniae 8825 and its transconjugant were sensitive

Fig. 30. Agarose gel electrophoresis of plasmid DNA from the E.coli J53-2 transconjugant of K.pneumoniae 8825 along with standard size plasmids.



A. Lambda restricted with Hind III; B. R6K; C. R1; D. Plasmid isolated from E.coli transconjugant of K.pneumoniae 8825.

Table 44. Antibiotic susceptibilities of strains.

	Minimum inhibitory concentrations (mg/L)		
Beta-lactam	K.pneumoniae 8825	K.pneumoniae 8825 X E.coli J53-2	E.coli J53-2
Ampicillin	>250	>250	4
+ clav*	8	8	4
Carbenicillin	>250	>250	8
Cephaloridine	125	32	2
Cephalexin	125	64	8
Cefoxitin	4	4	4
Cefuroxime	32	125	4
Ceftazidime	125	64	<1
+ clav*	0.25	0.25	0.13
Cefotaxime	8	8	<1
+ clav*	<0.06	<0.06	<0.06
Ceftriaxone	16	4	<1
Aztreonam	125	32	<1
Imipenem	0.13	0.25	0.25

* in combination with clavulanic acid (2mg/L)

to ampicillin, ceftazidime and cefotaxime in the presence of clavulanic acid (2mg/L), inferring that the beta-lactamases responsible for resistance to these drugs were either of the TEM or SHV groups.

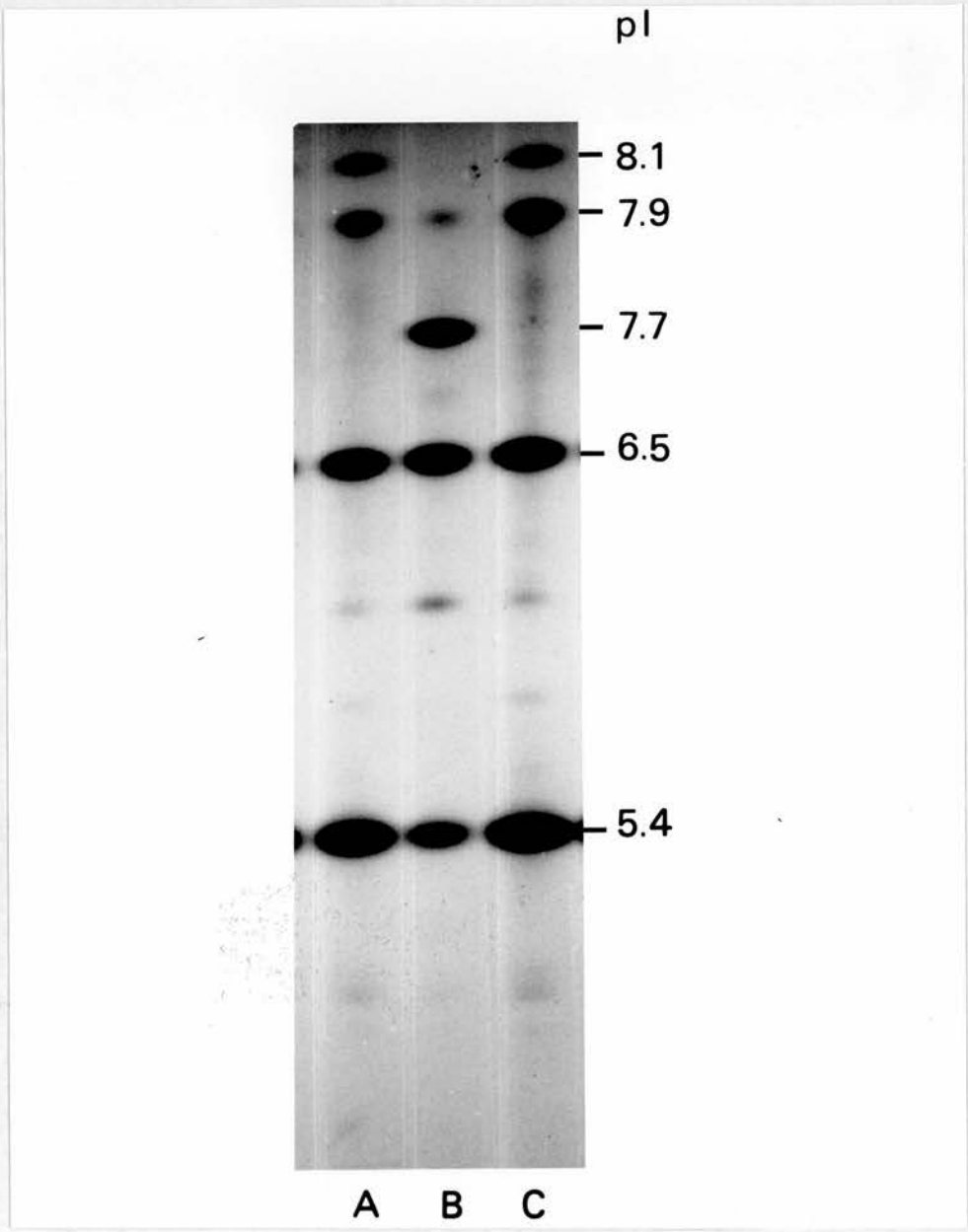
3.7.3. Identification of beta-lactamases.

The original K.pneumoniae isolate produced four beta-lactamases which focused at pI 5.4, pI 6.5, pI 7.7 and pI 7.9. The band at pI 5.4 aligned with TEM-1, the enzyme of pI 6.5 focused between TEM-3 (pI 6.3) and SHV-3 (pI 7.0) and the enzyme of pI 7.7 aligned with SHV-1/2. Crude enzyme preparations of the E.coli J53-2 transconjugants selected on carbenicillin and rifampicin or ceftazidime and rifampicin possessed the beta-lactamases which focused at pI 5.4, pI 6.5 and pI 7.9. However, they additionally produced another beta-lactamase which focused at pI 8.1 (fig. 31). This high pI band aligned directly with the E.coli J53-2 chromosomal beta-lactamase.

3.7.4. Preparation of beta-lactamase sample for FPLC System analysis.

As described in the methods section, a concentrate of sonicated cells from a 1 litre broth culture of the E.coli J53-2 transconjugant of K.pneumoniae 8825 was applied to a Sephadex G-75 column. Each fraction was examined for beta-lactamase activity. It was observed that, following purification by gel filtration, the intensities of the beta-lactamases of pI 7.9 and pI 8.1 were reduced. The fractions which exhibited beta-lactamase activity were pooled and dialysed against 50mM Tris-HCl buffer (pH 8.2) overnight.

Fig. 31. IEF of beta-lactamases produced by K.pneumoniae 8825 and its E.coli J53-2 transconjugant.



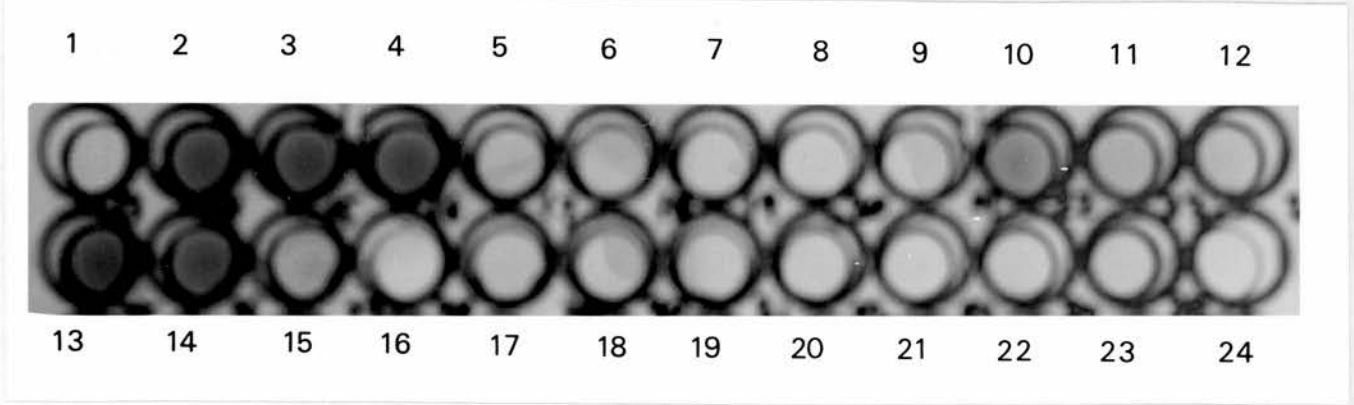
A. Beta-lactamases produced by the E.coli transconjugant of K.pneumoniae 8825 selected on ceftazidime and rifampicin; B. Beta-lactamases produced by K.pneumoniae 8825; C. Beta-lactamases produced by the E.coli J53-2 transconjugant of K.pneumoniae 8825 selected on carbenicillin and rifampicin.

3.7.5. FPLC System analysis.

Separation of the beta-lactamases was carried out with the Pharmacia FPLC System consisting of a LCC 500 Plus Controller, UV-M Monitor, and FRAC 100 with an HR5/5 Mono Q column. The column was equilibrated with 50mM Tris-HCl buffer (pH 8.2). Separation was achieved by elution with a linearly increasing concentration of sodium chloride (in the above buffer) to a maximum concentration of 1M.

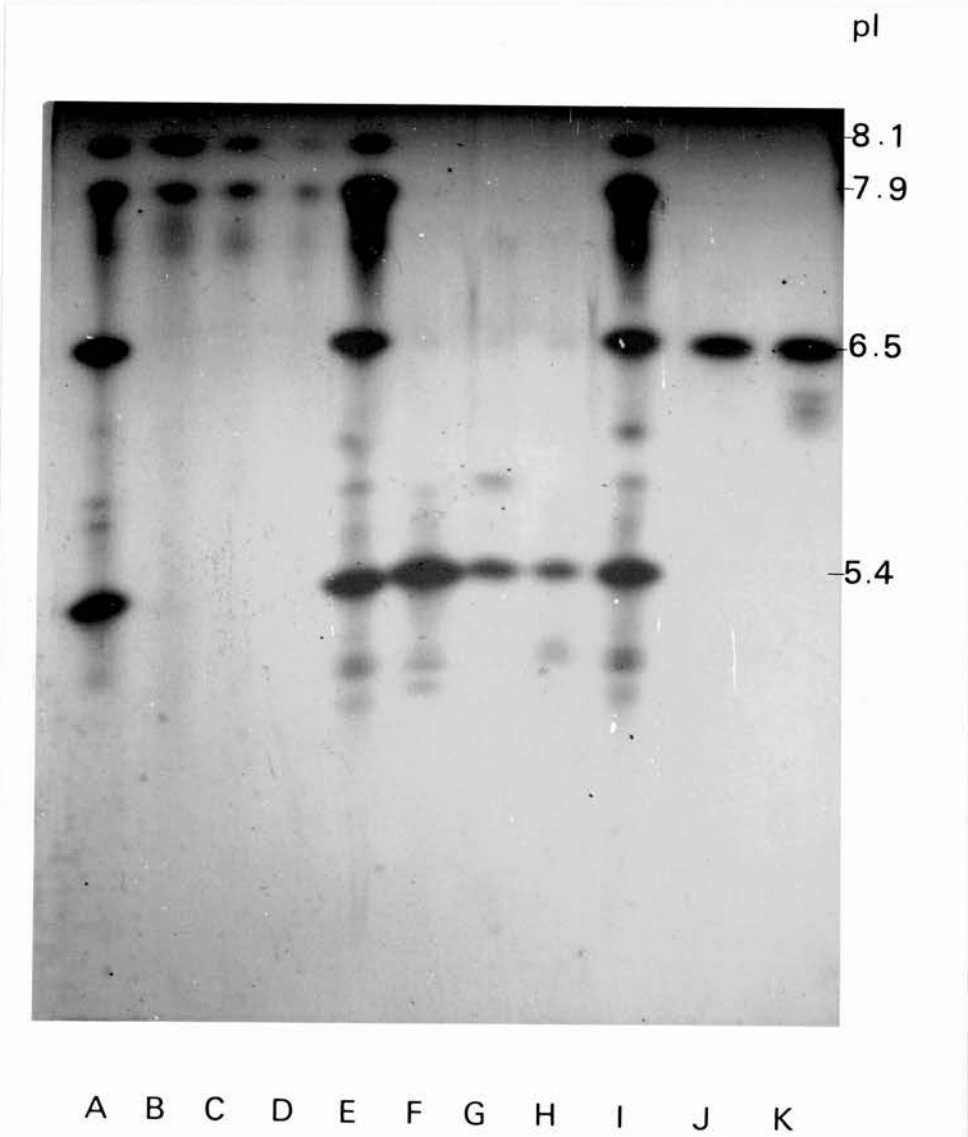
Two ml of the Sephadex G-75 purified beta-lactamase sample were separated through the Mono Q column in each run. Approximately, 34 fractions (1ml) were collected from each 30 minute separation. Each aliquot was tested for beta-lactamase activity by the chromogenic cephalosporin nitrocefin spot test. This process was repeated until sufficient quantities of the different enzymes were obtained. In each FPLC System separation, beta-lactamase activity was detected in the following fractions: 2, 3, and 4 (the void volume), 11/10, and 13/14 (fig. 32). When these fractions were examined by IEF the beta-lactamases of pI 7.9 and pI 8.1 were eluted in fractions 2, 3, 4, the enzyme of pI 6.5 in fractions 10/11, and the beta-lactamase of pI 5.4 in fractions 13/14 (fig. 33). The FPLC trace shows the position where each of the fractions were eluted, and demonstrates the relatively huge quantities of other proteins present in this partially purified beta-lactamase preparation (fig. 34). All the fractions, from the individual separations, containing the same beta-lactamase were combined so that sufficient enzyme could be characterised biochemically.

Fig. 32. Microtitre plate showing nitrocefin spot tests of the fractions eluted from the FPLC System.



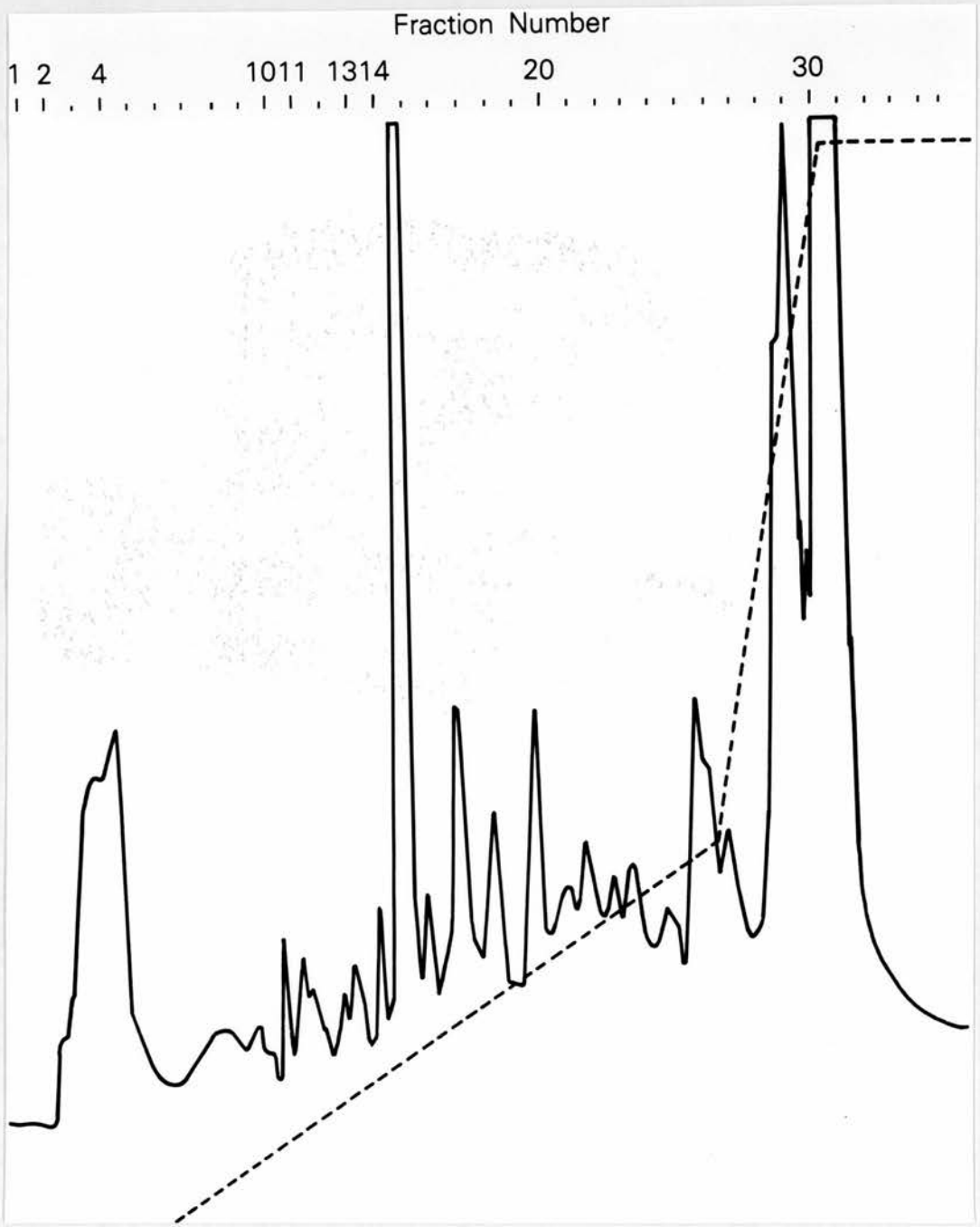
The dark wells denote beta-lactamase activity where the nitrocefin has been hydrolysed and turned from yellow to red. Nitrocefin spot tests of FPLC System fractions (numbers denote the number of the fraction eluted from the FPLC System).

Fig. 33. IEF gel of beta-lactamases separated by FPLC System.



A. Beta-lactamases produced by the E.coli J53-2 transconjugant of K.pneumoniae 8825 purified by Sephadex gel filtration; B. Fraction 2 eluted from FPLC System; C. Fraction 3; D. Fraction 4; E. Crude beta-lactamase preparation of the E.coli J53-2 transconjugant of K.pneumoniae; F. Fraction 14 eluted from FPLC System; G. Fraction 13; H. Fraction 15; I. As for E; J. Fraction 10 eluted from FPLC System; K. Fraction 11.

Fig. 34. FPLC System trace for the separation of the beta-lactamases produced by the E.coli J53-2 transconjugant of K.pneumoniae 8825.



The broken line represents the increase in sodium chloride concentration. The solid line represents protein concentration, measured at 280nm.

3.7.6. Characterisation of beta-lactamases.

The hydrolytic activity of each of the three enzymes against six different beta-lactam antibiotics are shown in table 45. The beta-lactamase which focused at pI 5.4 aligned with TEM-1, it also had no hydrolytic activity against ceftazidime or cefotaxime and was therefore assumed to be TEM-1. The beta-lactamase of pI 6.5 had hydrolytic activity against cefuroxime, cefotaxime, ceftazidime and had a greater activity for cephaloridine than TEM-1. The fractions containing enzymes of pI 7.9 and pI 8.1 had a similar pattern of hydrolytic activities but the activity against the newer cephalosporins must result from the enzyme of pI 7.9 and not the upper E.coli J53-2 chromosomal beta-lactamase band, as control studies have shown that this chromosomal enzyme has no hydrolysing activity against these beta-lactam antibiotics. The beta-lactamase of pI 7.9 was designated as DJP-1.

Table 45. *Relative rates of hydrolysis of FPLC System fractions.

Beta-lactam / Fraction: / pI:	+Pooled	2-4 7.9 & 8.1	10-11 6.5	13-14 5.4 (TEM-1)
Ampicillin	100.0	100.0	100.0	100.0
Carbenicillin	16.0	29.0	18.0	17.0
Cephaloridine	5.9	114.0	125.0	20.0
Cefuroxime	2.9	4.3	17.0	UM
Cefotaxime	4.2	17.0	9.2	UM
Ceftazidime	1.5	4.6	14.0	UM

*Values expressed as a percentage of the value for ampicillin.

UM = unmeasurable due to insufficient hydrolysis.

+Pooled fractions = combined hydrolytic activity of Sephadex G-75 extract before purification by FPLC System.

3.8. Studies on the satellite bands of beta-lactamases.

The isoelectric focusing of most beta-lactamases yield a main beta-lactamase band along with smaller bands of slightly different pIs from the main band. These additional bands have been referred to as beta-lactamase satellite bands. Simpson and Plested (1983) showed that the different satellite bands of beta-lactamases produced by Citrobacter diversus and Branhamella catarrhalis had similar substrate profiles, molecular weights and susceptibilities to inhibitors. However, there is little other documentation on the nature of these beta-lactamase bands. The experiments discussed in section 3.8.1. led to an extensive investigation of beta-lactamase satellite bands.

3.8.1. Investigation of the inducibility of the TEM-5 beta-lactamase.

K.pneumoniae CF504 produced the TEM-1, TEM-5 and SHV-1 beta-lactamases. The genes encoding TEM-1 and TEM-5 were transferred to an E.coli J62-2 recipient strain. Both K.pneumoniae CF504 and the E.coli transconjugant (designated E.coli Nb) were grown at different concentrations of ceftazidime to establish whether TEM-5 was an inducible beta-lactamase.

a. Procedure implemented to study the induction of TEM-5.

One hundred ml nutrient broth cultures of K.pneumoniae CF504 and E.coli Nb were grown overnight in an orbital shaker at 37^0C. Thirty ml of each of the overnight cultures were added to two separate flasks containing 300ml of pre-warmed nutrient broth. These cultures were left on the orbital shaker until the optical density of each culture reached 0.7 OD_{500}. E.coli Nb reached this absorbance after 3 hours and 50 minutes, whereas the K.pneumoniae reached an absorbance of 0.8 in 2 hours. Fifty ml of sterile nutrient broth were added to six 250ml conical flasks. Table 46 shows the volume of a 6.4mg/ml ceftazidime solution which was added to each flask to obtain the desired range of ceftazidime concentrations. Finally, 50ml of the E.coli Nb culture were added to each of the six flasks. This procedure was repeated with K.pneumoniae CF504. These cultures were then incubated in the orbital shaker for 2 hours. The cells from each broth culture were then harvested by centrifugation and crude beta-lactamase preparations were made from each of the cell pellets (as described in section 2.7). The protein concentrations of each of the 12 different enzyme samples were determined and their beta-lactamases examined by IEF.

b. Isoelectric focusing of crude beta-lactamase preparations.

The volume of beta-lactamase sample loaded onto the IEF gel was standardized so that an equal amount of protein was added from each of the

Table 46. Composition of media used in induction experiments and the absorbance reading of each culture prior to centrifugation.

Strain.	Volume of 6.4mg/ml ceftazidime solution added (ml).	Concentration of ceftazidime in flask after culture added (mg/L).	Absorbance of culture when harvested (OD_{500}).
E.coli Nb	-	-	0.80
E.coli Nb	0.064	4	0.75
E.coli Nb	0.125	8	0.70
E.coli Nb	0.250	16	0.70
E.coli Nb	0.500	32	0.70
E.coli Nb	1.000	64	0.70
K.pneumoniae CF504	-	-	0.85
K.pneumoniae CF504	0.125	8	0.80
K.pneumoniae CF504	0.250	16	0.80
K.pneumoniae CF504	0.500	32	0.71
K.pneumoniae CF504	1.000	64	0.70
K.pneumoniae CF504	2.000	125	0.71

beta-lactamase preparations obtained from the different growth conditions. The IEF gel is shown in fig. 35. K.pneumoniae CF504 appeared to express a larger SHV-1 band as the concentration of ceftazidime was increased (not shown). However, no change in the intensities of the TEM-1 or TEM-5 bands were observed in the preparations originating from either K.pneumoniae CF504 or E.coli Nb.

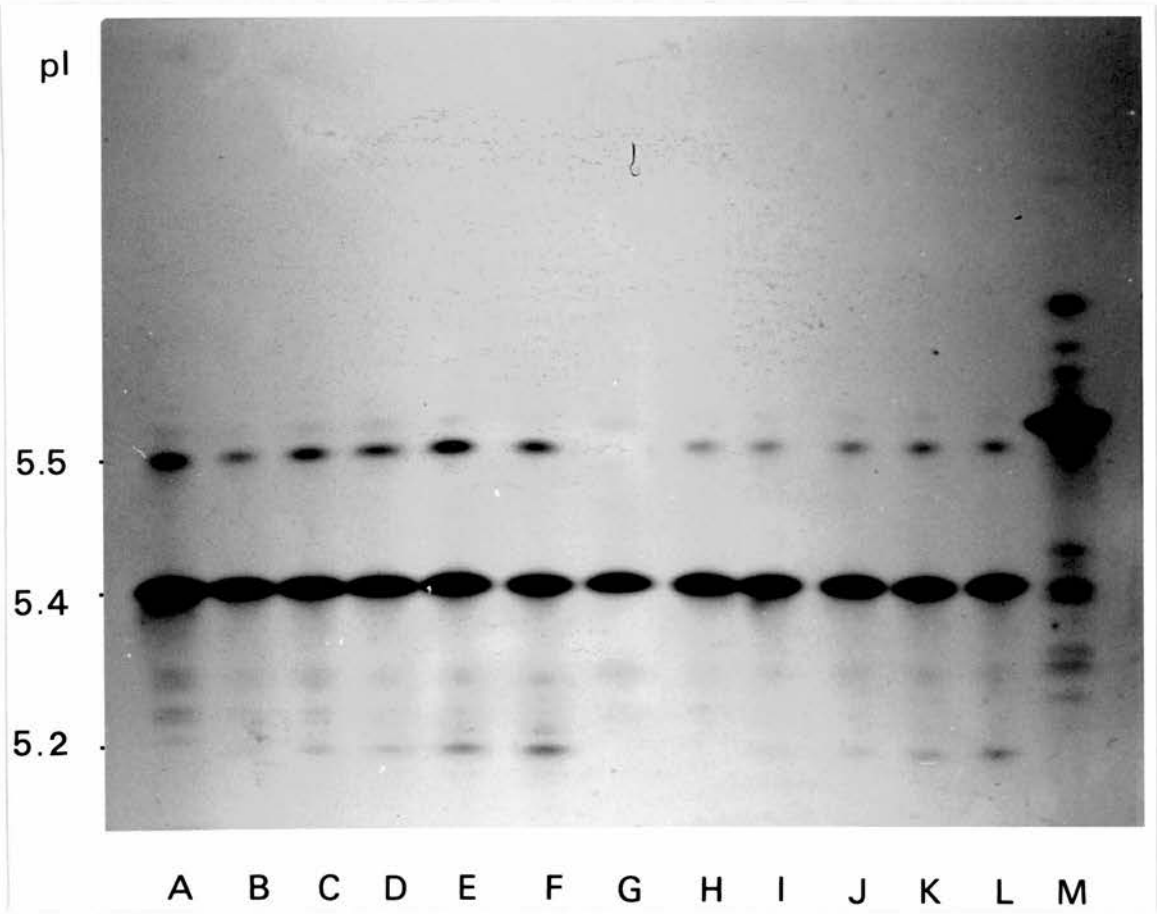
However, as the concentration of ceftazidime in the broth cultures increased there was a reciprocal increase in the intensity of a beta-lactamase band of pI 5.2, this phenomenon was identified with both the K.pneumoniae CF504 and E.coli Nb (fig. 35).

c. Antibiotic sensitivities.

In the following experiment, E.coli Nb was grown in a range of ceftazidime concentrations as described in section 3.8.1a. but, just before the cells were harvested, 5ml aliquots of each of the different cultures were removed and their MICs of ceftazidime were directly determined. A loopful from each of these 5ml aliquots was plated onto a nutrient agar plate and incubated overnight at 37°C. The MICs of ceftazidime were also performed on each of these strains.

The MICs of ceftazidime for each of these strains showed no significant differences (table 47).

Fig. 35. IEF of beta-lactamases produced by K.pneumoniae CF504 and E.coli Nb grown with increasing concentrations of ceftazidime (narrow range IEF).



Track M = TEM-2

Track No.	A	B	C	D	E	F	G	H	I	J	K	L
Strain.	E.coli Nb						TEM-1	K.pneumoniae CF504				
Conc. of CAZ in broth (mg/L).	-	4	8	16	32	64	-	-	16	32	64	125
Protein content of enzyme preparation (mg/ml).	17	14	17	15	16	19		13	13	16	14	14
Volume of sample loaded onto gel (ul).	10	12	10	11	11	9		10	10	8	9	9
B-lactamases present. TEM-5	+	+	+	+	+	+		+	+	+	+	+
TEM-1	+	+	+	+	+	+		+	+	+	+	+
Induced band			+	+	+	+			+	+	+	+

Table 47. MICs of ceftazidime for E.coli Nb strains grown at different concentrations of ceftazidime.

Concentration of ceftazidime in original broth culture (mg/L).	Minimum inhibitory concentration of ceftazidime (mg/L).	
	MICs performed directly on broth culture.	MICs performed on strains plated out on agar.
0	16	16
4	16	16
8	16	16
16	16	16
32	16	16
64	8	16
125	16	16
250	16	8

d. Kinetic characteristics of beta-lactamase preparations.

The beta-lactamases produced by the E.coli Nb at each of the ceftazidime concentrations were assayed for their ability to hydrolyse ceftazidime and cephaloridine. All of these beta-lactamase preparations had similar rates of hydrolysis against ceftazidime, when hydrolysis of cephaloridine was taken as 100%. The values ranged between 3.6% and 4.3% with no significant relationship with the increase in ceftazidime concentration (table 48). In addition to this no significant trend was observed for the specific activities of each beta-lactamase preparation for ceftazidime or cephaloridine.

These experiments concluded that TEM-5 was not an inducible beta-lactamase but a satellite band was induced as the concentration of ceftazidime was increased.

3.8.2. Investigation of beta-lactamase satellite bands.

Experiments were set up to determine if satellite bands induced in the experiments described in section 3.8.1. were actually caused by ceftazidime interaction with the beta-lactamase proteins produced by E.coli Nb or K.pneumoniae CF504.

Table 48. Brief biochemical characterisation of beta-lactamases produced by E.coli Nb grown at different concentrations of ceftazidime.

Concentration of CAZ in original culture (mg/L).		0	4	8	16	32	64	125	250
Rate of hydrolysis of CAZ (rate with CER = 100%).		3.7	4.0	3.9	3.8	3.6	3.9	4.2	4.3
Specific activities*.	CER	6.85	9.04	6.89	8.27	8.14	6.34	4.16	4.29
	CAZ	0.25	0.36	0.26	0.31	0.30	0.24	0.18	0.19

CAZ = ceftazidime.
CER = cephaloridine.
*measured in nmoles of substrate hydrolysed/minute/mg of protein.

The beta-lactamase sample obtained from the E.coli Nb culture, grown in the absence of ceftazidime, did not possess the satellite band of approximate pI 5.2. This preparation was divided into six 0.1ml aliquots. Table 49 shows the volume of a 10mg/ml solution of ceftazidime which was added to each 0.1ml aliquot to achieve a range of beta-lactamase samples containing an increasing concentration of ceftazidime. Each of these enzyme samples were immediately applied to an IEF gel. The volume of each preparation loaded onto the gel was adjusted to take into account the dilution caused by the addition of the ceftazidime solution.

The IEF gel (fig. 36) illustrates that the addition of ceftazidime to the beta-lactamase samples promoted the same satellite band which was induced by growing E.coli Nb in increasing concentrations of ceftazidime.

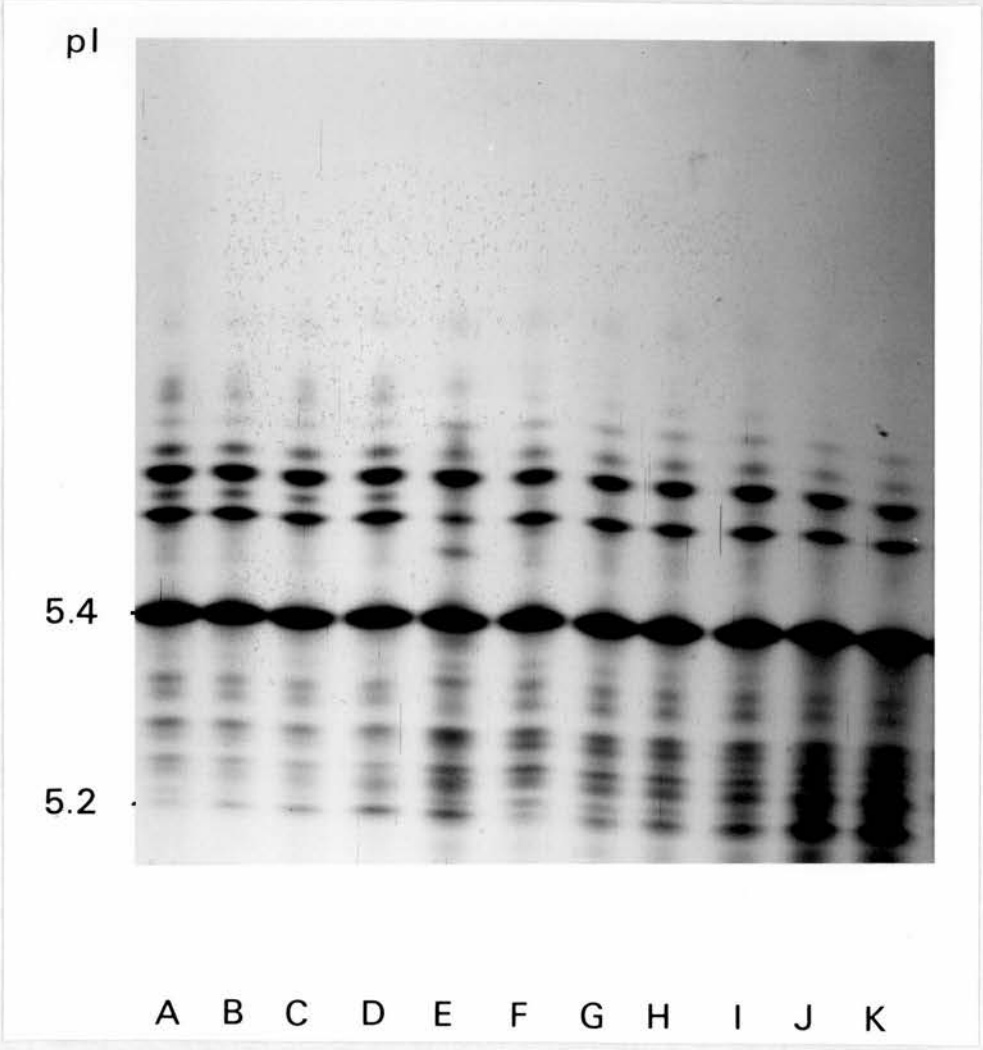
a. Effects of ceftazidime on the IEF of TEM-1.

The procedure described above was repeated with the TEM-1 beta-lactamase derived from E.coli J53-2, 2136E. The amounts of a 40mg/ml solution of ceftazidime added to each aliquot of enzyme, and the volumes loaded on to the IEF gel are shown in table 50. The IEF illustrated that ceftazidime had induced a satellite band from TEM-1 which had the same pI as the band induced from the beta-lactamases of E.coli Nb (fig. 37).

Table 49. Composition of enzyme samples electrophoresed in fig. 36.

Volume of 10mg/ml ceftazidime solution added to sample (ul).	Concentration of ceftazidime in enzyme sample (mg/ml).	Volume loaded onto IEF gel (ul).
0	0	3.0
1	0.1	3.0
2	0.2	3.1
4	0.4	3.1
10	0.9	3.3
20	1.7	3.6
100	5.0	6.0

Fig. 36. IEF gel illustrating how ceftazidime causes the production of an extra satellite band.



Tracks A-E are beta-lactamases prepared from E.coli J62-2 transconjugant of K.pneumoniae 2977E grown in increasing concentration of ceftazidime, A. 0, B. 4, C. 8, D. 16, E. 32 (mg/L of ceftazidime).

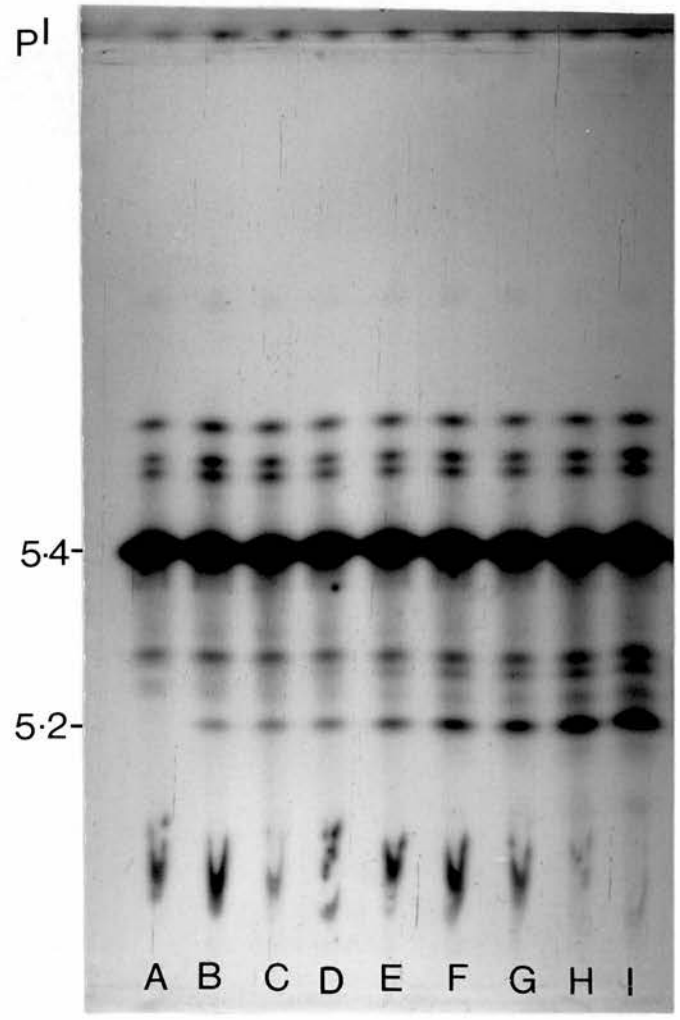
Tracks F-L are beta-lactamase preparations from the E.coli J62-2 transconjugant of K.pneumoniae 2977E containing increasing concentrations of ceftazidime, F. 0.1, G. 0.2, H. 0.4, I. 0.9, J. 1.7, K. 5.0 (mg/ml of ceftazidime).

The TEM-5 band (pI 5.5) appeared to dissociate into two bands after storage of the beta-lactamase preparation.

Table 50. Composition of TEM-1 beta-lactamase samples electrophoresed in fig. 37.

Volume of 40mg/ml ceftazidime solution added to sample (ul).	Concentration of ceftazidime in TEM-1 sample (mg/ml).	Volume loaded onto IEF gel (ul).
0.0	0.0	3.0
0.8	0.3	3.0
1.7	0.7	3.1
3.1	1.2	3.1
6.3	2.4	3.2
12.5	4.4	3.4
25.0	8.0	3.8
50.0	13.3	4.5
100.0	20.0	6.0

Fig. 37. The effect of CAZ on the IEF of TEM-1.



Prep.	Concentration of CAZ in enzyme prep. (mg/ml).
A.	0.0
B.	0.3
C.	0.7
D.	1.2
E.	2.4
F.	4.4
G.	8.0
H.	13.0
I.	20.0

b. Effects of incubation on the induction of beta-lactamase satellite bands.

A TEM-1 beta-lactamase preparation containing 5mg/ml was divided into two aliquots, one of these aliquots was left at room temperature and the other was incubated at 37°C for 2 hours. IEF analysis of these two samples revealed that the incubation period had resulted in the induction of a larger satellite band.

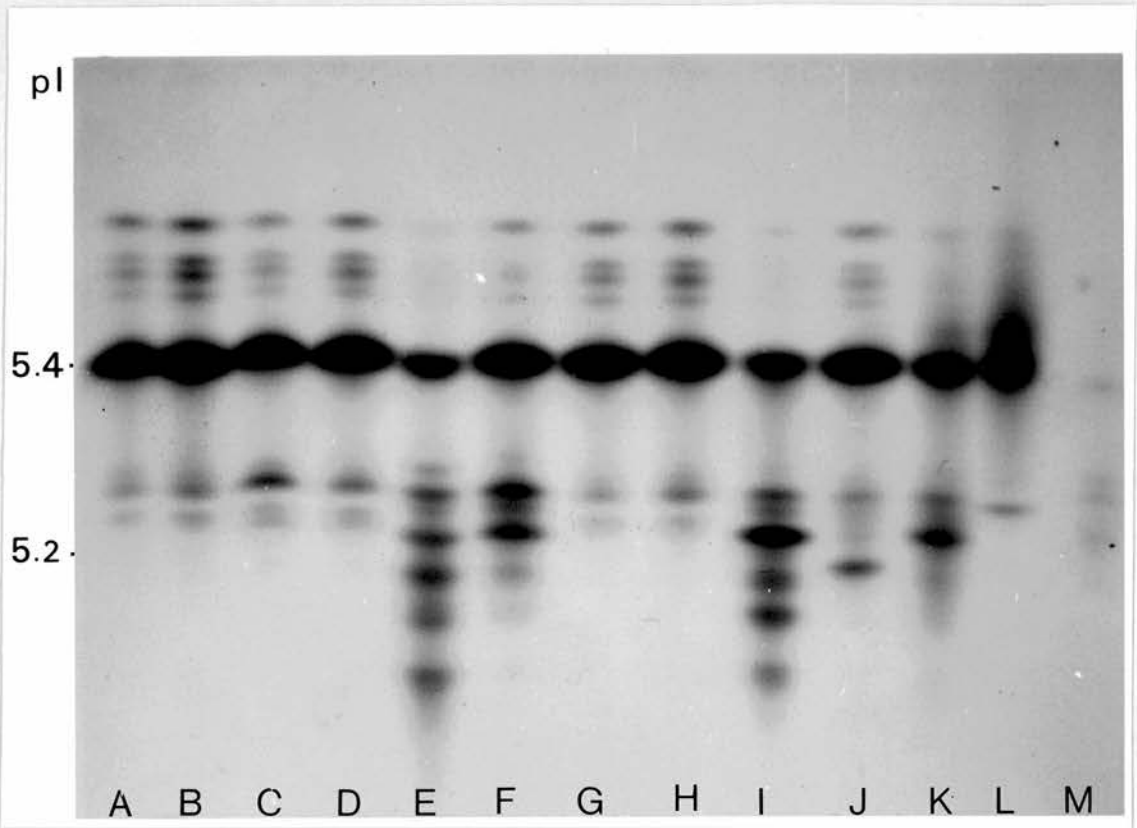
c. Effects of different beta-lactams on the TEM-1 beta-lactamase.

A TEM-1 beta-lactamase sample derived from E.coli J53-2, 2136E was divided into 13 0.1ml aliquots. 10ul of a 70mg/ml solution of 12 different beta-lactam antibiotics were added to each of the aliquots to obtain a final ceftazidime concentration of 6.4mg/ml. These preparations, including the TEM-1 aliquot containing no ceftazidime, were incubated at 37°C for 2 hours. Each aliquot (5ul) was then loaded onto an IEF gel. Fig. 38 shows that each beta-lactam induced slightly different satellite bands when compared with the TEM-1 control (containing no beta-lactam).

d. Effects of the addition of ceftazidime to other TEM derived 3GC hydrolysing beta-lactamases.

Beta-lactamase preparations of 16 TEM derived beta-lactamases were prepared as described in section 2.7. The beta-lactamase activity of each

Fig. 38. The effect of different beta-lactams on the isoelectric focusing of TEM-1.



Track.	Beta-lactam added to enzyme prep.	Conc. of beta-lactam in enzyme prep (mg/ml).
A.	Penicillin	6.4
B.	Ampicillin	6.4
C.	Methicillin	6.4
D.	Carbenicillin	6.4
E.	Cephaloridine	6.4
F.	Cefoxitin	6.4
G.	No beta-lactam	0.0
H.	Cephradine.	6.4
I.	Cefuroxime	6.4
J.	Ceftazidime	6.4
K.	Cefotaxime	6.4
L	Nitrocefin	6.4
M	Clavulanic acid	6.4

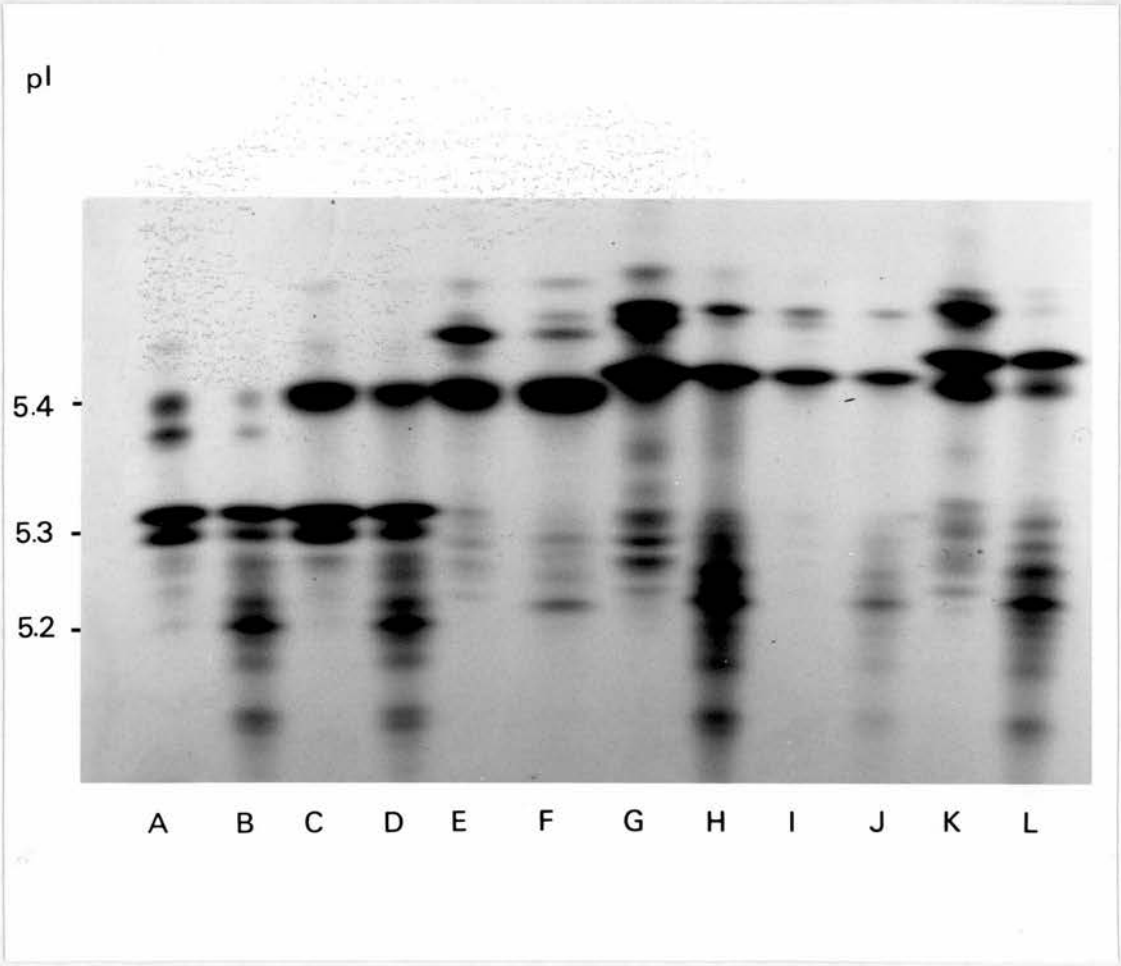
sample was determined by the nitrocefin spot test method (section 2.8) and this determined the volume loaded onto the IEF gel. Two 0.1ml aliquots were removed from each preparation and 0.01ml of a 70mg/ml solution of ceftazidime was added to one of the aliquots to give a final ceftazidime concentration of 6.4mg/ml. Both 0.1ml aliquots were then incubated at 37°C for 2 hours.

An equal volume of both aliquots, from each of the different beta-lactamases, was examined by IEF. With the exception of the TEM-3 and TEM-4 beta-lactamases, ceftazidime induced a satellite band in the region of pI 5.2 with all the TEM derived beta-lactamases (figs. 39-41).

e. Examination of the satellite bands produced by non-TEM like beta-lactamases.

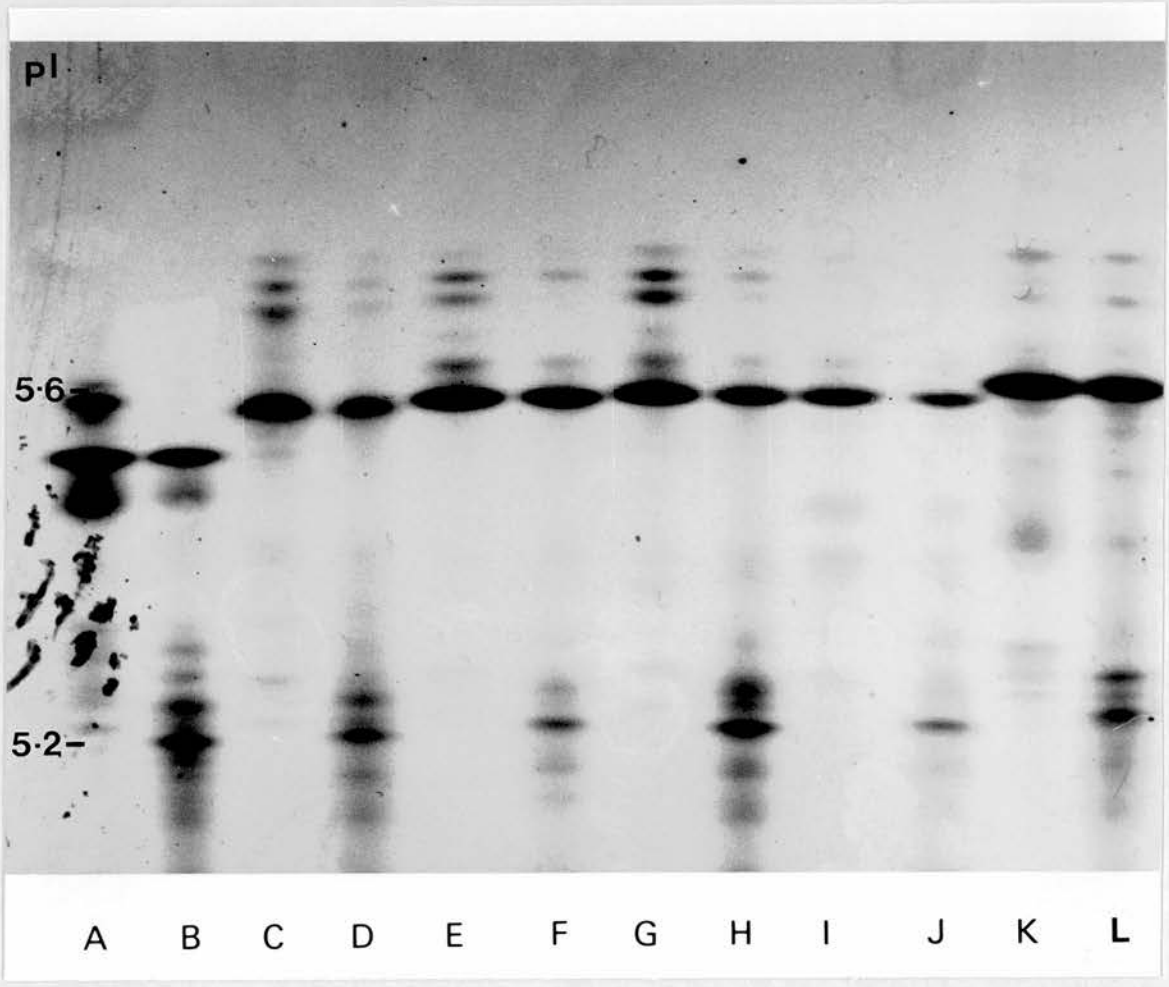
By implementing the same protocol as explained in section 3.8.2c the effects of ceftazidime on the induction of satellite bands from PSE-1, PSE-2, K-1 (chromosomal beta-lactamase from K.aerogenes 1082E), SHV-2, and SHV-3 beta-lactamases were investigated. Fig. 42 showed that ceftazidime induced no extra satellite bands from the PSE-1, or PSE-2 beta-lactamases. The addition of ceftazidime to the chromosomal klebsiella beta-lactamase, K-1, induced a beta-lactamase satellite band which was similar to the band induced from the TEM beta-lactamases. SHV-2 and SHV-3 produced extra satellite bands when mixed with ceftazidime but these were different to those bands induced by ceftazidime from the TEM beta-lactamases.

Fig. 39. Isoelectric focusing patterns of TEM like beta-lactamases, with and without ceftazidime (Mutant beta-lactamase A - TEM-7).



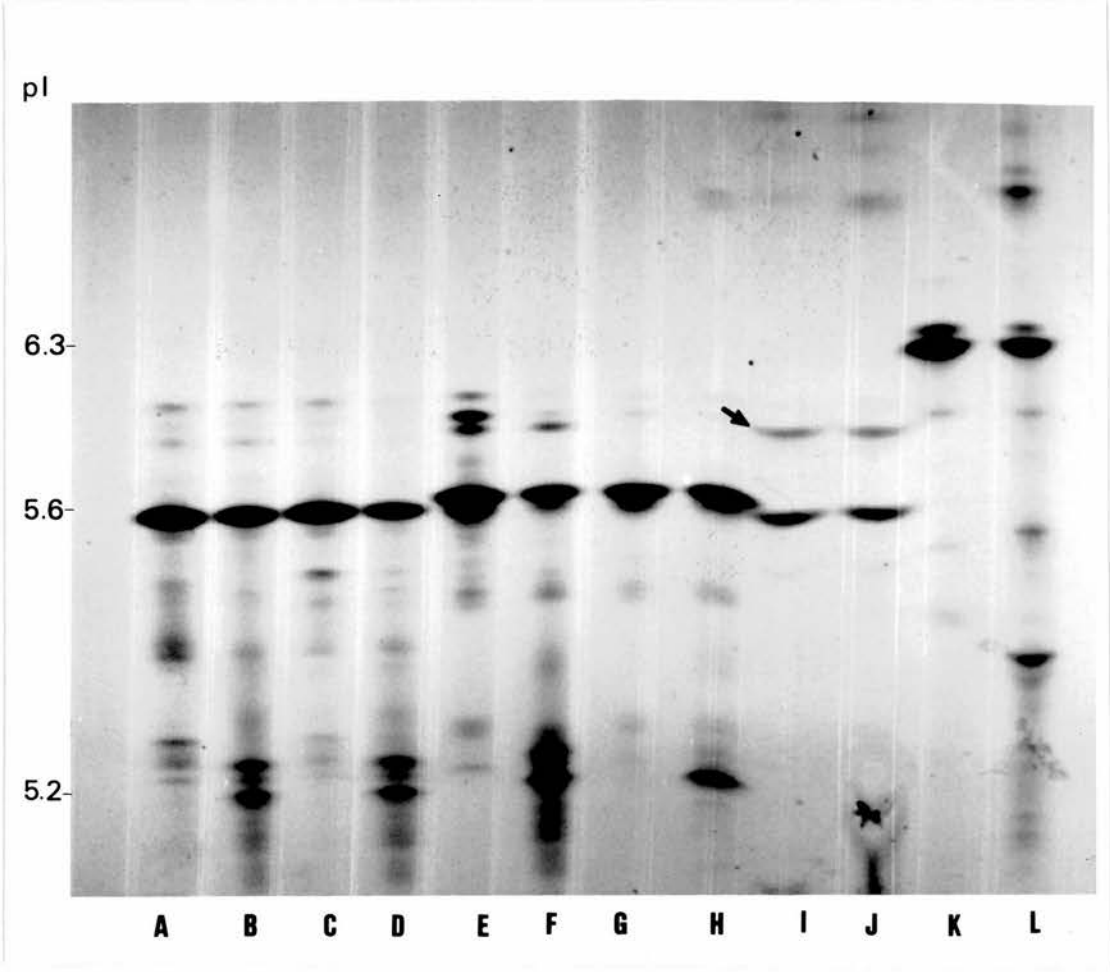
A. Mutant beta-lactamase A; B. Mutant beta-lactamase A + CAZ; C. TEM-E2; D. TEM-E2 + CAZ; E. TEM-1; F. TEM-1 + CAZ; G. TEM-E1; H. TEM-E1 + CAZ; I. Mutant beta-lactamase B; J. Mutant beta-lactamase B + CAZ; K. TEM-7; L. TEM-7 + CAZ (+ CAZ = 7mg/L ceftazidime.

Fig. 40. Isoelectric focusing patterns of TEM like beta-lactamases, with and without ceftazidime (TEM-7 - TEM-2).



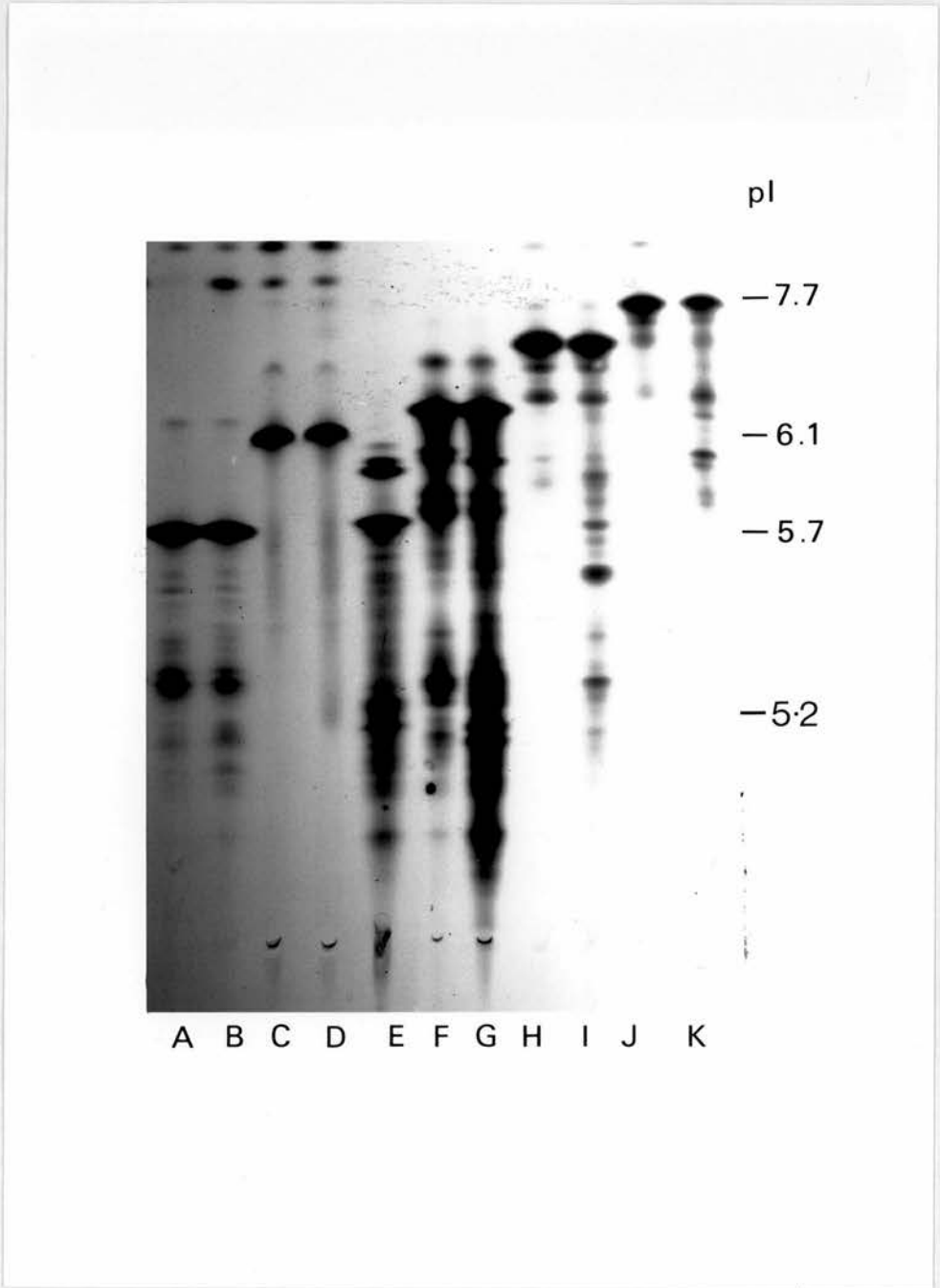
A. TEM-7; B. TEM-7 + CAZ; C. TEM-9; D. TEM-9 + CAZ; E. TEM-5; F. TEM-5 + CAZ; G. TEM-E3; H. TEM-E3 + CAZ; I. TEM-10; J. TEM-10 + CAZ; K. TEM-2; L. TEM-2 + CAZ.

Fig. 41. Isoelectric focusing patterns of TEM like beta-lactamases, with and without ceftazidime (TEM-2 - TEM-3).



A. TEM-2; B. TEM-2 + CAZ; C. Mutant beta-lactamase D; D. Mutant beta-lactamase D +CAZ; E. TEM-E4; F. TEM-E4 + CAZ; G. Mutant beta-lactamase D; H. Mutant beta-lactamase D + CAZ; I. TEM-4; J. TEM-4 + CAZ; K. CTX-1; L. CTX-1 + CAZ (the arrow denotes the TEM-4 band).

Fig. 42. IEF illustrating the effects of the addition of ceftazidime to non-TEM beta-lactamases.



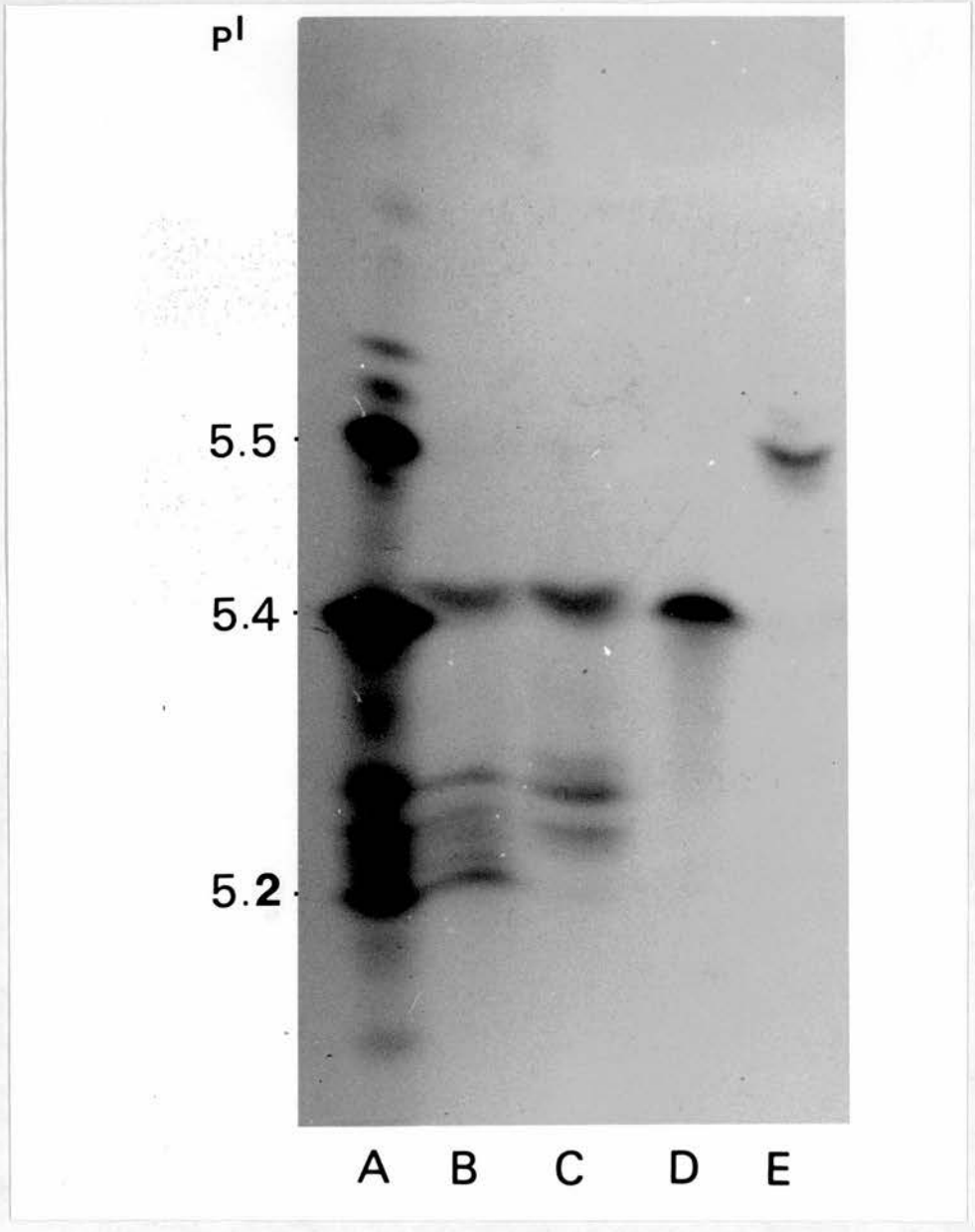
A. PSE-1; B. PSE-1 + CAZ; C. PSE-2; D. PSE-2 +CAZ; E. TEM-E4 +CAZ; F. K-1 (klebsiella chromosomal beta-lactamase); G. K-1 + CAZ; H. SHV-3; I. SHV-3 +CAZ; J. SHV-2; K. SHV-2 + CAZ.

f. Isolation of beta-lactamase satellite bands.

Fig. 43 track 1 shows the beta-lactamases produced by E.coli Nb grown in the presence of ceftazidime (125mg/L). The E.coli Nb strain produced TEM-1, TEM-5 and two prominent beta-lactamase satellite bands which focused below TEM-1. Satellite band 1 was the band induced by the presence of ceftazidime and has been fully described in the former sections. Satellite band 2 focused between satellite band 1 and TEM-1 with an approximate pI 5.3.

Each of these four bands were cut out of the IEF gel and separated from the polyacrylamide gel by electrodialysis (as described in section 3.4.2d). The purified TEM-1 and TEM-5 aligned with the TEM-1 and TEM-5 bands of the original beta-lactamase preparation in track 1. When the satellite band 1 was re-focused both the original satellite band and a band which focused with a similar pI to TEM-1 were observed, a similar phenomenon was demonstrated with the re-focusing of satellite band 2.

Fig. 43. Illustration of how electrodialysis can be used to purify beta-lactamase satellite bands from an analytical IEF gel.



A. TEM-5, TEM-1 and satellite bands 1 and 2 produced by E.coli Nb grown at 125mg/L ceftazidime; B. Satellite band 1 (induced by ceftazidime, pI 5.2) extracted from IEF gel by electrodialysis; C. Satellite band 2 extracted from IEF gel by electrodialysis (approximate pI 5.3); D. TEM-1 band extracted from IEF gel by electrodialysis; E. TEM-5 band extracted from IEF gel by electrodialysis.

4.0. DISCUSSION

It has been considered that the 3GC hydrolysing beta-lactamases occurred from the TEM and SHV-1 resistance genes. This section discusses the experiments which were performed to substantiate this theory, along with a review of all the 3GC hydrolysing beta-lactamases which have been identified.

4.1. Mutation experiments.

4.1.1. Comparison of mutants from this Thesis with those previously reported.

All the mutant 3GC hydrolysing enzymes obtained spontaneously from beta-lactamase producing strains which originally conferred no resistance to 3GCs are listed in table 51.

Table 51. Properties of 3GC hydrolysing beta-lactamases obtained spontaneously from TEM-1/2.

Host strain used in mutation	β-lactamase & plasmid used.	Selecting agent.	Mutation frequency.	Mutant designation. & pI.	MIC(mg/L). CTX	MIC(mg/L). CAZ	Reference
E.coli BM694	TEM-1 pBR322	CAZ	$5x10^{-9}$	TEM-101 5.3	0.06	4	Gutmann et al (1988)
E.coli J53-2	TEM-1 R6K	CAZ	$1x10^{-8}$	TEM-121-124 5.3	0.06	4	Sougakoff et al (1988)
E.coli BM694	TEM-2 R6K	CAZ	$5x10^{-9}$	TEM-201 5.41	0.06	32	Gutmann et al (1988)
E.coli J53-2	TEM-2 RP4	CAZ	$3x10^{-9}$	TEM-221 5.41	0.12	16	Sougakoff et al (1988b)
E.coli J53-2	TEM-2 RP4	CTX	$3x10^{-9}$	TEM-226-229 5.6	1	1	Sougakoff et al (1988b)
K.pneumoniae CF124	TEM-2 pCF24	CAZ	$1.6x10^{-9}$	TEM-222-225 5.41	0.12	8	Sougakoff et al (1988b)

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Table 51. Properties of 3GC hydrolysing beta-lactamases obtained spontaneously from TEM-1/2, PSE-4 and CAZ-1o.

Host strain used in mutation	β-lactamase & plasmid used.	Selecting agent.	Mutation frequency.	Mutant designation. & pI.	MIC(mg/L). CTX	MIC(mg/L). CAZ	Reference
K.pneumoniae	CAZ-1o	CAZ	$1x10^{-8}$	CAZ-hi-mutant 6.5	0.25	32	Vuye et al (1989)
E.coli J62-2 Nc	TEM-1 100kb	CAZ	$1x10^{-9}$	A 5.3 (doublet)	0.06	16	This Thesis
E.coli J62-2 Nc	TEM-1 100kb	CAZ	$1x10^{-9}$	B 5.41	<0.06	8	This Thesis
E.coli J53-2 2137E	TEM-2 RP4	CAZ	$5x10^{-8}$	C 5.6	<0.06	4	This Thesis
E.coli J53-2 2137E	TEM-2 RP4	CAZ	$1x10^{-9}$	D 5.61	<1	8	This Thesis
E.coli J53-2 AR273	PSE-4 R1818	CAZ	$1x10^{-7}$	E 5.3	<0.06	8	This Thesis

Where possible, the MICs values correspond to the resistance conferred by the mutant enzymes in E.coli K12 derivatives.

Previous reports of 3GC resistant mutants arising spontaneously from TEM-1, have detailed only one type of mutant, called TEM-101 by Gutmann et al. (1988) and TEM-121 to TEM-124 by Sougakoff et al. (1988). Both of these enzymes confer an MIC of ceftazidime of 4mg/L and have a pI of 5.3. This enzyme has a similar pI to Mutant enzyme A but it does not focus as two distinct bands of equal intensity. Other reported mutant enzymes, which were derived from TEM-2 were called TEM-201 by Gutmann et al. (1988) and TEM-221 to TEM-225 by Sougakoff et al.(1988), these enzymes had the same pI as TEM-7. Mutant enzyme B has a pI below TEM-7 and was obtained from TEM-1, illustrating that Mutant enzyme B is clearly different from other mutant enzymes previously reported.

Mutant beta-lactamase C and TEM-226 - TEM-229 (Sougakoff et al., 1988) were both obtained from TEM-2 and had pIs of 5.6. However, Mutant beta-lactamase C confers a greater resistance to ceftazidime than TEM-226 - 229 which suggests that these two mutant enzymes may not be identical.

Mutant beta-lactamase D was obtained spontaneously from TEM-2 and focused marginally above TEM-2 at pI 5.61. Consequently, it was different from any of the other previously reported mutant beta-lactamases.

Mutant beta-lactamase E was obtained from a non-TEM beta-lactamase. This implies the worrying possibility that other non-TEM beta-lactamases may be capable of mutating to create beta-lactamases which can confer transferable resistance to 3GCs.

4.1.2. Transferability of plasmids encoding Mutant beta-lactamases A to E.

Mutant beta-lactamase A and B were freely transferable, although at low frequency. The ceftazidime resistance conferred by Mutant beta-lactamase C could not be transferred when kanamycin was the selecting agent. However, ceftazidime resistant colonies, producing Mutant beta-lactamase C, were detected when ceftazidime was the selecting agent. As these colonies occurred at a very low frequency, it is possible that they were mutant colonies rather than true transconjugants. Therefore, the gene encoding Mutant beta-lactamase C was very possibly chromosomally mediated.

Transfer experiments with Mutant beta-lactamases D and E, each revealed much higher transfer frequencies when the selection was performed with kanamycin than when performed with ceftazidime. No transconjugants were detected when kanamycin and ceftazidime were used in combination as the selecting agents. Consequently, it appears that ceftazidime kills the transconjugants before they can produce sufficient quantities of the ceftazidimase to protect themselves from the antibacterial effects of the drug. However, it must be considered that the difference in transfer frequency may be the result of the presence of two plasmids, one of which has remained undetected.

4.1.3. Selection of mutant enzymes conferring high level resistance to 3GCs.

The experiments performed to obtain a mutant enzyme conferring high level resistance were unsuccessful. Similarly, it was not possible to obtain a mutant enzyme from TEM-E1 which would confer both cefotaxime resistance as well as ceftazidime resistance. However, Vuye et al. (1989) have shown that a clinically derived 3GC hydrolysing beta-lactamase, CAZ-lo, can mutate spontaneously to CAZ-hi which confers up to 16 times more resistance to ceftazidime. This work also showed that CAZ-hi is produced by clinical isolates.

4.1.4. Relevance of spontaneous mutation experiments.

Table 52 illustrates the similarities between mutant beta-lactamases and clinically derived beta-lactamases. Consequently, these experiments have illustrated the possible origins of TEM-7, TEM-E1, TEM-E2 and TEM-E4. Mutant beta-lactamase C and CAZ-lo have identical pIs and confer similar resistances. It is therefore probable that the two enzymes were the same. This illustrates that CAZ-lo may have evolved spontaneously from a TEM-2 producing organism.

Table 52. Mutant beta-lactamases which are similar to clinically derived beta-lactamases.

Beta-lactamase obtained by spontaneous mutation of TEM-1/2.	Corresponding beta-lactamase produced by clinical isolates.	Reference.
TEM-201	TEM-7	Gutmann et al., 1988
TEM-221	TEM-7	Sougakoff et al., 1988b
TEM-222 - 225	TEM-7	Sougakoff et al., 1988b
Mutant beta-lactamase A	TEM-E2	This Thesis
Mutant beta-lactamase B	TEM-E1	This Thesis
Mutant beta-lactamase C	CAZ-1o	This Thesis
Mutant beta-lactamase D	TEM-E4	This Thesis
*CAZ-hi (mutant)	CAZ-hi	Vuye et al., 1989

* obtained from CAZ-1o.

4.1.5. The use of mutagens to obtain 3GC hydrolysing beta-lactamases.

The beta-lactamases obtained from TEM-1 and TEM-2 were only selected at low levels of the drug and confer only a low level of resistance to ceftazidime. Therefore, there must be another factor responsible for the occurrence of those TEM-like enzymes which confer higher and broader levels of 3GC resistance.

The initial isolation of some of the high level resistance TEM enzymes were from patients undergoing a cocktail of drug treatment including methotrexate and metronidazole. The effects of methotrexate and metronidazole on the selection of 3GC hydrolysing beta-lactamases were investigated but neither of these drugs gave any conclusive results. However, further experimentation may reveal that other anticancer drugs or drug combinations are the additional factors required to select for TEM-like beta-lactamases which give higher levels of 3GC resistance. It may be that only powerful mutagens yield mutant beta-lactamases which confer high level resistance to 3GCs. Indeed, it has been shown that CAZ-hi could be obtained from CAZ-lo with more ease when using N-methyl-N'-nitro-N-nitrosoguanidine (Vuye et al., 1989).

4.2. Clinically derived 3GC hydrolysing beta-lactamases.

All the 3GC hydrolysing beta-lactamases which have been previously reported, along with those characterised in this Thesis, are summarised in table 53.

As explained in the Introduction there have been a number of reports of 3GC hydrolysing beta-lactamases which have derived from the TEM beta-lactamase gene. These enzymes have been designated TEM-3, TEM-4 and so on. However, at the time when this work was performed there was confusion in the literature over the next available TEM number. Consequently, to avoid different beta-lactamases being assigned the same TEM number, the "TEM-E" series of beta-lactamases was used. This nomenclature denotes that the beta-lactamase is TEM-like and characterised in Edinburgh. It is hoped that this will avoid the situation which arose with TEM-10, this enzyme was originally referred to as TEM-8 (Quinn et al., 1988; Chanal et al., 1989a), but later re-named TEM-10 (Quinn et al. 1989).

4.2.1. The TEM-E1 beta-lactamase.

This enzyme is different to any of the previously reported 3GC hydrolysing beta-lactamases, and has not been identified in any other strain, other than the E.coli 7891 isolated in Belgium in 1987.

Table 53. The 3GC hydrolysing beta-lactamases.

β-lactamase. (pI)	Year of initial isolation.	Original Isolate.	Original location.	Subsequent reports in different areas.	Approx. No. isolates reported to produce the enzyme.	References.
TEM-E1 (5.41)	1987	E.coli	Belgium	None cited	1	This Thesis
TEM-E2 (5.3-doublet)	1982	K.oxytoca	UK	USA-Two reports of a similar enzyme.	1 (confirmed)	This Thesis Smith et al. 1989 Jiang et al. 1989
TEM-E3 (5.55)	1987	E.coli	UK	USA-Two strains, enzyme designated TEM-10. UK-Royal Marsden Hos.	5	This Thesis Quinn et al. 1989
TEM-E4 (5.61)	NS	S.marcescens	Belgium	None cited	1	This Thesis
DJP-1 (8.?)	1988	K.pneumoniae	India	None cited	2	This Thesis
BIL-1 (8.8)	1989	E.coli	Pakistan	None cited	1	This Thesis
TEM-3 (6.3)	1984	K.pneumoniae	France	France-Toulouse, St.Etienne and Paris	>490	Sirot et al. 1988 Petit et al. 1990 Brun Buission et al. 1987.

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Table 53. The 3GC hydrolysing beta-lactamases.

β-lactamase. (pI).	Year of initial isolation.	Original Isolate.	Original location.	Subsequent reports in different areas.	Approx. No. of isolates reported to produce the enzyme.	References.
TEM-4 (5.9)	1986	E.coli	France	None cited	1	Paul et al. 1989
TEM-5 (5.55)	1987	K.pneumoniae	France	None cited	14	Petit et al. 1988 Chanal et al. 1989b
TEM-6 (5.9)	1986	E.coli	West Germany	None cited	15	Bauernfeind et al. 1987 & 1989
TEM-7 (5.41)	NS	C.freundii	France	None cited	1	Gutmann et al. 1988
TEM-9 (5.5)	NS	K.pneumoniae	UK	None cited	3	Spencer et al. 1987
CAZ-2 (TEM) (6.0)	1987	K.pneumoniae	France	None cited	19	Chanal et al. 1988a Chanal et al. 1989b
CAZ-3 (TEM) (5.3)	1987	K.pneumoniae	France	None cited	1	Sirot et al. 1989
CAZ-6 (TEM) (6.5)	1988	K.pneumoniae	France	None cited	10	Chanal et al. 1989b
CAZ-7 (TEM) (6.3)	1988	K.pneumoniae	France	None cited	6	Chanal et al. 1989b

continued on next page.

Table 53. The 3GC hydrolysing beta-lactamases.

β-lactamase. (pI)	Year of initial isolation.	Original Isolate.	Original location.	Subsequent reports in different areas.	Approx. No. of isolates reported to produce the enzyme.	References.
SHV-2 (7.7)	1983	K.ozaenae	West Germany	Chile, China, Tunisia, Greece Egypt, Switzerland France & USA	NS	Kliebe et al. 1985 Jacoby et al. 1988 Shannon et al. 1990 Labia et al. 1988 Jarlier et al. 1988 Thomson et al. 1990
SHV-3 (7.0)	1985-7	K.pneumoniae	France	None cited	35	Jarlier et al. 1988 Nicolas et al. 1988
SHV-4 (CAZ-5) (7.8)	1987	K.pneumoniae	France	Clermont-Ferrand , & Paris.	50	Sirot et al. 1989 Bure et al. 1988
SHV-5 (CAZ-4) (8.2)	1987	K.pneumoniae	Chile	Greece, France.	3	Gutmann et al. 1989 Shannon et al. 1990 Sirot et al. 1989
FEC-1 (8.2)	NS	E.coli	Japan	None cited	1 (isolated from experimental dog)	Matsumoto et al 1988
CAZ-1o (5.6)	1988	K.pneumoniae (main spp. isolated)	Belgium	None cited	17	Claeys et al. 1989

continued on next page.

Table 53. The 3GC hydrolysing beta-lactamases.

β-lactamase. (pI)	Year of initial isolation.	Original Isolate.	Original location.	Subsequent reports in different areas.	Approx. No. of isolates reported to produce the enzyme.	References.
CAZ-hi (6.5)	1988	K.pneumoniae (main spp. isolated)	Belgium	None cited	5	Claeys et al. 1989
FUR (7.5)	1988	K.pneumoniae (main spp. isolated)	Belgium	None cited	>100	Claeys et al. 1989
pMG 225 (5.55)	1988	K.pneumoniae	USA	None cited	2 (novelty not confirmed)	Jacoby et al. 1988
No name (5.7-5.9)	1986	Ent.cloacae	UK	None cited	2	Corkill et al. 1989
MJ-1 (5.35)	NS	K.oxytoca	France	None cited	1	Deschaseaux et al. (1988)
MJ-2 (5.55)	NS	C.amalonaticus	France	None cited	1	Deschaseaux et al. (1988)

4.2.2. The TEM-E2 beta-lactamase.

TEM-E2 was produced along with TEM-1, from which it was separated by the quick and easy method of electrodialysis. It was shown that Mutant beta-lactamase A has very similar properties to TEM-E2. The E.coli J53-2 strain producing Mutant beta-lactamase A exhibited a similar 3GC resistance profile to the E.coli J53-2 transconjugant producing TEM-E2 and TEM-1. Therefore, as the mutant strain exhibited virtually no TEM-1 activity, it is apparent that the TEM-1 did not significantly contribute towards ceftazidime resistance.

MIC studies and isoelectric focusing have revealed that TEM-E2 is probably the same beta-lactamase as CAZ-3 (results not shown) but the isolation of the strain producing TEM-E2 predates the discovery of CAZ-3 by five years.

The first plasmid mediated resistance to 3GCs was identified in Germany in 1983 (Knothe et al., 1983). This transferable resistance was later found to be conferred by SHV-2. Therefore, as the original clinical isolate producing TEM-E2 was isolated in 1982 the appearance of TEM-E2 represents not only the earliest example of a transferable TEM-like beta-lactamase, but also the first plasmid encoded beta-lactamase to confer resistance to any of the new broader spectrum cephalosporins.

When K.oxytoca 5445 was first isolated the ceftazidime resistance was

quoted as "not being beta-lactamase mediated because only TEM-1 was present". This illustrates that 3GC hydrolysing TEM-derived beta-lactamases may have been in bacterial populations long before they were actually identified, but they evaded correct identification because they have pIs very similar to TEM-1/2.

4.2.3. The TEM-E3 beta-lactamase.

Strains producing TEM-E3 have been isolated in two London Hospitals in 1987. A year after these strains were identified TEM-E3 was identified in the USA, designated TEM-10 by Quinn et al. (1989). The similarities of the TEM-E3 beta-lactamases were confirmed by their resistance profiles and standard biochemical assays. These tests also successfully distinguished TEM-E3 from TEM-5 and TEM-9.

It is hard to conclude whether the occurrences of TEM-E3 in the two different London hospitals were independent of each other because little is known about the patient at the Royal Marsden Hospital from which Enterobacter cloacae 7923 was isolated. It is not known whether the patient had any indirect or direct contact with the North Middlesex Hospital. Moreover, although the strains encoding TEM-E3 from the different London hospitals were distinctly different, the plasmids encoding the two TEM-E3 beta-lactamases were very similar in size and they could be related. It is more probable that the occurrence of TEM-E3 in the

USA was an independent event as the plasmid encoding the TEM-E3 enzyme was significantly different from either pUK722 or pUK723.

4.2.4. The TEM-E4 beta-lactamase.

This beta-lactamase is different from any of the other previously reported 3GC hydrolysing beta-lactamases and has not been identified in any other strain other than the strain of Serratia marcescens 7919 isolated in Belgium.

4.2.5. Properties of TEM-E1, TEM-E2, TEM-E3 and TEM-E4.

TEM-E1 to TEM-E4 show strong similarities with TEM-1 and many of the other 3GC hydrolysing TEM-like beta-lactamases. Furthermore, TEM-E1, TEM-E2 and TEM-E4 can all be obtained spontaneously from a TEM-1 or TEM-2 producing organism, which is strong evidence that all three of these enzymes were TEM-derived. Also TEM-E1, TEM-E3 and TEM-E4 hybridise with a TEM-E1 probe which hybridises with TEM-1 but not with SHV-1 or OXA-1 genes. There is a 68% homology between the amino acid sequences of TEM and SHV (Barthelemy et al. 1988a). Therefore, the probe is fairly specific to TEM sequences. The mutation and probing experiments confirm that the genes encoding TEM-E1 to TEM-E4 are highly homologous with the nucleotide sequence of TEM-1/2.

4.3. K.pneumoniae 8825 and the use of Fast Protein Liquid Chromatography System (FPLC System) to purify beta-lactamases.

This work has shown that Fast Protein Liquid Chromatography System is the most powerful method to date in the separation of multiple beta-lactamases produced by the same strain. Its rapid, complete separation of the enzymes enabled the biochemical characterisation of each individual beta-lactamase and permitted an assessment of how each beta-lactamase contributed to the range of beta-lactam resistances expressed by the host strain. It was thus possible to show that two of the enzymes produced by the clinically derived K.pneumoniae 8825 strain confer resistance to cefuroxime, cefotaxime, ceftriaxone and ceftazidime. Evaluation of the biochemical profile of each of these enzymes strongly suggests that the beta-lactamase pI 7.9 (beta-lactamase DJP-1) is a novel 3GC hydrolysing enzyme. However, the plasmid mediated beta-lactamase of pI 6.5 may be the same as CAZ-hi (Vuye et al., 1989) or CAZ-6 (Chanal et al., 1989b), both of which have a reported pI of 6.5. The majority of transferable 3GC resistance has arisen in France, Germany, and the U.K. where broad spectrum cephalosporin usage is high. However, these two broad spectrum enzymes were found to be produced by a strain isolated in India where the use of these newer cephalosporins is much lower. The occurrence of these two beta-lactamases is the first report of transferable 3GC resistance in either India or even Asia. It is also the first example of two broad spectrum beta-lactamases

encoded by the same plasmid.

4.4. The BIL-1 beta-lactamase.

The original clinical isolate which produced BIL-1 contained a number of plasmids. BIL-1 was shown to be transferable from this strain to a number of other recipient strains from a range of different species (work performed by the Antibiotic Reference Laboratory, PHLS Colindale). However, following a series of conjugation experiments involving the transfer of the BIL-1 gene, a transconjugant which expressed BIL-1, was found to possess a single 28 Mdal plasmid. This plasmid could not be transferred to another E.coli recipient. This suggested that BIL-1 was encoded by a plasmid which was not freely transferable and possibly relied on the presence of other plasmids to mobilise its transfer.

Previously, all the other plasmid mediated beta-lactamases which confer resistance to 3GCs have been shown to have derived from the ubiquitous TEM-1/2 and SHV-1 resistance genes. All of these TEM- or SHV- derived beta-lactamases are sensitive to clavulanic acid and hydrolyse penicillins and certain cephalosporins.

However, BIL-1 differs from TEM-1/2 or SHV derived beta-lactamases for the following reasons;

a. It confers transferable resistance to clavulanic acid, cefoxitin as well as all generations of cephalosporins.

b. It is resistant to inhibition by clavulanic acid.

c. It does not hydrolyse ampicillin, cefuroxime, ceftazidime or cefotaxime although it confers resistance to all these beta-lactams.

d. Ampicillin, cefuroxime and ceftazidime (at concentrations less than 10uM) inhibit the hydrolytic activity of BIL-1.

BIL-1 closely resembles the isoelectric focusing characteristics and biochemical hydrolysis profiles of the chromosomally mediated beta-lactamases of Enterobacter cloacae (Seeberg et al., 1983) and E.coli (Minami et al., 1980b). Therefore, BIL-1 is the first example of a beta-lactamase which confers transferable resistance to all generations of cephalosporins, cefoxitin, and clavulanic acid. This work also illustrates the first report of a gene encoding a Class C-like (Ambler, 1980), or a Class I chromosomal beta-lactamase (Richmond and Sykes, 1973) which has migrated into plasmid DNA.

The plasmid-carriage of the BIL-1 gene and the *in vitro* transfer studies to bacteria of different species raises the worrying possibility that this resistance gene may well be capable of migrating into diverse genera of clinical bacteria by *in vivo* transfer. Although no direct patient-to-patient spread of the strain or the resistance gene was observed it is possible that this could take place, especially if patients who are prone to infection are in close proximity. Therefore, it is suggested that patients transferred from outside the UK and harbouring bacteria with unknown resistance genes should be kept separate from patients prone to infection.

Shannon *et al.* (1990) expressed a similar concern when they suspected that strains producing 3GC hydrolysing SHV-like beta-lactamases, isolated from patients at St. Thomas Hospital London, were acquired by the patients in Egypt and Greece.

4.6. Classification of plasmid mediated 3GC hydrolysing beta-lactamases.

The 3GC hydrolysing beta-lactamases can be categorised into three groups by virtue of their efficiency of hydrolysis values for ceftazidime and cefotaxime relative to ampicillin or penicillin. This method of classification was chosen because it takes into account both the binding and the hydrolysis capabilities of the enzymes with ceftazidime and

cefotaxime. These two beta-lactams were selected because they were the two substrates most consistently tested by other workers and they produced a relatively varied effect with each of beta-lactamases listed in table 53.

As there are not efficiency of hydrolysis values for all the 3GC beta-lactamases not all the 3GC beta-lactamases have been classified in this scheme. Those enzymes which have had their hydrolytic capabilities measured only in terms of Vmax or rate of hydrolysis are listed in a separate table.

4.6.1. Group I (table 54).

All of these enzymes hydrolyse both cefotaxime and ceftazidime with very low efficiency. In most cases they hydrolyse cefotaxime with slightly greater efficiency than ceftazidime. This is a paradox as all the beta-lactamases confer far greater resistance to ceftazidime than cefotaxime. This phenomenon will be discussed in greater detail in section 4.6.5.

Table 54. Group I

	TEM-E1	TEM-E2	TEM-E4	TEM-7	CAZ-3 (TEM-E2)
Benzylpenicillin					100
Ampicillin	100	100	100	100	
Ceftazidime	1.1 (32)	0.7 (32)	0.1 (16)	0.4 (16)	0.3 (32)
Cefotaxime	2.5 (0.13)	1.5 (0.25)	0.8 (<1)	0.3 (0.06)	0.4 (0.5)
Reference	a	a	a	b	c

Table 55. Group II

	TEM-9	TEM-10	TEM-E3 (NMH)	CAZ-7
Benzylpenicillin				100
Ampicillin	100	100	100	
Ceftazidime	29 (250)	33 (64)	20 (125)	6.1 (256)
Cefotaxime	20 (2)	16 (0.5)	12 (1)	1.8 (4)
Reference	a	a	a	c

See end of table 56 for references.

MICs shown in parenthesis; in all cases MIC values are for E.coli K12 transconjugants of the original clinical isolates.

4.6.2. Group II (table 55).

These beta-lactamases all hydrolyse ceftazidime more efficiently than cefotaxime and confer high level resistance to ceftazidime. However, TEM-9 and the TEM-E3 beta-lactamase all have significant efficiency of hydrolysis values for cefotaxime but confer almost no resistance to the drug.

4.6.3. Group III (table 56).

All these enzymes hydrolyse cefotaxime more efficiently than ceftazidime. However, the majority of the beta-lactamases confer a greater resistance to ceftazidime than cefotaxime (see section 4.6.5.).

4.6.4. Those beta-lactamases not classified in Groups I-III.

Some 3GC hydrolysing beta-lactamases have only been characterised by their Vmax or relative rates of hydrolysis of different beta-lactam substrates. These enzymes are listed in table 57. As discussed previously such data does have deficiencies but can be used in rough approximations of the specificities of different beta-lactamases.

DJP-1 hydrolyses ceftazidime, cefotaxime and cefuroxime, but the strain producing DJP-1 produces one other 3GC hydrolysing beta-lactamase. Therefore, it is not possible to deduce the MICs conferred by just this

Table 56. Group III.

	TEM-3	TEM-5	CAZ-2	CAZ-6	SHV-2	SHV-4 (CAZ-5)	SHV-5 (CAZ-4)	FEC-1
Penicillin	100	100	100	100		100	100	
Ampicillin					100			100
Ceftazidime	2 (16)	17 (64)	11 (128)	17 (512)	1 (4)	3 (128)	5.6 (128)	0.06 (13)
Cefotaxime	18 (8)	23 (16)	51 (2)	21 (8)	10 (4)	16 (32)	25 (8)	67 (200)
Reference	d	d	c	c	e	f	f	g

MICs shown in parenthesis; in all cases MIC values are for E.coli K12 transconjugants of the original clinical isolates.

References.

a This Thesis
b Gutmann et al. 1988
c Chanal et al. 1989b
d Chanal et al. 1988b
e Gutmann et al. 1989
f Sirot et al. 1989
g Matsumoto et al. 1988

Table 57. Vmax or relative rate of hydrolysis data for those 3GC hydrolysing beta-lactamases not in Groups I-III.

	DJP-1	BIL-1	TEM-4	TEM-6	SHV-3	CAZ-1o	CAZ-hi	FUR	MJ-1	MJ-2	No name
Benzylpenicillin			100@	100@	100@				100+	100+	
Ampicillin	100+	UM									
Cephaloridine	114	50*	232	203	367	100+	100+	100+	95	<0.2	
Cefuroxime	4.3	UM (125)				0.5 (1-2)	1.0 (2)	53 (128)			
Ceftazidime	4.6	UM (64)	10 (16)	55 (128)	<1 (4)	1.0 (2-4)	36 (32)	<0.5 (2)			UM (16-32)
Cefotaxime	17	UM (16)	300 (8)	12 (1)	67 (4)	1.0 (0.03)	2.3 (0.25)	5.8 (1)	19	10	(<0.5)
Aztreonam		(16)	<1 (2)	11 (64)	<1 (2)	<0.5 (0.06)	<0.5 (8)	<0.5 (2)			
Reference	a	a	b	b	c	d	d	d	e	e	f

MICs shown in parenthesis; where possible MIC values are for E.coli K12 transconjugants of the original clinical isolates. @ = Relative Vmax values; * = Efficiency of hydrolysis value relative to nitrocefin; + = Relative rates of hydrolysis. UM = unmeasurable because hydrolysis of substrate too low.

References

a, This Thesis; b, Paul et al. (1989); c, Bure et al. 1988; d, Vuye et al. (1989); e, Deschaseaux et al. (1988); f, Corkill et al. (1989).

enzyme. TEM-4 and SHV-3 appear to have a higher hydrolytic activity with cefotaxime than ceftazidime and may be Group III 3GC hydrolysing beta-lactamases. Conversely, CAZ-hi and TEM-6 have a greater hydrolytic activity with ceftazidime. Both these enzymes confer a greater resistance to ceftazidime than to cefotaxime. This suggests that these enzymes may be Group II 3GC hydrolysing beta-lactamases.

CAZ-lo has low activity with the 3GCs tested and confers greater resistance to ceftazidime than to cefotaxime. This implies that CAZ-lo is a Group I beta-lactamase.

FUR has a much higher rate of hydrolysis of cefuroxime than ceftazidime or cefotaxime, and confers high level transferable resistance to cefuroxime. This enzyme may represent the start of a new series of plasmid mediated beta-lactamases.

The values listed for MJ-1 and MJ-2 and the enzyme characterised by Deschaseaux et al. (1988) suggests that all these enzymes confer transferable resistance to 3GCs, but insufficient data are available to make any other conclusions.

4.6.5. Correlation between MICs and efficiency of hydrolysis of ceftazidime and cefotaxime.

The majority of these beta-lactamases either hydrolyse cefotaxime more efficiently than ceftazidime or hydrolyse cefotaxime and ceftazidime with fairly similar efficiencies. However, most of the 3GC hydrolysing beta-lactamases confer a greater resistance to ceftazidime than cefotaxime. This suggests that cefotaxime is more permeable to the bacterial cell than ceftazidime and can reach its antibacterial target more efficiently.

Nikaido (1989) verified this hypothesis when he showed that cefotaxime diffused into liposomes reconstituted with E.coli Omp F or Omp C proteins at higher relative rates than ceftazidime. Nikaido et al. (1990) also showed that cefotaxime permeated intact E.coli cells at a greater rate than ceftazidime and that cefotaxime permeated liposomes reconstituted with Enterobacter cloacae F porin at greater rates than ceftazidime. Likewise, cefotaxime also had a higher permeability coefficient for intact Ent.cloacae cells than ceftazidime (Nikaido et al., 1990). Although the outer membrane proteins of the E.coli transconjugants used for MICs in tables 54-57, were not known in any great detail, Nikaido's results prove that cefotaxime is more permeable than ceftazidime. This helps to explain the paradox concerning the efficiency of hydrolysis values and MIC values for ceftazidime and cefotaxime.

4.6.6. Correlation between sequences of the TEM enzymes and their hydrolytic activities.

Many of the 3GC hydrolysing beta-lactamases have been sequenced (reviewed by Philippon et al., 1989) and, like TEM-3, they differ from either TEM-1/2 or SHV-1 by only a few amino acid residues. The majority of these amino acid changes are distant from the active serines which represent the catalytic centres of the SHV-1 and TEM-1 beta-lactamases. However, these slight changes allow these new enzymes to hydrolyse 3GC, which the former TEM-1/2 or SHV-1 beta-lactamases cannot achieve. As there is a significant homology between the amino acid sequences of the Staphylococcus aureus PC1 beta-lactamase and the TEM beta-lactamase it has been postulated that the tertiary structure of the TEM protein is very similar to the three dimensional structure of the PC1 enzyme which has been deduced by Herzberg and Moult (1987). This has enabled the amino acid changes which facilitate hydrolysis of 3GCs to be visualised on a three dimensional model of the TEM enzyme. Therefore, although the amino acid changes seemed distant from the active serines, when presented as a linear sequence, the tertiary folding of the TEM-1/2 beta-lactamase illustrated that these changes were actually in close proximity to the active serines of the TEM beta-lactamases.

At the moment it is difficult to correlate a particular amino acid change with a particular type of hydrolysis. For example it is not known which amino acid change is essential for a highly efficient hydrolysis of

ceftazidime. As more TEM or SHV enzymes are sequenced this will supply a wealth of information which will build up a greater understanding of the active sites of this new series of beta-lactamases.

However, it must be noted that there is only 30% homology between the amino acid sequences of the S. aureus PC1 enzyme and TEM-1 (Arakawa et al., 1986). Therefore, accurate assumptions regarding the active sites of these new enzymes can only be made when studying the three dimensional structure of the TEM beta-lactamase.

4.6.7. Consideration of other factors which may be responsible for the resistances conferred by the 3GC hydrolysing beta-lactamases.

The work of Sougakoff et al. (1988a) on TEM-3 showed that the amino acid changes may not be the only factors responsible for the resistances conferred by 3GC hydrolysing beta-lactamases.

TEM-3 differs from TEM-2 by only two amino acid residues (table 58). Table 59 shows the resistances conferred by each of these amino acid changes with different promoters. Promoter Pa+Pb is identical to the promoter encoding TEM-3 and promoter P3 is the beta-lactamase promoter of pBR322 which is 10 times less efficient than the Pa+Pb promoter. These results show that the two amino acid changes in TEM-3 are not sufficient for the high level resistance to 3GCs. A strong promoter initiating transcription

Table 58. Comparison of the amino acid sequences of TEM-2 and TEM-3.

Amino acid residue No.	104	238
Beta-lactamase		
TEM-2	glutamate (glu)	glycine (gly)
TEM-3	lysine (lys)	serine (ser)

Table 59. The effects of promoters on the 3GC resistance conferred by TEM-3.

Amino acid changes at 104 and 238.	lys-104 ser-238	lys-104 gly-238	glu-104 ser-238	lys-104 gly-238
Promoter.	Pa+Pb	P3	P3	Pa+Pb
MIC cefotaxime	>64	0.125	1	1
MIC ceftazidime	>64	2.0	0.5	32

Taken from Sougakoff et al. (1988)[a].
The numbering system is that of Ambler, 1980.

of the structural gene for the new enzyme is also required (Sougakoff et al. 1988).

4.7. 3GC hydrolysing beta-lactamases - are they currently a problem ?

There have been about 60 plasmid mediated beta-lactamases characterised since 1974; surprisingly almost half this number have been 3GC hydrolysing beta-lactamases characterised in only the last five years. However, out of the 28 beta-lactamases listed in table 53 only 7 have been reported to be expressed by more than 10 strains. 3GC hydrolysing beta-lactamases have created few problems in the UK and, on every reported case in this country there has been almost no spread of the strain or resistance plasmid. In contrast the 3GC hydrolysing beta-lactamases, and in particular TEM-3, have created major problems in French hospitals. The emergence of TEM-3 in France appeared not only related to the selective pressures of 3GCs but also to those of amikacin or netilmicin (Sirot et al. 1988). So the emergence and spread of new TEM enzymes may not be directly related to the increase in use of 3GCs. Although these outbreaks initially create huge problems, Brun-Buisson et al. (1987) showed that the attack rate of a TEM-3 producing, multiresistant K.pneumoniae was reduced from 6% to 1% in six months. This was achieved by a regime of hand washing and use of disposable gloves for handling urine from infected or colonised patients.

4.8. 3GC hydrolysing beta-lactamases - are they a future threat to the use of 3GCs.

The 3GC hydrolysing beta-lactamases have created the most severe problems in France. Indeed, the majority of initial reports of the enzymes in table 53 have come from French hospitals. In the UK, which has not had major problems with 3GC hydrolysing beta-lactamases, far less 3GCs are used than in France. Therefore, it is logical to suggest that as the future use of 3GCs increases because of the increased resistance to first and second generation cephalosporins, so the occurrence of both novel and established 3GC hydrolysing beta-lactamases will increase also. However, it is also possible to obtain strains producing ceftazidimases and cefotaximases in countries where 3GCs are not widely available and where selective pressure is low. For example BIL-1 and K.pneumoniae 8825, which produces two 3GC hydrolysing beta-lactamases, both came from third world countries where 3GC availability is restricted by cost.

As discussed previously, the 3GC hydrolysing SHV and TEM beta-lactamases can be obtained spontaneously from SHV-1 and TEM-1/2 beta-lactamases respectively. Therefore, any strain which produces TEM-1 or SHV-1 are potential producers of 3GC hydrolysing beta-lactamases. Table 5 (Introduction) shows that TEM-1 and SHV-1 are two of the most ubiquitous beta-lactamases found in bacterial populations. Moreover, calculations from a survey conducted by Wiedemann et al. (1989) on 802 clinical

isolates, showed that 17% of clinical isolates produce either TEM-1/2 or SHV-1. Therefore, a quarter of all clinically derived strains are potential producers of 3GC hydrolysing beta-lactamases. Consequently, it is surprising that those beta-lactamases such as TEM-7, TEM-E1, and TEM-E4, which have been shown to be obtainable from TEM-1/2 spontaneously, are not more prevalent. Conversely, the worldwide reports of SHV-2 may be explained by the fact that SHV-2 can be obtained spontaneously from SHV-1.

The occurrence of novel plasmid mediated 3GC hydrolysing enzymes appear to crop up as "chance mutations" and, in most cases, they do not spread throughout bacterial populations. The spread of one of these enzymes through bacterial populations appears to depend more on the pathogenicity of the strains involved, stability of the plasmid encoding the beta-lactamase, the other resistance markers on the plasmid and the consistency of the selective pressure to stabilise the resistance plasmid (not necessarily a 3GC). If all these factors are satisfied then a particular 3GC hydrolysing beta-lactamase could cause a significant threat to 3GC therapy .

Another worrying possibility is that the 3GC hydrolysing beta-lactamases may have the selective advantage to become as successful as their predecessors. The evidence to date does not suggest this but, if the use of 3GCs increases significantly, then the situation may well change. It could also be hypothesised that because of the similarities with other non-3GC hydrolysing beta-lactamases, many of these ceftazidimases and

cefotaximases may have remained undetected in current bacterial populations. Lastly, all the SHV and TEM derived beta-lactamases are very sensitive to beta-lactamase inhibitors such as clavulanic acid, sulbactam or YTR 830, and special emphasis has been placed on this fact in many publications (Kitzis et al., 1988; Paul et al., 1989; Gutmann et al., 1989). However, if BIL-1 represents a new series of plasmid mediated beta-lactamases then both the future of beta-lactams and beta-lactamase inhibitors will be under threat.

4.9. Studies on satellite bands of beta-lactamases.

This work illustrated that the mixing of beta-lactam antibiotics with beta-lactamases produced beta-lactamase satellite bands. Extraction of TEM satellite bands, induced by the addition of ceftazidime, from an isoelectric focusing gel and the subsequent re-focusing of this preparation revealed not only the satellite band but also a band which focused in close proximity to TEM-1. It is probable that this was TEM-1, but, because of irregularities in the polyacrylamide gel, it did not align exactly with the TEM-1 from the original preparation. Therefore, during the extraction of the satellite protein it had probably reverted back to the TEM-1 protein. This suggested that both these proteins must be very similar in structure. This provides the proof for the hypothesis that beta-lactamase satellite bands differ only very slightly from the main beta-lactamase protein.

It is probable that on mixing the beta-lactam with the beta-lactamase, some of the beta-lactam molecules bind to the beta-lactamase causing some distortion in its three dimensional structure. It is proposed that these distorted proteins may have slightly different physicochemical constants to the main beta-lactamase protein which is why they reveal themselves as satellite bands with different pIs from the main beta-lactamase band.

It was shown that different beta-lactams caused satellite bands of different pIs to be produced. It was surprising that ceftazidime and cefotaxime did not produce the same satellite band, as it would be expected that they would bind to similar positions on the TEM-1 protein and cause a similar distortion. However, it may be that different beta-lactams bind to different sites on the same beta-lactamase or that the range of beta-lactams studied all bind at the same site but cause different types of distortions to the protein.

It was noted that when ceftazidime was added to most TEM-like beta-lactamases it produced the same satellite band of pI 5.2. When ceftazidime was added to a selection of non-TEM beta-lactamases this satellite band was not normally seen. However, it was seen that the klebsiella chromosomal beta-lactamase, K-1, produced a similar satellite band at pI 5.2 but this may result from the fact that there is significant amino acid homology between K-1 and TEM-1 (Emanuel et al., 1986). Consequently, the addition of ceftazidime to a beta-lactamase preparation can help to verify whether the enzyme is TEM derived.

4.10. Conclusions.

1. It has been shown that some of the 3GC hydrolysing beta-lactamases produced by clinical isolates are obtainable spontaneously from TEM-1/2 beta-lactamases. This strongly implies how they originated in bacterial populations.

2. The characterisation of TEM-E1, TEM-E2, TEM-E3, TEM-E4, DJP-1, and BIL-1 has shown that all these beta-lactamases confer transferable resistance to 3GCs. At the time when these enzymes were characterised they were novel to any of the previously reported beta-lactamases.

3. Klebsiella oxytoca 5445 was isolated in 1982 and shown to produce TEM-E2. This suggests that plasmid mediated resistance to 3GCs existed in bacterial populations many years before it was actually reported.

4. The 3GC hydrolysing beta-lactamases can be classified into three groups by virtue of their efficiency of hydrolysis of ceftazidime or cefotaxime.

Group I.

Those beta-lactamases which hydrolyse both cefotaxime and ceftazidime with low efficiency.

Group II.

Those beta-lactamases which hydrolyse ceftazidime with greater efficiency than cefotaxime.

Group III.

Those beta-lactamases which hydrolyse cefotaxime with greater efficiency than ceftazidime.

5. Electrodialysis of beta-lactamases from polyacrylamide gel and Fast Protein Liquid Chromatography System are both major advances for the efficient purification and separation of multiple beta-lactamases produced by the same strain.

6. It has been proved that the proteins which produce satellite bands on isoelectric focusing of beta-lactamases can revert back to the parent protein. Also the binding of beta-lactams to beta-lactamases cause the production of specific satellite bands.

Transferable resistance to 3GC at this present time does not appear to be a major clinical problem. However, the next five years will reveal the true nature of this threat and whether any of these enzymes have the capabilities to become as prevalent as the TEM-1/2 or SHV-1 beta-lactamases.

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FEMS Microbiology Letters 59 (1989) 97–100
Published by Elsevier

FEM 03544

TEM-E1: a novel β-lactamase conferring resistance to ceftazidime

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Received 12 December 1988
Accepted 6 January 1989

Key words: β-lactamase; TEM; Ceftazidime; Resistance

1. SUMMARY

A novel β-lactamase, conferring resistance to ceftazidime, has been identified to be encoded by a 31 kb plasmid (pUK720) in a clinical *E. coli* strain isolated in Belgium. The β-lactamase, new designated TEM-E1, has a p*I* of approximately 5.4 and lies in between the iso-electric focused bands of the β-lactamases TEM-1 and TEM-7. The TEM-E1 β-lactamase has a similar molecular weight of 22000 to the TEM-1 and it is also inhibited by clavulanic acid. However, the TEM-E1 enzyme differs from TEM-1 by its low rates and efficiency of hydrolysis for ceftazidime and cefotaxime, TEM-E1 has similar efficiency of hydrolysis values for ceftazidime and cefotaxime, but only confers resistance to ceftazidime.

2. INTRODUCTION

There have been a number of recent reports on the novel plasmid-mediated β-lactamases TEM-3 to TEM-7, RRH-1, and CAZ-2 [1,2,3]. Although some of these enzymes are known, by nucleotide sequence analysis, to have evolved from TEM-1/TEM-2 β-lactamases [1,4,5], they differ from these two prototype enzymes because they confer resistance to third generation cephalosporins. This communication reports a novel TEM-like enzyme, identified in a clinical *E. coli* strain isolated in Belgium, which confers resistance to ceftazidime and is distinct from the other 3GC hydrolysis enzymes previously reported.

3. MATERIALS AND METHODS

Antibiotic sensitivity tests and plasmid transfers were determined on solid media as before [6]. The minimum inhibitory concentrations (MIC) for ampicillin, ceftazidime and cefotaxime were also measured in the presence of clavulanic acid (2 mg/l) or sulbactam (2 mg/l). The method of Takahashi and Nagano [7] was used to extract, separate and visualize plasma DNA. β-lactamase preparations were obtained from 1 litre cultures of the ceftazidime resistance transconjugant strain *E. coli* J53-2. The enzyme was identified by analytical isoelectric focusing [8] with sonicated bacterial extracts. β-lactamase activity, substrate profile, Michaelis-Menten kinetics and the effect of inhibitors were determined by spectrophotometric assays [9–11]. The molecular weight of the β-lactamase was determined on a calibrated Sephadex G075 column (2 $cm^2 \times 90$ cm) eluted with 25 mM sodium phosphate buffer (pH 7.0) at 16 ml/h [12].

4. RESULTS

The clinical strain *E. coli* 7891 was isolated from the urine of a patient attending a hospital in Belgium in 1987. The ceftazidime resistance gene could be transferred easily into the rifampicin-resistant strain *E. coli* J53-2. This transconjugant was resistant to ceftazidime (MIC = 32 mg/l at 10^5 cfu) but sensitive to all the other third generation cephalosporins tested.

4.1. Conjugation experiments

E. coli J5302 transconjugants were selected on agar containing ceftazidime (4mg/l) and rifampicin (50 mg/l) after overnight mixed incubation of the donor and recipient strains. When the original clinical isolate, *E. coli* 7891, was examined for plasmic DNA, to DNA bands were identified (sizes 31 kb, and 3 kb) and these two bands were also seen in the ceftazidime resistant transconjugant. The ceftazidime-resistance gene was believed to be located on the 31 kb plasmid which we have designated pUK720.

4.2. Antibiotic sensitivity

The MIC data shows that both the clinical strain and the *E. coli* J5302 transconjugant were ceftazidime resistant, which is the only additional resistance conferred when compared to a TEM-1 producing strain (Table 1). The strains expressing the novel enzyme were seen to be sensitive to ceftazidime in the presence of clavulanic acid (2 mg/l) or sulbactam (2 mg/l). Clavulanic acid was the more efficient inhibitor when used in combination with ampicillin, but the two inhibitors seemed to be equally effective when third generation cephalosporins were used as substrates.

4.3. Isoelectric Focusing

Then β-lactamase produced by *E. coli* 7891 and the *E. coli* J5302 transconjugant were examined by analytical isoelectric focusing (IEF) employing a broad range ampholine (pH 3.5–10). Both strains produced identical enzymes of low p*I* which were barely distinguishable from the TEM-1 β-lactamase. The β-lactamase band was, however, quite clearly distinguishable from the other transferable third generation cephalosporin hydrolysing enzymes. The enzyme from strain 7891 was re-examined by IEF with a 1 : 1 mixture of pH 4–6 and pH 3.5–10 ampholines to increase the sensitivity. The TEM-1, TEM-2 and TEM-7 β-lactamases were used as standard p*I* markers and the novel β-lactamase was found to focus between TEM-1 and TEM-7 enzymes (Fig. 1).

Table 1

Antibiotic susceptibilities of strains

β-lactam	Minimum inhibitory concentrations (mg/l)			
	E. coli 7891	*E. coli* 7891 ×J53-2	**E. coli* J53-2 2136E	*E. coli* J53-2
Ampicillin	> 250	> 250	> 250	4
A + clav	4	2	2	2
B + sulb	32	8	~ 250	4
Methicillin	> 250	> 250	> 250	8
Carbenicillin	> 250	> 250	> 250	4
Cephaloridine	64	32	8	4
Cephalexin	16	8	4	8
Cefoxitin	8	1	1	2
Cefuroxime	16	2	2	2
Ceftazidime	16	32	< 0.06	0.13
A + clav	0.5	0.25	< 0.06	0.13
B + sulb	1	0.25	< 0.06	< 0.06
Cefotaxime	0.25	0.13	< 0.06	< 0.06
A + clav	< 0.06	< 0.06	< 0.06	< 0.06
B + sulb	< 0.06	< 0.06	< 0.06	< 0.06
Ceftriaxone	0.25	0.13	< 0.06	< 0.06
Aztreonam	1	0.13	< 0.06	< 0.06
Imipenem	0.25	0.5	0.13	0.25
Augmentin	32	16	16	8

A + clav = in combination with clavulanic acid (2 mg/l);
B + sulb = in combination with sulbactam (2 mg/l).
* *E. coli* J53-2 2136 E produces TEM-1.

4.4. Molecular weight determination

A crude extract of the ceftazidime resistant transconjugant was applied to a sephadex G-75 column, calibrated with the standard proteins ovalbumin, chymotrypsinogen and cytochrome *C*. The size of the novel enzyme was 22 000 which was indistinguishable from the TEM-1 nβ-lactamase when estimated by the same method.

4.5. Kinetic characteristics

The substrate profiles of the TEM-1 β-lactamase and that from the *E. coli* J53-2 transconjugant of 7891 are shown in Table 2. The profiles

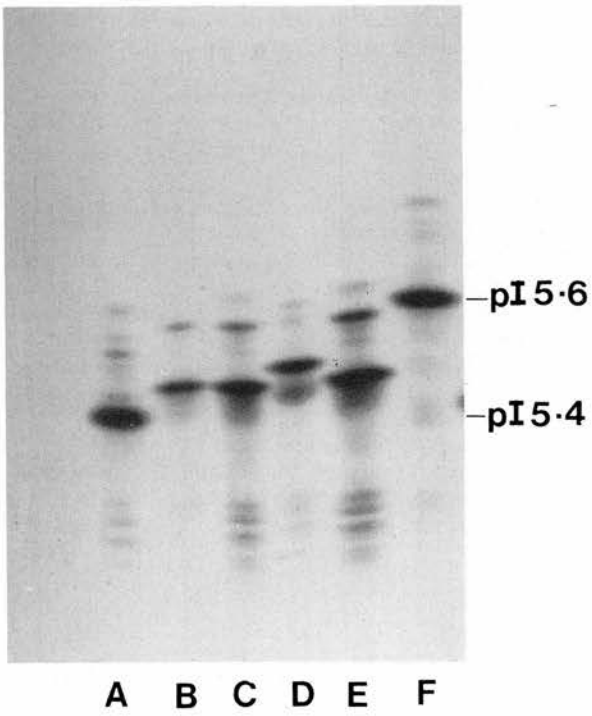

Fig. 1. Iso-electric focusing pattern of the plasmic encoded β-lactamase derived from strain 7891, compared with the enzymes TEM-1, TEM-2 and TEM-7 over a narrow pH range. A. *E. coli* J53-2 producing TEM-1; B. *E. coli* 7891 producing TEM-E1; C. *E. coli* J53-2 transconjugant of *E. coli* 7891 producing TEM-E1; D. *E. coli* BM694 producing TEM-7; E. as C. F. *E. coli* J53-2 producing TEM-2.

are similar for carbenicillin, ampicillin, and cephaloridine. However, the novel enzyme showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme

Table 2

* Relative rates of hydrolysis of β-lactamases TEM-E1 and TEM-1

β-lactam substrate	β-lactamase	
	TEM-E1	TEM-1
Ampicillin	100	100
Carbenicillin	29.6	10.9
Cephaloridine	42.1	18
Cefuroxime	0.25	0.014
Cefotaxime	1.48	0.014
Ceftazidime	0.31	0.017

* Rate for ampicillin = 100%.

Table 3

* Efficiency values for TEM-E1 and TEM-1 (relative V_{max}/K_m)

β-lactam substrate	β-lactamase	
	TEM-E1	TEM-1
Ampicillin	100	100
Carbenicillin	18	18
Cephaloridine	134	110
Cefotaxime	2.5	0.16
Ceftazidime	1.1	N.D.

N.D. not done because break down was insufficient.
* Efficiency with ampicillin = 100%.

showed no hydrolysis of either substrate. The K_m, V_{max} and the relative efficiency of hydrolysis (V_{max}/K_m, ampicillin = 100%) values for TEM-1 and TEM-E1 for five β-lactam substrates are shown in Table 3. The efficiency of hydrolysis of ceftazidime and cefotaxime by TEM-E1 were very similar; equal to or less than 2.5% of the rate for ampicillin.

4.6. Inhibition studies

When assayed for the hydrolysis of nitrocefin, the novel β-lactamase was sensitive to inhibition by clavulanic acid (ID_{50} = 0.63 μM).

5. DISCUSSION

The properties of the enzyme from strain 7891 and, in particular, its isoelectric focusing pattern confirm that this plasmid-mediated enzyme from Belgium is not identical to any of the other transferable β-lactamases responsible for third generation cephalosporin resistance [1]. The kinetic parameters of enzyme TEM-E1 were very similar to those of the TEM-7 β-lactamase [13] and the rates and efficiency of hydrolysis of ceftazidime and cefotaxime observed for both these enzymes are low but very similar. However, both the enzyme derived from 7891 and the TEM-7 β-lactamase confer much higher resistance to ceftazidime than cefotaxime. It was found that TEM-7 enzyme and the β-lactamase from strain 7891 could be effectively distinguished by their different isoelectric focusing positions and thus we have called the new enzyme TEM-E1.

ACKNOWLEDGEMENTS

We thank the Science and Engineering Research Council for the CASE studentship for DJP and F. Goldstein for the TEM-7 producing strain.

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J. Med. Microbiol. — Vol. 32 (1990), 131–134

0022–2615/90/0032–0131/$10.00

Characterisation of a unique ceftazidime-hydrolysing β-lactamase, TEM-E2

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Summary. A strain of *Klebsiella oxytoca*, originally isolated in Liverpool in 1982, has been found to produce a novel transferable β-lactamase, TEM-E2. This enzyme confers resistance to ceftazidime and focused as a doublet band with an iso-electric point (pI) of 5·3. The strain also produced the TEM-1 β-lactamase. Both TEM-1 and TEM-E2 β-lactamases were encoded by a transferable 103 kb plasmid; these two enzymes also had similar molecular weights, were inhibited by clavulanic acid, and hydrolysed ampicillin, carbenicillin and cephaloridine at similar rates. However, unlike the TEM-1 enzyme, the TEM-E2 β-lactamase hydrolysed ceftazidime and cefotaxime with similar efficiency, although it conferred much greater resistance to ceftazidime in the host strain. This is the earliest documented example of a TEM-like enzyme which confers transferable resistance to ceftazidime and related cephalosporins.

Introduction

When cefotaxime and ceftazidime were first introduced, they were resistant to hydrolysis by most of the plasmid-mediated β-lactamases that were known at that time, including the TEM-1 and TEM-2 enzymes.[1,2] However, there have since been several reports of β-lactamases, which differ from TEM-1 and TEM-2 by only a few amino acids, that are able to hydrolyse these and related cephalosporins.[3] Between 1984 and June 1987, 490 strains producing TEM-3 (which exhibits high activity against cefotaxime) were isolated in France.[4] In 1987 and 1988 the novel enzymes TEM-4, 5, 6, 7, 9 and CAZ-2 were identified;[5–7] all appear to be related to the TEM group of β-lactamases. We now describe a ceftazidime-resistant strain of *Klebsiella oxytoca*, isolated in 1982, which produces a β-lactamase that hydrolyses ceftazidime and cefotaxime and shows strong similarities with TEM-1.

Materials and methods

Bacterial strains

K. oxytoca strain 5445 was isolated from the blood and cerebrospinal fluid of a baby in the Neonatal Intensive Care Unit of Liverpool Maternity Hospital on Feb. 5, 1982. Rifampicin-resistant *Escherichia coli* J53-2 was used as the standard recipient strain in the conjugation experiments.[8] *E. coli* J53-2 transconjugants were selected on agar containing rifampicin 100 mg/L and ceftazidime 4 mg/L after overnight incubation of a mixture of the donor and recipient strains.[9]

Antibiotic sensitivities and plasmid analysis

Minimum inhibitory concentrations (MICs) of β-lactam antibiotics were determined by the agar incorporation method in Iso-Sensitest Agar (Oxoid), with a bacterial inoculum of 10^5 cfu. In certain experiments, clavulanic acid or sulbactam was included in the medium at a concentration of 2 mg/L. The method of Takahashi and Nagano[10] was used to extract, separate and visualise plasmid DNA.

β-Lactamase studies

Bacteria were grown overnight in 1 L of Nutrient Broth No. 2 (Oxoid) containing ceftazidime 4 mg/L, and β-lactamase preparations were obtained from sonicated extracts of the concentrated bacterial pellet.[11] The β-lactamases were identified by analytical iso-electric focusing (IEF) of these extracts;[12] β-lactamases PSE-4, TEM-1, TEM-2 and TEM-7 were used as standard pI markers. β-Lactamase activity, substrate profile, Michaelis-Menten kinetics and the effect of inhibitors were determined by spectrophotometric assays according to

Received 5 June 1989; revised version accepted 29 Nov. 1989.
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published methods.[13–15] The relative molecular mass (M_r) of the β-lactamase was determined on a Sephadex G-75 column (2 $cm^2 \times 90$ cm) eluted with 25 mM phosphate buffer (pH 7·0) at a flow rate of 16 ml/h.[16] Ovalbumin, chymotrypsinogen and cytochrome C were used as standard M_r markers.

Purification of the TEM-E2 β-lactamase by electrodialysis

A crude β-lactamase solution, prepared from the *E. coli* J53-2 transconjugant of *K. oxytoca* 5445, was spread on to an analytical IEF polyacrylamide gel containing pH 6–8 and pH 4–6 ampholines (LKB Pharmacia) in a 1:1·5 ratio. Care was taken not to cover the area of the gel where the β-lactamase bands focused. After focusing, a 1-cm wide strip of filter paper soaked in a solution of nitrocefin (0·5 mg/ml) was placed from the cathode to the anode of the gel to identify the β-lactamase bands. The portion of the gel containing the TEM-E2 enzyme was cut out and placed in dialysis tubing with a minimal amount of 25 mM sodium phosphate buffer (pH 7·0). The dialysis sack was placed in the cathode reservoir of a Bio Rad Mini Sub Cell with 25 mM phosphate buffer (pH 7·0) as running buffer. A charge of 150 V was applied for 10 min. The dialysis sack was then removed and the polyacrylamide gel was discarded. The purity of the enzyme remaining in the sack was analysed by IEF.

Results

Conjugation experiments

The ceftazidime-resistant transconjugants of *E. coli* J53-2 were obtained by conjugation with *K. oxytoca* 5445 at 37°C for 6 h. Analysis of the plasmid DNA in the transconjugant strains showed a single large plasmid band of 103 kb, designated pUK721, similar to that found in the original clinical strain.

Antibiotic sensitivities

K. oxytoca 5445 and the *E. coli* J53-2 transconjugant were resistant to ceftazidime but sensitive to cefotaxime and ceftriaxone. Indeed, ceftazidime resistance was the most significant additional β-lactam resistance conferred by plasmid pUK721 when compared with a TEM-1 β-lactamase producing strain (table I). Susceptibilities to ceftazidime were restored in the presence of clavulanic acid (2 mg/L) or sulbactam (2 mg/L). However, clavulanic acid was more efficient than sulbactam in reducing MICs of ampicillin.

Table I. Antibiotic sensitivities of test strains

Antibiotic	Minimum inhibitory concentrations (mg/L)			
	K. oxytoca 5445	*E. coli* J53-2 × 5445	*E. coli* J53-2 2136E*	*E. coli* J53-2
Ampicillin	>250	>250	>250	4
+clav	16	8	2	2
+sulb	125	250	>250	4
Carbenicillin	>250	>250	>250	8
Cephaloridine	250	64	8	4
Cephalexin	16	8	4	4
Cefoxitin	16	4	1	2
Cefuroxime	16	4	2	2
Ceftazidime	64	32	<0·06	0·13
+clav	1	0·25	<0·06	0·13
+sulb	2	0·5	<0·06	<0·06
Cefotaxime	0·5	0·25	<0·06	<0·06
+clav	<0·06	<0·06	<0·06	<0·06
+sulb	<0·06	<0·06	<0·06	<0·06
Ceftriaxone	0·5	0·13	<0·06	<0·06
Aztreonam	2	1	<0·06	<0·06
Imipenem	0·25	0·25	0·13	0·25

+clav, in combination with clavulanic acid 2 mg/L;
+sulb, in combination with sulbactam 2 mg/L.
*Strain 2136E produces TEM-1.

β-Lactamase studies

Analytical IEF with a broad range ampholine (pH 3·5–10) revealed that *K. oxytoca* 5445 and the *E. coli* J53-2 transconjugant produced β-lactamases of low pI. IEF with a 1:1 mixture of pH 4–6 and pH 3·5–10 ampholines showed that the clinical isolate and the *E. coli* transconjugant each produced a band which co-focused with TEM-1 and a novel β-lactamase which focused as a doublet band at pI 5·3 (figure). This novel β-lactamase, TEM-E2, was clearly distinguishable from any of the other ceftazidime-hydrolysing β-lactamases.[17] The TEM-E2 doublet band produced by *K. oxytoca* 5445 was separated from the TEM-1 band by electrodialysis from the IEF polyacrylamide gel (figure).

The V_{max} and K_m values of the TEM-E2 and TEM-1 enzymes are shown in table II. Neither enzyme had any measurable activity against cefuroxime. TEM-1 had no activity against ceftazidime but showed some affinity for cefotaxime. TEM-E2 showed activity against both substrates but had a greater affinity for cefotaxime. Both enzymes showed similar relative efficiency of hydrolysis of ampicillin, carbenicillin and cephaloridine (table II). However, the novel enzyme paradoxically hydrolysed ceftazidime less efficiently than cefotax-

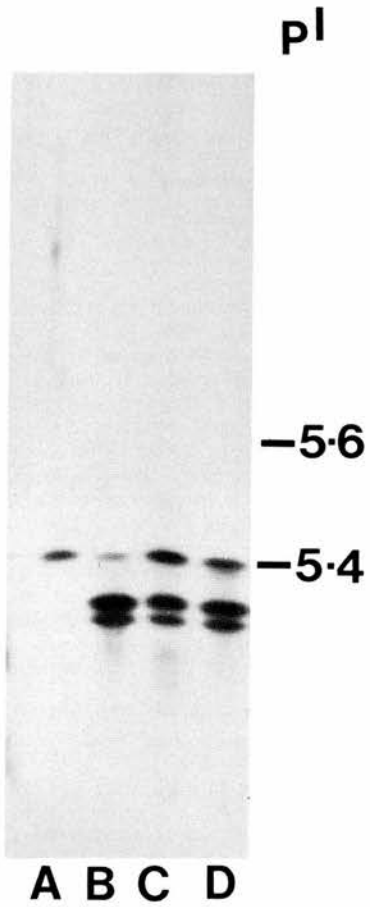

Figure. IEF of β-lactamases. **A**, *E. coli* J53-2 producing TEM-1; **B**, TEM-E2 β-lactamase purified from TEM-1 by electrodialysis from IEF gel; **C**, *E. coli* J53-2 transconjugant of *K. oxytoca* 5445 producing TEM-1 and TEM-E2; **D**, *K. oxytoca* 5445 producing TEM-1 and TEM-E2.

ime. Hydrolysis of nitrocefin by TEM-E2 was inhibited by clavulanic acid (ID_{50} 0·8 μM). The M_r of TEM-E2, purified by electrodialysis from an analytical IEF gel, was 23 500. Under the same experimental conditions, the M_r of the TEM-1 enzyme was 22 000. However, this difference may not be significant. The crude TEM-E2 enzyme preparation (i.e., with the TEM-1 enzyme) was analysed by gel filtration and fractions were collected from either side of the peak of β-lactamase activity; examination of each of these fractions by IEF showed that they all contained the TEM-1 band and the doublet band, thus confirming their identical M_r.

Discussion

The novel β-lactamase, TEM-E2, is different from any of the previously reported enzymes that hydrolyse cephalosporins of the cefotaxime type. Its kinetic characteristics appear similar to those of other TEM-like enzymes such as TEM-E1[18] and TEM-7[19] in that it hydrolysed cefotaxime and ceftazidime with similar, low efficiencies although it conferred resistance only to ceftazidime. The novel enzyme showed similarities with TEM-1, from which it could be separated by electrodialysis. An enzyme with very similar properties to TEM-E2 has been obtained from a TEM-1 producing strain by spontaneous mutation.[17] This strongly suggests that TEM-E2 has been derived from the TEM-1 β-lactamase. Moreover, as the mutant strain exhibited virtually no TEM-1 activity, it is apparent that the TEM-1 enzyme did not contribute towards ceftazidime resistance. The first example of plasmid-mediated resistance to cefotaxime was identi-

Table II. Kinetic constants of TEM-E2 compared to those of TEM-1

Antibiotic	TEM-E2			TEM-1		
	K_m (μM)	V_{max}*	V_{max}/K_m†	K_m (μM)	V_{max}*	V_{max}/K_m†
Ampicillin	119	100	100	167	100	100
Carbenicillin	83	23	32	100	11	18
Cephaloridine	500	87	20	167	23	23
Cefuroxime	UM	UM	UM	UM	UM	UM
Cefotaxime	181	0·84	0·55	286	0·06	0·04
Ceftazidime	500	0·87	0·21	UM	UM	UM

UM, unmeasurable.
*Relative to ampicillin as 100%.
†Efficiency of hydrolysis relative to ampicillin as 100%.

fied in Germany in 1983.[20] This transferable resistance was later found to be conferred by the SHV-2 β-lactamase.[21] Since the *K. oxytoca* 5445 strain discussed in this study was isolated in 1982, TEM-E2 is the earliest example of a TEM-like enzyme which can hydrolyse ceftazidime, and also the first plasmid-encoded β-lactamase to confer resistance to any of the new broader-spectrum cephalosporins. The study of this enzyme has also illustrated the use of a simple technique for the separation and purification of β-lactamases. The method may be particularly useful when the host strain produces enzymes with similar pI values.

We thank the Science and Engineering Research Council for the CASE studentship for D.J.P.

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Journal of Antimicrobial Chemotherapy (1989) **24,** 103–110

Mutants of the TEM-1 β-lactamase conferring resistance to ceftazidime

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Spontaneous ceftazidime resistant mutants were obtained from an *Escherichia coli* K12 J62-2 expressing the TEM-1 β-lactamase (mutation frequency = 10^{-9}). These mutants produced β-lactamases with similar molecular weights, kinetic parameters and isoelectric points (pI) to the β-lactamases produced by ceftazidime resistant clinical isolates which have recently been identified in this laboratory. Mutant enzyme A focused as a doublet band at pI 5·3 with an additional weak pI 5·4 band. The doublet co-focused with the TEM-E2 β-lactamase, produced by a ceftazidime resistant *Klebsiella oxytoca* isolate, which was originally obtained in a Liverpool hospital. Mutant enzyme B had a pI identical to the TEM-E1 β-lactamase produced by a ceftazidime resistant clinical isolate of *E. coli* found in Belgium. These results suggest that the two β-lactamases in the clinical strains may have come from simple mutations of the TEM-1 β-lactamase gene.

Introduction

Plasmid mediated β-lactamases conferring resistance to third generation cephalosporins were present as early as 1982 (Payne, Marriott & Amyes, submitted for publication). Since then TEM-3 to TEM-7, RHH-1 and CAZ-2 β-lactamases have been reported (Chanal *et al.*, 1988; Sougakoff *et al.*, 1988; Spencer *et al.*, 1988). Kinetic analyses of these enzymes have shown that they have distinct similarities to the TEM-1 and TEM-2 β-lactamases. Moreover, amino-acid sequencing performed on some of these enzymes has provided evidence that they differ from TEM-1 and TEM-2 enzymes by only a few amino-acid residues (Collatz *et al.*, 1988; Petit *et al.*, 1988*a*; Sougakoff *et al.*, 1988). It has been reported that TEM-1 and -2 enzymes can give spontaneous mutants that hydrolyse third generation cephalosporins (Gutmann *et al.*, 1988; Sougakoff *et al.*, 1988). In this study two novel mutant enzymes have been obtained directly from TEM-1 β-lactamase. These mutants differ from all those found in other studies but are identical to two novel β-lactamases which we have recently found in clinical isolates.

Materials and methods

Bacterial strains

Klebsiella oxytoca 5445 was isolated in 1982 at a Liverpool hospital and produces TEM-E2 β-lactamase. This is believed to be the earliest example of a plasmid mediated

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0305–7453/89/080103+08 $02.00/0

β-lactamase conferring resistance to third generation cephalosporins (Payne, Marriott & Amyes, submitted for publication). *Escherichia coli* 7891 was isolated from the urine of a patient attending a hospital in Belgium and produces the novel third-generation cephalosporin hydrolysing enzyme TEM-E1 (Payne, Marriott & Amyes, 1989). Rifampicin resistant *E. coli* K12 J53-2 (pro^- met^-) and *E. coli* K12 J62-2 (pro^- his^- trp^-) strains were used as recipients in conjugation experiments (Bachmann, 1972). An *E. coli* J62-2 transconjugant of *Klebsiella pneumoniae* CF504 (Petit *et al.*, 1988b) which produced only TEM-1 β-lactamase and not the TEM-5 enzyme present in the *K. pneumoniae* strain was used as the parent in the spontaneous mutation experiments.

Spontaneous mutation experiments

A 10-ml overnight nutrient broth culture (Oxoid No. 2) of *E. coli* K12 J62-2, harbouring the TEM-1 β-lactamase coding plasmid was harvested by centrifugation and resuspended in 1 ml of 25 mM phosphate buffer (pH 7·0). Aliquots (0·1 ml) of this suspension were inoculated on to ten Isosensitest Agar (Oxoid) plates containing 0·7 mg/l ceftazidime. This was the lowest ceftazidime concentration that eradicated all the ceftazidime sensitive bacteria at this abnormally high inoculum. Ceftazidime resistant colonies were purified on fresh ceftazidime-containing plates and the growth requirements of each selected colony were checked to verify that they were *E. coli* J62-2. The identity of the mutants was further checked by API 20E strips. These strains were then examined for any change in β-lactamase profile.

Antibacterial drug susceptibilities and plasmid analysis

Minimum inhibitory concentrations (MIC) of antibacterial drugs were determined on solid media at a cell concentration of 10^5 cfu as described previously by Amyes & Gould (1984). The MICs of ampicillin, ceftazidime and cefotaxime were additionally measured in the presence of clavulanic acid (2 mg/l) and sulbactam (2 mg/l). The method of Takahashi & Nagano (1984) was used to extract, separate and visualise plasmid DNA.

Conjugation experiments

In all mating experiments 0·1 ml of an overnight culture of the donor strain was mixed with 1 ml of an overnight culture of the recipient strain in 4·5 ml of nutrient broth, as described previously by Amyes & Gould (1984). This mixture was then incubated at 37°C for 6 h. *E. coli* J53-2 transconjugants of the two clinical stains were selected on agar containing rifampicin (50 mg/l) and ceftazidime (4 mg/l). *E. coli* J53-2 transconjugants of the TEM-1 mutants were selected on Davis-Mingioli minimal agar (Davis & Mingioli, 1950) containing proline (50 mg/l), methionine (50 mg/l) and ceftazidime (4 mg/l). All kinetic studies were performed on enzymes isolated from the ceftazidime-resistant *E. coli* J53-2 transconjugants.

β-Lactamase studies

β-Lactamase preparations were obtained from ultrasonicates of overnight Oxoid Isosensitest broth cultures containing ceftazidime (4 mg/l) (Simpson, Harper & O'Callaghan, 1980). The β-lactamases were identified by analytical isoelectric focusing (IEF)

(Matthew *et al.*, 1975) of bacterial sonicates. The β-lactamases TEM-1, TEM-2, TEM-7 and PSE-4 were used as standard pI markers. The enzymes were first examined on polyacrylamide gels containing a broad range ampholine (pH 3·5–10). They were subsequently re-examined on similar gels containing a 1:1 mixture of pH 4–6 and pH 3·5–10 ampholines, to provide a narrow pH range and increase sensitivity. Enzyme substrate profile, V_{max}, K_m, and inhibitor studies were determined by spectrophotometric assay (Sykes *et al.*, 1981; Eliasson & Kamme, 1985; Reid & Amyes, 1986). The relative molecular masses (M_r) of the β-lactamases were determined on a calibrated Sephadex G-75 column (2 $cm^2 \times 90$ cm) eluted with 25 mM phosphate buffer (pH 7·0) at 16 ml/h (Andrews, 1964).

Results

Preparation of TEM-1 β-lactamase parent strain

K. pneumoniae CF504 was a clinical strain isolated in Clermont-Ferrand, France. It was resistant to ceftazidime and cefotaxime and the gene encoding these resistances was located on a 150 kb plasmid (pCFF14) (Petit *et al.*, 1988*b*). Isoelectric focusing showed that this strain also produced TEM-1 β-lactamase as well as the TEM-5 enzyme responsible for the cephalosporin resistances. This strain was conjugated with the rifampicin-resistant *E. coli* J62-2 and transconjugants were selected on Isosensitest agar plates containing rifampicin and ceftazidime. The transconjugants were purified and their purity checked. Analysis of the plasmid DNA revealed that the transconjugant had lost the 150 Kb plasmid (pCFF14), which encodes the TEM-5 β-lactamase (Petit *et al.*, 1988*b*), and possessed only a 100 Kb plasmid along with two smaller plasmids. This transconjugant reverted to the normal characteristics of a TEM-1 β-lactamase producing *E. coli* J62-2. This strain was used as the parent in the spontaneous mutation experiments. The rationale for this was that there should be a higher chance of obtaining a third generation cephalosporin-hydrolysing mutant enzyme from a TEM-1 β-lactamase gene in a strain that was host to a TEM-like third generation cephalosporin-hydrolysing β-lactamase than a standard laboratory strain hosting only the TEM-1 β-lactamase gene.

Selection of mutants

The ceftazidime-resistant mutants, which appeared at a frequency of 1 in 10^9, were purified and examined for any change in β-lactamase profile. In all the colonies studied, ceftazidime resistance was associated with the mutation of the TEM-1 β-lactamase gene to produce one of two different β-lactamases (mutant enzymes A and B). We examined 20 colonies and the mutant β-lactamases they produced. This revealed that the ratio of occurrence of enzyme A to enzyme B was 4:1. The genes encoding the mutant β-lactamases were transferred by conjugation into *E. coli* J53-2, with selection of the transconjugants on ceftazidime-containing plates. The transfer of the plasmids elevated the MIC of ceftazidime for the *E. coli* J53-2 recipient more than 60-fold, from 0·13 to 8–16 mg/l (Table I). The transferability of the resistance determinant confirmed that the mutation had occurred to a plasmid encoded gene and not on the chromosome. Both the original mutants and their J53-2 transconjugants possessed the 100 Kb plasmid, but the two smaller plasmid bands were seen only in the original mutant strains.

Table I. MICS of β-lactams for *E. coli* strains harbouring mutant β-lactamases and β-lactamases from clinical strains

β-lactam antibiotic	MIC (mg/l) Mutant A J62-2 mutant	Mutant A J53 transconjugant	Mutant A 5445[c] xJ53	Mutant B J62-2 mutant	Mutant B J53 transconjugant	Mutant B 7891[d] xJ53	Controls J62-2	Controls J53-2
Ampicillin	>250	>250	>250	>250	>250	>250	4	4
Amp + clav[a]	ND	2	8	ND	2	2	ND	2
Amp + sulb[b]	ND	4	250	ND	4	8	ND	4
Carbenicillin	>250	>250	>250	>250	>250	>250	4	8
Cephaloridine	8	16	64	32	16	32	2	4
Cephalexin	4	4	8	4	4	8	4	4
Cefoxitin	2	1	4	1	1	1	2	2
Cefuroxime	2	0.5	4	2	1	2	2	2
Ceftazidime	4	16	32	4	8	32	<0.06	<0.13
Ctaz + clav[a]	ND	0.13	0.25	ND	0.06	0.25	ND	0.13
Ctaz + sulb[b]	ND	<0.06	0.5	ND	0.13	0.25	ND	<0.06
Cefotaxime	0.06	0.06	0.25	<0.06	<0.06	0.13	<0.06	<0.06
Ctax + clav[a]	ND	<0.06	<0.06	ND	<0.06	<0.06	ND	0.06
Ctax + sulb[b]	ND	<0.06	<0.06	ND	<0.06	<0.06	ND	<0.06
Ceftriaxone	<0.06	0.13	0.13	<0.06	<0.06	0.13	<0.06	<0.06
Aztreonam	0.25	0.5	1	0.13	0.5	0.5	<0.06	0.13
Imipenem	0.13	0.5	0.25	0.13	0.5	0.5	0.13	0.25
Amoxycillin/ clavulanate	8	16	32	16	16	16	8	8

[a] + clav = in combination with 2 mg/l clavulanic acid.
[b] + sulb = in combination with 2 mg/l sulbactam.
[c] Produces TEM-E2.
[d] Produces TEM-E1.
ND Not done.
Amp, Ampicillin; Ctaz, Ceftazidime, Ctax, Cefotaxime.

Isoelectric focusing

Mutant enzyme A focused as a doublet band at pI 5·3 with an additional weak band that cofocused with TEM-1 enzyme. The TEM-E2 β-lactamase expressed by the ceftazidime resistant isolate, *K. oxytoca* 5445 produced an identical doublet band at pI 5·3, but exhibited a much stronger TEM-1-cofocusing band. Mutant enzyme B showed distinct similarities to enzyme TEM-E1 produced by the clinical isolate *E. coli* 7891. Mutant enzyme B and TEM-E1 co-focused and had a pI marginally above the TEM-1 enzyme but below the TEM-7 β-lactamase (Figure 1).

Minimum inhibitory concentrations

E. coli J53-2 transconjugants expressing the mutant enzymes and the transconjugants expressing the clinically obtained extended spectrum β-lactamases were all resistant to ampicillin, carbenicillin, cephaloridine, ceftazidime, and aztreonam. However, they were sensitive to all other cephalosporins tested including cefotaxime (Table I). Clavulanic acid and sulbactam were similar in their activity when cefazidime was used as the principal antibiotic. However, clavulanic acid was seen to be the more efficient β-lactamase inhibitor when used in combination with ampicillin (Table I).

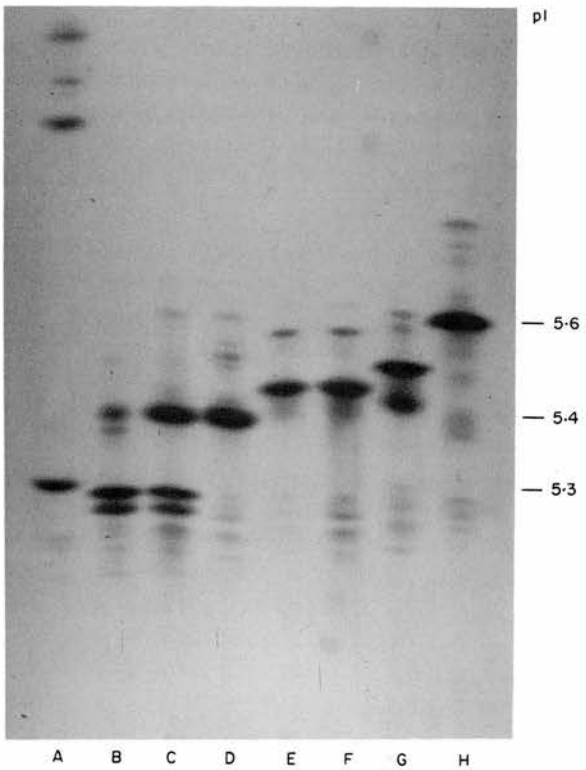

Figure 1. Isoelectric focussing patterns of mutant enzymes and β-lactamases from clinical isolates over a narrow pH range. A, PSE-4; B, mutant A; C, TEM-E2; D, TEM-1; E, mutant B; F, TEM-E1; G, TEM-7; H, TEM-2.

Table II. [a]Relative rates of hydrolysis and molecular masses of β-lactamases TEM-1, TEM-E1, TEM-E2, and mutant enzymes A and B

β-lactamase	M_r[b]	Relative rates of hydrolysis of β-lactams					
		ampicillin	carbenicillin	cephaloridine	cefuroxime	ceftazidime	cefotaxime
TEM-E1	22.0	100	29.8	42.1	0.25	0.31	1.48
Enzyme B	24.0	100	17.4	68	0.55	1.11	1.36
TEM-E2	25.5	100	19.3	41.6	0.22	0.23	0.58
Enzyme A	23.5	100	26.3	38.2	0.52	0.92	2.27
TEM-1	22.0	100	10.9	18	0.014	0.017	0.014

[a]Rate for ampicillin = 100%.
[b]M_r, Molecular mass.

Biochemical profiles of the mutant β-lactamases, TEM-E1, and TEM-E2

The two mutant enzymes and the two enzymes from clinical isolates all had molecular masses indistinguishable from the TEM-1 β-lactamase (Table II). The substrate profiles of these four enzymes are compared to TEM-1 in Table II. The profiles were similar to TEM-1 for ampicillin, carbenicillin and cephaloridine. However, both the mutant enzymes and the β-lactamases from clinical strains showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed no hydrolysis of either of these substrates. The relative V_{max} and the K_m values for these enzymes against a number of β-lactams were determined. These values were combined to give the relative efficiency of hydrolysis values (relative V_{max}/K_m, compared with ampicillin = 100%) which takes into account both the binding and the hydrolysis capabilities of the enzyme for a particular β-lactam. The relative efficiency of hydrolysis values for ceftazidime and cefotaxime by TEM-E1, TEM-E2, mutant A, and mutant B were very similar (Table III). All the enzymes were inhibited by clavulanic acid with I_{50}s in the region of 0·5–0·8 μM.

Discussion

Previous reports of third generation cephalosporin resistant mutants arising spontaneously from TEM-1, have detailed only one type of mutant, called TEM-101 by Gutmann *et al.* (1988) and TEM-121 to TEM-124 by Sougakoff *et al.* (1988), for which the MIC of ceftazidime was 4 mg/l and the β-lactamase pI 5·3. This enzyme had a

Table III. [a]Relative efficiency values for TEM-E1, TEM-E2, and the mutant enzymes A and B

β-Lactam antibiotic	β-Lactamases				
	TEM-E1	enzyme B	TEM-E2	enzyme A	TEM-1
Ampicillin	100	100	100	100	100
Carbenicillin	18	7	9	16	18
Cephaloridine	134	76	78	81	110
Ceftazidime	1.1	2.1	0.69	3.3	ND
Cefotaxime	2.5	0.9	1.5	6.1	0.16

[a]Efficiency with ampicillin = 100%.
ND, Not done because breakdown was insufficient.

similar pI to mutant enzyme A but it did not focus as two distinct bands of equal intensity. Other reported mutant enzymes, derived from TEM-2, were called TEM-201 by Gutmann *et al.* (1988) and TEM-221 to TEM-225 by Sougakoff *et al.* (1988). These enzymes had the same pI as TEM-7. Mutant enzyme B differed in having a pI below TEM-7 and in being obtained from TEM-1. Thus mutant enzyme B was clearly different from other mutant enzymes previously reported. The mutant enzymes and the clinically derived enzymes all hydrolysed both ceftazidime and cefotaxime at similar rates and efficiencies but, surprisingly, only conferred resistance to ceftazidime and not cefotaxime. Amino-acid sequencing has shown that TEM-4 and TEM-5 are more similar to TEM-1 than TEM-2 (Petit *et al.*, 1988*a*) and, consequently, these two enzymes are believed to have been derived from TEM-1. However, the sequences of the TEM-3 and TEM-7 enzymes (Collatz *et al.*, 1988; Sougakoff *et al.*, 1988) include the one amino-acid change which distinguishes TEM-2 from the TEM-1 enzyme and they are therefore thought to have evolved from the TEM-2 β-lactamase. This study has shown how two β-lactamases, indistinguishable from similar enzymes present in clinical strains, have been obtained *in vitro* from a TEM-1 producing organism, suggesting that TEM-E1 and TEM-E2 had similarly evolved from TEM-1.

Acknowledgements

We thank the Science and Engineering Research Council for the CASE studentship for DJP, and F. Goldstein for a TEM-7 producing strain and D. Sirot for the TEM-5 producing organism.

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(*Received 23 January 1989; revised version accepted 13 April 1989*)

FEMS Microbiology Letters 69 (1990) 195–200
Published by Elsevier

FEMSLE 04006

Separation of plasmid-mediated extended spectrum β-lactamases by fast protein liquid chromatography (FPLC system)

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Received 23 January 1990
Revision received 5 February 1990
Accepted 6 February 1990

Key words: FPLC; β-Lactamase; TEM; Cefotaxime; Ceftazidime resistance

1. SUMMARY

We have devised a reliable procedure for the separation of three β-lactamases of isoelectric focusing points (p*I*), 5.4, 6.5, and 7.9 by Fast Protein Liquid Chromotography (FPLC System). All of these enzymes were transferable and originated from a ceftazidime and cefotaxime resistant *Klebsiella pneumoniae* isolated in Bombay, India. The complete separation of the enzymes, achievable by this method, allowed each of the different individual β-lactamases to be characterised biochemically. This analysis revealed that the enzymes of p*I* 6.5 and p*I* 7.9 hydrolysed ceftazidime and cefotaxime, and were responsible for the resistance of *K. pneumoniae*, and its *Escherichia coli* J53-2 transconjugant to third generation cephalosporins. The enzyme of p*I* 5.4 was the TEM-1 β-lactamase. The β-lactamase of p*I* 7.9 appears quite different from any previously reported third generation cephalosporin hydrolysing β-lactamase, and consequently given the preliminary designation DJP-1. This is also the first example of extended spectrum hydrolysing β-lactamases found in Asia.

2. INTRODUCTION

There have been recent reports of transferable β-lactamases which confer resistance to broad spectrum cephalosporins [1–8]. These enzymes have been derived from the SHV and TEM β-lactamase genes [1]. In some cases these new enzymes are produced along with other transferable β-lactamases of different isoelectric points and sometimes these distinct β-lactamases are encoded by the same plasmid. Consequently, the investigation and characterisation of these enzymes would prove to be very difficult as they must be completely separated from the other β-lactamases. As many of these enzymes have similar molecular sizes, separation techniques based on the enzyme's ionic charge have been employed. This can be

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achieved by techniques such as preparative isoelectric focusing (IEF) which was used to isolate TLE-2 from TEM-1 and SHV-1 [9] and electro-dialysis which was used to separate TEM-E2 from TEM-1 [8]. However, these methods are time consuming and the amount and purity of the enzyme recovered is unsatisfactory. We have developed a Fast Protein Liquid Chromatography (FPLC System) technique for the rapid separation of three different β-lactamases produced by a ceftazidime and cefotaxime resistant *Escherichia coli* J53-2 transconjugant of a *Klebsiella pneumoniae* strain. In the past reverse phase High Performance Liquid Chromatography (HPLC) techniques have been used to purify β-lactamases such as SHV-1 [10], and FPLC System has been implemented to purify an inducible β-lactamase produced by *Proteus vulgaris* [11], and a β-lactamase from *Clostridium butyricum* [12], but neither procedure has ever been used to separate three plasmid encoded β-lactamases produced by the same strain.

3. MATERIALS AND METHODS

3.1. Strains

Klebsiella pneumoniae 8825 was isolated from a lymphoma patient at the Tate Memorial Hospital, Bombay, India. The rifampicin resistant *E. coli* J53-2 was used as the recipient strain in conjugation experiments.

3.2. Conjugation experiments

One ml of an overnight nutrient broth (Oxoid) culture of *E. coli* J53-2 and 0.1 ml of an overnight culture of *K. pneumoniae* were incubated with 4.5 ml of nutrient broth for 6 h at 37 °C. The *E. coli* J53-2 transconjugants were selected on agar containing ceftazidime (1 mg/l) and rifampicin (50 mg/l) or carbenicillin (100 mg/l) and rifampicin (50 mg/l).

3.3. Antibiotic susceptibilities

Minimum inhibitory concentrations (MIC) of antibacterial drugs were determined on Diagnostic Sensitivity Test Agar (Oxoid) at a concentration of 10^5 cfu as described previously by Amyes and Gould (1984) [13]. The MICs of ampicillin, cefotaxime and ceftazidime were additionally measured in the presence of clavulanic acid (2 mg/l).

3.4. β-lactamase preparation

One litre of nutrient broth containing ceftazidime (4 mg/l) was inoculated with the *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 and grown overnight at 37 °C. The cells were harvested by centrifugation for 15 min at $6000 \times g$. The bacterial pellets were washed in 25 mM sodium phosphate buffer (pH 7.0) and the centrifugation was repeated as described before. One ml of 25 mM sodium phosphate buffer (pH 7.0) was then added to the final pellet and the cells were resuspended to give 3 ml of cell suspension which was disrupted by ultrasound [14]. The cell lysate was cleared by centrifugation for one hour at $32\,000 \times g$. All of this crude β-lactamase preparation was applied to a Sephadex G-75 gel filtration column ($2\ cm^2 \times 90$ cm) and eluted with 25 mM sodium phosphate buffer (pH 7.0) at 15 ml/h [15]. The column was calibrated with chymotrypsinogen, cytochrome *c*, and ovalbumin. The fractions which exhibited β-lactamase activity were pooled and dialysed against 50 mM Tris-HCl buffer (pH 8.2) overnight.

3.5. β-lactamase identification

The β-lactamases were identified by analytical IEF on polyacrylamide gels [16] containing a 1 : 1 ratio of pH 3.5–10 and pH 4–6 ampholines (LKB). The substrate profiles of the β-lactamases were determined by assaying their hydrolytic activity against fixed concentrations of six different β-lactam drugs [14]. The rates of hydrolysis were expressed relative to ampicillin.

3.6. Fast protein liquid chromatography

Separation of the β-lactamases was carried out with the Pharmacia FPLC System consisting of LCC 500 Plus Controller, UV-M Monitor, FRAC 100 with an HR5/5 Mono Q column. The column was equilibrated with 50 mM Tris-HCl buffer (pH 8.2). Two ml of the β-lactamase sample (from the pooled peak fractions obtained by gel filtration with the Sephadex G-75 column) were added to the column. Separation was achieved by elution

with a linearly increasing concentration of sodium chloride (in the above buffer) to a maximum concentration of 1 M. Approximately, 34 fractions (1 ml) were collected from each 30 min separation. Each aliquot was tested for β-lactamase activity by the chromogenic cephalosporin nitrocefin spot test [17].

3.7. Preparation of plasmid DNA

The method of Takahashi and Nagano [18] was used to isolate and visualize the plasmid DNA of the *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825. The plasmid samples were run on an 0.5% agarose gel with plasmids R1 (89 kb), R6K (38 kb) as standards for large plasmids.

4. RESULTS

4.1. Conjugation experiments and plasmid analysis experiments

E. coli J53-2 transconjugants of *K. pneumoniae* 8825 were detected on agar plates containing ceftazidime or carbenicillin. The frequency of transfer on the ceftazidime and rifampicin was 1.18×10^{-6} per donor cell, and the transfer frequency for the selection on carbenicillin and rifampicin was 3.53×10^{-6} per donor cell. Analysis of the plasmid DNA in the *E. coli* J53-2 transconjugant revealed plasmids of 100 kb and 2.5 kb (Fig. 1). This suggested that the β-lactamase genes conferring resistance to carbenicillin and ceftazidime were either one and the same or they were located on the same plasmid.

4.2. Antibiotic susceptibilities

The *K. pneumoniae* 8825 strain was resistant to all the penicillins and first, second, and third generation cephalosporins, which were tested, with the exception of cefoxitin. All these resistance determinants were transferred to the *E. coli* J53-2 in the conjugation experiments (Table 1). In addition, the clinical strain and the transconjugant were both sensitive to imipenem. The *K. pneumoniae* 8825 and its transconjugant were sensitive to ampicillin, ceftazidime and cefotaxime in the presence of clavulanic acid (2 mg/l), inferring that the

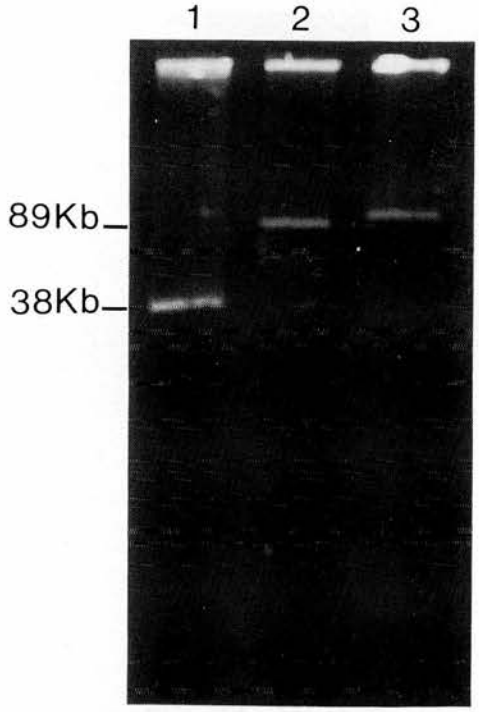

Fig. 1. Agarose gel electrophoresis of plasmid DNA from the *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 along with standard size plasmids. 1. R6K; 2. R1; 3. Plasmid isolated from *E. coli* transconjugant of *K. pneumoniae* 8825.

Table 1

Antibiotic susceptibilities of strains

β-lactam	Minimum inhibitory concentrations (mg/l)		
	K. pneumoniae 8825	*K. pneumoniae* 8825 × *E. coli* J53-2	*E. coli* J53-2
Ampicillin	> 250	> 250	4
+ clav *	8	4	4
Carbenicillin	> 250	> 250	8
Cephaloridine	125	32	2
Cephalexin	125	64	8
Cefoxitin	4	4	4
Cefuroxime	32	125	4
Ceftazidime	125	64	< 1
+ clav *	0.25	0.25	0.13
Cefotaxime	8	8	< 1
+ clav *	< 0.06	< 0.06	< 0.06
Ceftriaxone	16	4	< 1
Aztreonam	125	32	< 1
Imipenem	0.13	0.25	0.25

* In combination with clavulanic acid (2 mg/l).

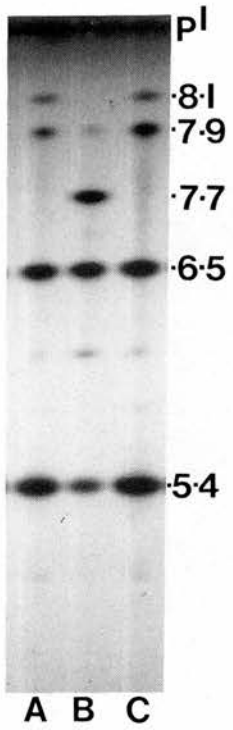

Fig. 2. IEF of β-lactamases produced by *K. pneumoniae* 8825 and its *E. coli* J53-2 transconjugant. A. β-lactamases produced by the *E. coli* transconjugant of *K. pneumoniae* 8825 selected on ceftazidime and rifampicin; B. β-lactamases produced by *K. pneumoniae* 8825; C. β-lactamases produced by the *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 selected on carbenicillin and rifampicin.

β-lactamases responsible for resistance to these drugs were either of the TEM or SHV groups.

4.3. Identification of β-lactamases

The original *K. pneumoniae* isolate produced four β-lactamases which focused at p*I* 5.4, p*I* 6.5, p*I* 7.7 and p*I* 7.9. The band at p*I* 5.4 aligned with TEM-1, the enzyme of p*I* 6.5 focused between TEM-3 (p*I* 6.5) and SHV-3 (p*I* 7.0), and the enzyme of p*I* 7.7 aligned with SHV-1/2. Crude enzyme preparations of the *E. coli* J53-2 transconjugants selected on either carbenicillin and rifampicin or ceftazidime and rifampicin possessed the β-lactamases which focused at p*I* 5.4, p*I* 6.5, and p*I* 7.9. However, they additionally produced another β-lactamase which focused at p*I* 8.1 (Fig. 2). This high p*I* band aligned directly with the *E. coli* J53-2 chromosomal β-lactamase. When the crude preparation of the transconjugant strain selected on rifampicin and ceftazidime was separated through the Sephadex G-75 column the intensities of the β-lactamases of p*I* 7.9 and p*I* 8.1 were reduced.

4.4. Fast protein liquid chromatography

Two ml of the Sephadex G-75 purified β-lactamase sample were separated through the Mono Q column in each run. This process was repeated until sufficient quantities of the different enzymes were obtained. In each FPLC System separation, β-lactamase activity was detected in the following fractions: 2, 3 and 4 (the void volume), 10/11, and 13/14. When these fractions were examined by IEF the β-lactamases of p*I* 7.9 and p*I* 8.1 were eluted in fractions 2, 3 and 4, the enzyme of p*I* 6.5 in fractions 10/11, and the β-lactamase of p*I* 5.4 in fractions 13/14 (Fig. 3). All the fractions, from the individual separations, containing the same β-lactamase, were combined so that sufficient enzyme could be characterised biochemically.

4.5. Characterisation of β-lactamases

The hydrolytic activity of each of the three enzymes against six different β-lactam antibiotics are shown in Table 2. The β-lactamase which focused at p*I* 5.4 aligned with TEM-1, it also had no hydrolytic activity against ceftazidime or cefotaxime and was therefore assumed to be TEM-1. The β-lactamase of p*I* 6.5 had hydrolytic activity against cefuroxime, cefotaxime, ceftazi-

Table 2

Relative rates of hydrolysis of FPLC system fractions *

β-lactam	Fraction:	2–4	10–11	13–14
	p*I* of β-lactamases:	7.9 and 8.1	6.5	5.4 (TEM-1)
Ampicillin		100	100	100
Carbenicillin		29	18	17
Cephaloridine		114	125	20
Cefuroxime		4.3	17	UM
Cefotaxime		17	9.2	UM
Ceftazidime		4.6	14	UM

* Rate for ampicillin = 100%; UM = unmeasurable due to insufficient hydrolysis.

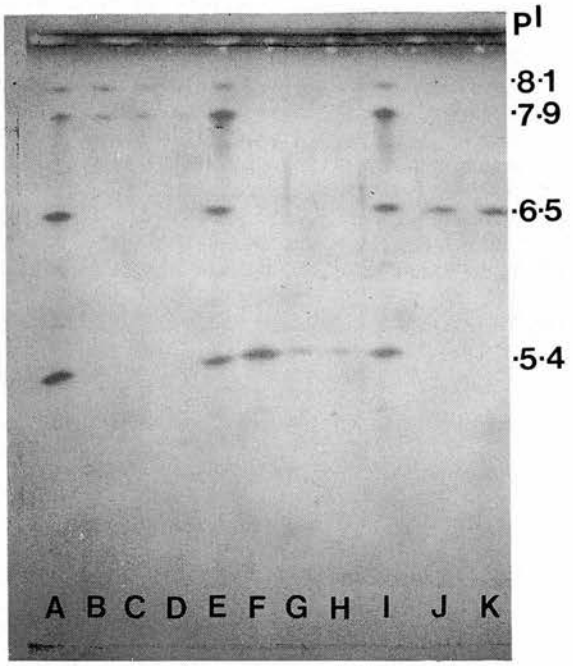

Fig. 3. IEF gel of β-lactamases separated by FPLC System. A. β-lactamases produced by *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 purified by G-75 Sephadex gel filtration; B. Fraction 2 eluted from FPLC System; C. Fraction 3; D. Fraction 4; E. Crude β-lactamase preparation of *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825; F. Fraction 14 eluted from FPLC System; G. Fraction 13; H. Fraction 15; I. as for E; J. Fraction 10 eluted from FPLC System; K. Fraction 11.

dime, and had a greater activity for cephaloridine than TEM-1. The fractions containing enzymes of p*I* 7.9 and p*I* 8.1 had a similar pattern of hydrolytic activities but the activity against the newer cephalosporins must result from the enzyme of p*I* 7.9 and not the upper *E. coli* J53-2 chromosomal β-lactamase band, as control studies have shown that this chromosomal enzyme has no hydrolysing activity against these β-lactam antibiotics.

5. DISCUSSION

These results have shown that Fast Protein Liquid Chromatography has been the most powerful method to date in the separation of multiple β-lactamases produced by the same strain. Its rapid, complete separation of the enzymes enabled the biochemical characterisation of each individual β-lactamase and permitted an assessment of how each β-lactamase contributed to the range of β-lactam resistances expressed by the host strain. We were thus able to show that two of the enzymes produced by the clinically derived *K. pneumoniae* 8825 strain confer resistance to cefuroxime, cefotaxime, ceftriaxone, and ceftazidime. Evaluation of the biochemical profile of each of these enzymes strongly suggests that the β-lactamase p*I* 7.9 (β-lactamase DJP-1) is a novel third generation cephalosporin hydrolysing enzyme. However, the plasmid mediated β-lactamase of p*I* 6.5 may be the same as CAZ-hi [4], or CAZ-6 [2], both of which have a reported p*I* of 6.5. The majority of transferable third generation cephalosporin resistance has emergence in France, Germany, and the U.K. where broad spectrum cephalosporin usage is high. However, these two broad spectrum enzymes were found to be produced by a strain isolated in India where the use of these newer cephalosporins is much lower. The occurrence of these two β-lactamases is the first report of transferable third generation cephalosporin resistance in India and Asia, and also the first example of two broad spectrum β-lactamases encoded by the same plasmid.

ACKNOWLEDGEMENTS

We thank the Science and Engineering Research Council for the CASE award for DJP, Michaela Torrance of Pharmacia LKB Biotechnology for the FPLC System, and Dr Kelker for sending the strains from the Tate Memorial Hospital, Bombay, India.

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LACK OF TRANSFERABLE THIRD GENERATION CEPHALOSPORIN RESISTANCE IN SCOTLAND.

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Four hundred and thirteen Gram-negative strains, which appeared resistant to cefuroxime by disk sensitivity testing, were isolated from patients of the Royal Infirmary, Edinburgh, Scotland over a period from January 1986 to January 1989. This collection of strains excluded Pseudomonas spp.. Seventy-nine of these strains had MICs of ceftazidime, cefotaxime or ceftriaxone of 4mg/L or greater. The β-lactamases produced by 54 of these third generation cephalosporin (3GC) resistant strains were examined by analytical isoelectric focusing. This same group of 3GC resistant organisms were also tested to see if they exhibited transferable resistance to either ceftazidime or cefotaxime.

This study revealed that none of the 54 3GC resistant strains conferred transferable resistance to either ceftazidime or cefotaxime. Indeed, none of these strains produced any of the TEM or SHV derived β-lactamases which have recently been reported to confer transferable resistance to 3GCs. Consequently, the 3GC resistance conferred by strains in this survey most probably resulted from the production of chromosomal cephalosporinases. This survey yielded only one novel chromosomal β-lactamase which was produced by Xanthomonas maltophilia and focused as 3 distinct bands of pI 5.2 to 5.6 and possessed different characteristics to the L-1 or L-2 β-lactamases of X.maltophilia. In conclusion this survey has illustrated that TEM or SHV derived β-lactamases which mediate transferable resistance to 3GCs do not appear to be threatening the future use of 3GCs in Scotland.

WESTERN PACIFIC CONGRESS ON INFECTIOUS DISEASES & CHEMOTHERAPY

KUALA LUMPUR, MALAYSIA 21 - 24 FEBRUARY 1989

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MUTANTS OF THE TEM-1 β-LACTAMASE CONFERRING RESISTANCE TO THIRD GENERATION CEPHALOSPORINS

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There have been recent reports that a new series of plasmid-mediated β-lactamases, which confer resistance to third generation cephalosporins (3GC), have been derived from the TEM-2 enzyme. In this study, two types of ceftazidime (CAZ) resistant mutants have been obtained spontaneously from an E. coli J62-2 expressing the TEM-1 enzyme (mutation frequency = 10^{-9}). These mutants produce β-lactamases with similar molecular weights, kinetic parameters and iso-electric points (pI) to novel β-lactamases produced by CAZ resistant clinical isolates which have recently been identified in this laboratory. The first mutant enzyme focuses as a doublet band at pI 5.3 with an additional weak TEM-1 band (pI 5.4). This doublet co-focuses with a β-lactamase produced by a CAZ resistant Klebsiella pneumoniae, which was originally isolated at a Liverpool hospital, although the clinical strain had a stronger TEM-1 band. The second mutant produced an enzyme with a pI identical to an enzyme produced by a CAZ resistant E. coli strain isolated in Belgium; both these β-lactamases focus between TEM-1 and TEM-7. These results purport the view that some plasmid-encoded β-lactamases, which confer resistance to 3GC, have evolved from the simple mutation of the TEM-1 β-lactamase resistance genes rather than the TEM-2 gene.

Transferable cephalosporin resistance not inhibited by clavulanate in *Escherichia coli*

SIR,—We report the isolation of a strain of *Escherichia coli* resistant to penicillins and cephalosporins because of a plasmid-encoded beta-lactamase that was not inhibited by clavulanic acid. The organism was isolated in London from raw area swabs and a biopsy specimen from a patient with 35% burns. The patient had been treated in Pakistan with cefotaxime and amikacin for at least 15 days before transfer to the UK. The strain was resistant to all beta-lactams tested (table), except imipenem (sensitive by disc test), and to chloramphenicol, tetracycline, and trimethoprim. On disc testing, addition of clavulanic acid did not restore susceptibility to ceftazidime. The strain was susceptible to aminoglycosides and ciprofloxacin. Following grafting, the patient made steady progress. Two episodes of clinical septicaemia were treated with imipenem and ciprofloxacin, respectively, although no organisms were isolated from blood cultures. The resistant strain was not isolated subsequently from his burns. The patient was in isolation throughout his two-month stay and secondary spread to other patients was not observed.

The strain contained three plasmids of 80, 45, and 35 MD. Resistance to beta-lactams was transferred readily to, and maintained stably in, *E coli, Enterobacter cloacae,* and *Klebsiella oxytoca* (table). These transconjugants acquired the 80 MD plasmid together with resistance to chloramphenicol and tetracycline. In addition, *Ent cloacae* transconjugants acquired the 35 MD plasmid and *E coli* transconjugants acquired plasmids which varied from 28 to 45 MD. These plasmids may have been deletions of plasmids carried by the original (donor) strain. These results suggest that the resistance to beta-lactams was encoded on the 80 MD plasmid. No transfer was detected to strains of *Proteus mirabilis* or *Serratia marcescens.*

The *E coli, K oxytoca,* and *Ent cloacae* transconjugants all produced an identical beta-lactamase of pI 8·8. This beta-lactamase (designated BIL-1) hydrolysed nitrocefin and cephaloridine, but not ampicillin, carbenicillin, cefuroxime, cefotaxime, and ceftazidime. Unlike the TEM-1 beta-lactamase, the hydrolytic activity of BIL-1 was inhibited by ampicillin, cefuroxime, and ceftazidime. BIL-1 was not inhibited by clavulanic acid since this compound did not render the *E coli* transconjugants sensitive to ampicillin, cefotaxime, or ceftazidime (table). BIL-1 was almost 4000 times more resistant to inhibition by clavulanic acid than TEM-1 (data not shown).

There have been reports of plasmid-mediated beta-lactamases that confer resistance to the third generation cephalosporins, but these are all derivatives of the ubiquitous TEM-1/2 and SHV-1 resistance genes[1] and are very sensitive to inhibition by clavulanic acid. Our data indicate that BIL-1 is unrelated to these enzymes. The characteristics of BIL-1 resemble more closely those of the chromosomally mediated beta-lactamases of *Ent cloacae*[2] and *E coli*.[3] We are unaware of other reports of a plasmid-encoded class C-like extended-spectrum cephalosporinase.[4] In addition, BIL-1 is the first example of a beta-lactamase conferring transferable resistance to all penicillins, to first, second, and third generation cephalosporins, and to clavulanic acid.

SUSCEPTIBILITY TO BETA-LACTAM ANTIBIOTICS OF E COLI AND TRANSFER OF RESISTANCE TO OTHER GENERA

Strain	Minimum inhibitory concentration (mg/l)*					
	Amp	Carb	Cefo	Ceft	Cefu	Pip
E coli						
BS (clinical strain)	64	256	>16	>16	>32	64
K12 (recipient)	≤4 (4)	≤16	≤0·25 (<0·06)	0·5 (0·13)	≤2	≤4
Transconjugant	64 (>32)	128	8 (>4)	>16 (>4)	>32	64
K oxytoca						
478 (recipient)	>64†	128†	≤0·25	≤0·25	4	16
Transconjugant	>64	128	2	4	32	64
Ent cloacae						
471 (recipient)	≤4	≤16	≤0·25	≤0·25	4	≤4
Transconjugant	>64	64	16	8	>32	16

†Chromosomally encoded resistance to penicillins.
*MIC in presence of 2 mg/l clavulanic acid shown in parentheses.
Amp = ampicillin, Carb = carbenicillin, Cefo = cefotaxime, Ceft = ceftazidime, Cefu = cefuroxime, Pip = piperacillin.

The transferability in vitro of this resistance to bacteria of different genera raises the possibility that similar dissemination might occur in vivo. This importation of an organism with transferable resistance to beta-lactams lends further weight to the view[5] that patients from abroad should be screened microbiologically, and that appropriate control measures should be instituted until they prove to be clear of such carriage.

We thank Mr J. A. Clarke for permission to report this case.

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Cyclophosphamide versus ifosfamide in paediatric oncology

SIR,—We concur with Dr Shaw and Dr Eden's (April 28, p 1022) comments about ifosfamide and cyclophosphamide use in paediatric oncology. For 15–20 years cyclophosphamide dose in widely used regimens in paediatric oncology has been 0·9–1·5 g/m² per course;[1-3] "intensive" regimens use 1·8 g/m² per course.[4,5] Typical ifosfamide doses are 6–10 g/m² per course,[7] which is the equivalent of 1·7–3·0 g/m² of cyclophosphamide—ie, up to 3·3 times the "standard" cyclophosphamide dose. It is well established that tumours that do not respond to low doses of alkylating agents regress when the dose of the same agents is increased. Hence, it should be no surprise that results are encouraging when ifosfamide 6–10 g/m² per course is substituted for cyclophosphamide 1·2 g/m² per course in otherwise equivalent regimens.[6]

Dr Jurgens and Dr Gobel (June 9, p 1399), commenting on Shaw and Eden's report, state that ifosfamide has an advantage over high-dose cyclophosphamide in that the latter produces greater bone-marrow toxicity and necessitates supportive treatment with cytokines.

We would point out that studies in children and young adults from our institution have demonstrated the feasibility of administraton of multiple courses of very high dose cyclophosphamide in conjunction with other cytotoxic agents. Thus, on the basis of previous experience,[7] we initiated in 1987 an induction protocol for patients with poor-risk neuroblastoma that included four consecutive courses of cyclophosphamide 140 mg/kg (ie, 4200 mg/m²), doxorubicin 45 mg/m², and vincristine.[8] (This cyclophosphamide dose is 2·3 times the intensive dose and up to 4·7 times the standard dose.) To achieve maximum dose intensity (high doses, short term interval), courses were started when the neutrophil count reached 500/μl and platelets were over 100 000 μl. Most courses began by day 21. Grade 3–4 myelosuppression occurred, yet, as evidence of the relative stem-cell sparing effect of cyclophosphamide, bone-marrow harvested after the four courses reconstituted haemopoiesis after subsequent myeloablative therapy with autologous bone-marrow rescue. Extramedullary toxicities were mild. This last finding, as well as analyses demonstrating the

Resistance to antibacterial drugs in commensal bacterial flora in South India

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In 1984 a study was carried out at the Christian Medical College Hospital (CMCH) in Vellore, Tamil Nadu, South India to investigate resistance to antibiotics in pathogens causing urinary tract infections. This investigation reported high levels of resistance to ampicillin and trimethoprim. Resistance to ampicillin (Minimum Inhibitory Concentration (MIC) >10mg/L) was found in 77% of Escherichia coli isolated (Nandivada & Amyes 1987) and 64% were resistant to trimethoprim (MIC > 10mg/L)(Young et al 1986).

In 1989, we returned to CMCH to establish if the reservoirs of the antibiotic resistance genes were actually located in the commensal faecal flora of the healthy population. Two cohorts of volunteers were recruited: an urban group (U) from the people of the town of Vellore (pop. 150,000) and a rural group (R) from villages situated about 40km from the town. Three villages were used; Kilvayattarankuppam - a roadside village with good access to the town, Kavanur - a riverside village with moderate access and Melmoil - a village in the foothills with poor communications. In both study groups faecal specimens were taken from volunteers who were at least five years old, apparently healthy and had received no antibacterial chemotherapy in the previous week. Each specimen (92 from the urban study and 122 from the rural group) was initially examined for the presence of large Gram-negative rods (not Pseudomonas) which were resistant to trimethoprim, ampicillin, chloramphenicol or nalidixic acid. The faecal specimens were plated out onto a Maconkey-like agar containing each of these drugs (at 10mg/L). There was almost universal carriage of enterobacteria resistant to trimethoprim (U=98.3%, R=100%), ampicillin (U=98.9%; R=97.5%) and chloramphenicol (U=96.8%; R=97.5%). There was neither any significant difference between the proportions in the urban and rural study areas nor between proportions from the individual villages, despite the varying degree of remoteness. On the other hand, the carriage of nalidixic acid resistance was considerably lower. However, it was significantly more common in the urban population (29.7%) than in the rural (11.2%) (χ^2 = 7.84; 0.01>p>0.001).

Further study on the trimethoprim resistant strains has revealed that 86% of them were highly resistant (MIC >1000mg/L), which is indicative of a plasmid origin of the gene. However, only a minority of these highly resistant strains were capable of transferring trimethoprim resistance. None of the nalidixic acid resistant strains tested were resistant to ciprofloxacin (1mg/L) despite the free availability of this drug in India.

These results provide evidence that the commensal gut flora of the human population, in this part of India, acts as a reservoir for antibiotic resistance genes which may then be acquired by pathogens. Such high carriage rates of resistant commensals may be stimulated by the free availability of antibiotics "over the counter" without prescription. Antibacterial drugs, bought in Vellore, were examined by bio-assay and absorbance spectrum analysis and found to be virtually identical to equivalent drugs obtainable in the UK and USA.

We are grateful to the Royal Society and the British Society for Antimicrobial Chemotherapy for funding this research visit.

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TEM-E4: A β-lactamase which confers transferable resistance to ceftazidime

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When third generation cephalosporins (3GCs), such as ceftazidime and cefotaxime, were first introduced they were resistant to hydrolysis by all the plasmid-mediated β-lactamases known at that time. However, in the last four years a number of plasmid mediated β-lactamases have evolved which have the ability to hydrolyse ceftazidime and cefotaxime and, consequently, promote transferable resistance to 3GCs (Philippon et al 1989). Many of the 3GC hydrolysing β-lactamases have been shown to have evolved from the ubiquitous TEM-1/2 and SHV-1 resistance genes.

The ceftazidime resistant isolate of Serratia marcescens 7919 was isolated in Belgium in 1987. Conjugation of strain 7919 with Escherichia coli J53-2 resulted in ceftazidime-resistant E. coli J53-2 transconjugants. Analysis of the plasmid DNA in these transconjugant strains revealed a single plasmid band of 56Kb, which was designated pUK724. This plasmid was also visualized in the S. marcescens strain. With the exception of imipenem, the S. marcescens 7919 strain was resistant to all penicillins as well as first, second and third generation cephalosporins but the E. coli J53-2 transconjugant was resistant only to ceftazidime and remained sensitive to all the other third and second generation cephalosporins tested. The ceftazidime and ampicillin resistances, expressed by the E. coli transconjugant, were diminished with the addition of clavulanic acid (2mg/L). This illustrated that the β-lactam resistance of the E. coli J53-2 transconjugant of S. marcescens 7919 must be β-lactamase mediated. Iso- electric focusing of bacterial extracts and visualisation with the chromogenic cephalosporin, nitrocephin revealed that both the original S. marcescens isolate and the E. coli J53-2 transconjugant produced a novel β-lactamase, designated TEM-E4. This enzyme focused marginally above the TEM-2 (pI 5.6) and below the TEM-6 (pI 5.85) enzyme. In comparison with standards, TEM-E4 was allocated a pI of 5.61. The TEM-E4 β-lactamase exhibited low rates of hydrolysis for ceftazidime and cefotaxime although it hydrolysed cefotaxime more efficiently than ceftazidime. It was also shown that TEM-E4 and TEM-1 had similar molecular weights and ID_{50} values for clavulanic acid.

In separate experiments, challenging E. coli J53-2 (RP4), a TEM-2 β-lactamase producing strain, with ceftazidime (2mg/L) resulted in spontaneous mutants capable of resisting the drug. In these mutants, the TEM-2 enzyme had mutated so that it was now capable of hydrolysing ceftazidime (Mutant β-lactamase D). Close comparison of Mutant β-lactamase D and TEM-E4 showed that they had similar resistance profiles to β-lactam antibiotics, they had identical pIs and similar kinetic constants for five different β-lactam substrates. These results infer that TEM-E4 resulted from direct mutation of the TEM-2 gene. Further evidence for this has been obtained by DNA-DNA hybridization studies which showed that the TEM-E4 gene hybridised with a radiolabelled TEM gene probe.

In conclusion, the TEM-E4 β-lactamase is a unique plasmid-encoded enzyme, capable of hydrolysing 3GCs, which is present in clinical bacteria. TEM-E4 could be obtained by spontaneous mutation of the common TEM-2 gene and seems to have derived directly from it.

We thank the SERC for the CASE studentship to DJP.

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Tn3701, PROTOTYPE OF A CLASS OF COMPOSITE CONJUGATIVE GENETIC ELEMENTS IN STREPTOCOCCI.

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The plasmid-free Streptococcus pyogenes strain A454 contains a conjugative element, Tn3701, encoding resistance to erythromycin (Em), tetracycline (Tc), and minocycline (Mn) (1). We have mapped a 50-kilobase (kb) chromosomal region of A454 corresponding to the internal part of Tn3701. Tn3701 includes a 19.7-kb structure, designated Tn3703, on which the EmTcMn determinants were localized. Tn3703 which is very similar in structure to the conjugative transposon Tn916 (2), is capable of transposition but is not conjugative. By hybridization experiments, we revealed strong homology between Tn3701 and the conjugative chromosomal elements Ω (cat-tet) (3) and Tn3951 (4) described in Streptococcus pneumoniae strain BM6001 and in Streptococcus agalactiae strain B109, respectively. Like Tn3701, Ω(cat-tet) and Tn3951 are composite elements containing in their internal region a structure similar to Tn916. Moreover, among eleven antibiotic resistant plasmid-free clinical isolates tested (10 streptococci of groups A,B and G and one S. pneumoniae) three showed homology to Tn3701 and four to Tn916, suggesting that these two types of conjugative chromosomal elements are common in plasmid-free antibiotic resistant streptococci.

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610/OS31

TWO NOVEL PLASMID MEDIATED B-LACTAMASES WHICH CONFER RESISTANCE TO CEFTAZIDIME

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There have been a number of recent reports on the plasmid mediated B-lactamases TEM-3 to TEM-7. These enzymes confer resistance to third generation cephalosporins (3GC) and are believed to have evolved from TEM-1/TEM-2. We have identified a further two novel plasmid mediated B-lactamases which confer resistance to 3GC.
The first was derived from Klebsiella pneumoniae and isolated in 1982 at a Liverpool hospital. This is believed to be the earliest example of a plasmid mediated B-lactamase conferring resistance to a 3GC. This strain contains a 103 kb plasmid which encodes TEM-1 (pI 5.4) and the novel B-lactamase (doublet at pI 5.3). The second B-lactamase was expressed by an E.coli strain isolated in Belgium. It is encoded by a 60 kb plasmid and has a pI between TEM-1 (pI 5.4) and TEM-7 (pI 5.41). Both the original isolates and their respective E.coli J53-2 transconjugants had much higher MICs of ceftazidime (32mg/L at 10^5 cfu) than for cefotaxime (0.25-0.5mg/L at 10^5 cfu). However,the relative efficiency of hydrolysis (Vmax/Km [ampicillin = 100%]) of ceftazidime and cefotaxime were in the order of 1-2% for both enzymes. The rates and efficiency of hydrolysis observed for these two enzymes for ampicillin, carbenicillin, and cephaloridine were also similar to TEM-1. Both enzymes were inhibited by clavulanic acid and had molecular weights similar to TEM-1. This enforces the view that these two enzymes (along with the 3GC hydrolysing

Reprints from ABSTRACTS OF THE
ANNUAL MEETING OF THE AMERICAN SOCIETY
FOR MICROBIOLOGY, 1990
13–17 May 1990
Anaheim, CA.

A-70 Comparison of TEM-E3 and TEM-10 β-lactamases.

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Transferable resistance to ceftazidime has been found in a South London Hospital (SLH) and the North Middlesex Hospital (NMH). The β-lactamases responsible (TEM-E3) were shown to have identical isoelectric points (pI) and similar Vmax, Km and efficiency of hydrolysis values for six β-lactam substrates. These assays were performed in parallel with TEM-10, a β-lactamase recently identified in the USA, and demonstrated that TEM-10 and TEM-E3 were identical. MIC data showed that E.coli J53-2 trans-conjugants producing TEM-10, TEM-E3 (NMH), or TEM-E3 (SLH) were resistant to ceftazidime (64-125 mg/L), aztreonam (32-64 mg/L), and only slightly resistant to cefotaxime (0.5-1 mg/L). TEM-E3 and TEM-10 had a greater affinity for cefotaxime (Km 11-21 μM), than for ceftazidime (Km 100-167 μM) although they hydrolyse cefotaxime at a much slower rate (2-3% relative to the Vmax for ampicillin) than ceftazidime (30-40%). However, the efficiency of hydrolysis values for cefotaxime were relatively high (12-16% relative to ampicillin and 20-33% for ceftazidime) considering the enzyme confers no significant cefotaxime resistance. This study confirms that the same gene is now present in clinical bacteria in the USA and Europe and this β-lactamase differs from both TEM-5 and TEM-9.