THE CHROMOSOMAL BETA-LACTAMASES OF THE GENUS ACINETOBACTER

by

JOHN HOOD

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"Good science is no rosebed but the romance is still there. The thrill of discovery outweighs the drudgery, the despair at one's inadequacy, the fight for financial support, the setbacks and mistakes, the long hours and the nagging feeling of being overtaken. "

Max Perutz

DEDICATION

This thesis is dedicated to my family: Patricia, my wife and Katie, my daughter; George and May Hood, my father and mother; and May Cockburn, my mother-in-law. It was in *their* time that most of the work involved in carrying out this thesis took place. If it was not for their continued support and encouragement it would never have come to completion.

It is also dedicated to Robert Reid Gillies - who taught me much and is sorely missed.

ABSTRACT

Introduction

The evolution of the Host-Parasite relationship in terms of the fascinating interaction of the evolution of the host and parasite over the last 50 years is considered. This includes the evolution of new therapies such as intensive care and new drugs. The shifts in the sort of pathogens causing nosocomial infection and the evolution of their resistance mechanisms to antimicrobial agents are described.

The various classification schemes for β-lactamases are reviewed. The evolution and origin of β-lactamases are discussed. The newer forms of resistance mediated to third generation cephalosporins are evaluated. Finally the introduction considers briefly the genus *Acinetobacter* with particular reference to its 'up and coming' status as a problematical nosocomial pathogen and the taxonomic chaos that surrounds it.

Results

Cefuroxime resistance survey

This deals with a survey of 200 strains of cefuroxime-resistant, Gram negative, oxidase negative aerobic bacilli, collected in the Royal Infirmary of Edinburgh. The predominant species within this group was *Enterobacter* spp. followed by *Acinetobacter spp.* The β-lactamases produced by these 200 strains were investigated using isoelectric focusing. It was apparent that most of the cefuroxime resistance was the result of 'typical' chromosomal β-lactamases. There was no evidence of transferable third generation cephalosporin resistance.

Hospital cefuroxime/gentamicin resistance study

This study employed computer records and looked at cefuroxime and gentamicin resistance over 5 years in Gram negative, oxidase negative aerobic bacilli (14,000 isolates) within 18 distinct units of the RIE. This showed a remarkable variation between units. Overall the resistance to cefuroxime was much higher than to

gentamicin. Resistance to cefuroxime, within the hospital, was much higher in the ITU areas. Over the five year period cefuroxime resistance was increasing (clearly significant in the Surgical hospital and ITU areas) and gentamicin resistance was decreasing (clearly significant in the Medical hospital) or stable. An attempt to relate the differences in resistance rates was made with respect to antimicrobial usage within these areas over a 1 year period coupled with the bed occupancy figures. No clear relationship was found but the possible reasons for these findings were discussed.

Acinetobacter chromosomal β-lactamases

These enzymes were studied with the aim of characterising them fully by means of pI, M_r, substrate profiles, kinetic data and inhibition studies. 9 enzymes were considered. It was found that these enzymes do not focus on conventional IEF polyacrylamide gels and therefore a novel technique was devised employing an agarose, urea and sorbitol (AUS) gel. Four ACE (*Acinetobacter* **C**hromosomal **E**nzyme) groups were elucidated. M_r estimates on conventional low pressure gel permeation showed that many of these enzymes seemed very large with M_r's of 500 - >1,000Kd. Biochemically their place as Bush group 1 (CEP-N) cephalosporinases was confirmed. Further studies using high performance ion exchange chromatography, employing FPLC, and Reversed polarity SDS-free electrophoresis on the PhastSystem, confirmed the groupings of the ACE 1, 3 and 4 enzymes, however ACE 1 and ACE 2 were found to be very similar.

A purification scheme was devised employing high performance ion exchange chromatography on FPLC, followed by gel permeation on FPLC and with SDS-PAGE on the PhastSystem. This revealed that these enzymes exist as different ionic variants of at least three molecular sizes, in the case of the ACE 1 enzyme produced by H63 the predominant size in this system was around 10-15Kd with variants at around 40Kd and 700Kd. Further purification techniques and more research were therefore required.

The position of the β-lactamase within the structure of the *Acinetobacter* cell was shown to be predominantly in the periplasm and not membrane bound.

Therefore these enzymes appear to be cephalosporinases which exist as aggregated proteins which partially explains their unusual behaviour in conventional gel permeation and polyacrylamide gel systems. Further directions for future research are discussed.

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DECLARATION

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

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"L'art du chercheur c'est d'abord de se trouver un bon patron" ,

Andre Lwoff.

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Full Papers

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ABBREVIATIONS

ACE	*Acinetobacter* **C**hromosomal **E**nzyme
AMP	ampicillin
AUS	agarose, urea & sorbital gel
AZ(AZT)	aztreonam
bp	base pair
CARB	carbenicillin
CAZ	ceftazidime
CED(CRD)	cephradine
CER	cephaloridine
CFX	cefoxitin
CIP	ciprofloxacin
CTR(ROC)	ceftriaxone
CXM	cefuroxime
FPLC	Fast Protein Liquid Chromatography R
GENT	gentamicin
IEF	isoelectric focusing
IMI	imipenem
Kd	kilodaltons
L	litres
M	molar
MIC	minimum inhibitory concentration
mM	millimolar
μM	micromolar
M_r	molecular size
NET	netilmicin
nmole	nanomole

PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
pI	isoelectric point
RIE	Royal Infirmary Edinburgh
SDM	stably derepressed mutants
SDS	sodium dodecyl sulphate

INTRODUCTION

PROLOGUE

In 1884, a nurse from King's College Hospital came under the care of Joseph Lister for the treatment of a deep gluteal abscess after she had been injured by a horse - drawn cart. He treated this locally with an extract of *Penicillium glaucum* (therefore presumably crude penicillin). The patient herself related this much later (Fraser - Moodie, 1971).

MacFarlane (1984) in his superb biography of Alexander Fleming mentions two occasions in which Fleming used crude penicillin to treat infections in his colleagues. Firstly, 1ml of "mould filtrate" was instilled into the antrum of a co-worker (Craddock) who had acute sinusitis. The result was inconclusive. Secondly he relates the successful treatment of a pneumococcal conjunctivitis in Dr K B Rogers a research assistant and key member of Fleming's rifle team just prior to an important competition. These cases were also mentioned by Selwyn (1980).

None of these cases were objectively assessed and published in reputable journals. Therefore the era of the serious therapeutic use of the Beta-lactam antibiotics commenced on the 12 February 1941 in the John Radcliffe Hospital, Oxford when Dr Charles Fletcher injected 200mg of penicillin into a 43 year old policeman.

This unfortunate man, who previously had been fit and healthy, had developed a primary infection with *Staphylococcus aureus* with a secondary infection with *Streptococcus pyogenes*. The infection which had started from a sore at the corner of his mouth, had within a month spread to involve severe

infections of both orbits,the lung, osteomyelitis, and multiple abscesses of his face and scalp. There had been little improvement with a course of sulphapyridine.

He then received, over a period of 5 days, a total dose of 4.4g of penicillin. This resulted in a dramatic improvement of his condition with complete resolution of the infections at most sites. At this point the stocks of penicillin ran out and although he remained stable for a further 10 days he subsequently deteriorated and died of overwhelming sepsis. (Abraham *et al.*,1941; Fletcher,1984). This is a cautionary tale of the devastating effect of infection in the pre-antibiotic era.

This case, however, gives us a taste of the bitter-sweet nature of this particular "success" story. The penicillin almost, but not quite, cured the policeman's infection before the supply ran out; however, it must have given them hope for the future which was shortly to be confirmed by independant clinical trials particularly those carried out on wounded soldiers in the North Africa Campaign of World War II (Pulvertaft, 1943). None of those involved in these early studies could have realised that by 1985 (in that year alone) 2,200 tonnes of penicillin were manufactured for human and veterinary use with a further 12,000 tonnes produced to provide the starting point for the manufacture of the semi-synthetic penicillins and cephalosporins (Rolinson, 1988).

So began an era in medical practice which was to have profound effects on enormous numbers of patients, both in terms of their morbidity and mortality. Never before nor since can a simple group of drugs have had such a profound impact on human disease; and continue to have that effect some fifty years later.

Garrod (1979) would perhaps dispute this, he felt that it was the discovery of prontosil in 1935 by Domagk that was "the outstanding therapeutic discovery of modern times". Donowitz and Mandell (1988) would disagree

stating that the accolade should go to the Beta-lactam drugs. They quote McDermott & Rogers (1982) who took the rather dramatic view that if one could cure all the cancers in the USA it would increase the life expectancy of the average American by two years while the introduction of antimicrobials had already raised the life expectancy by 10 years. "The agent most responsible for this remarkable effect is penicillin, the prototypic Beta-lactam antibiotic".

At this time we read of many new and important therapeutic breakthroughs which help and enhance the lives of many patients. These might include agents such as the Beta-blockers and H_2 antagonists. Many of these drugs at best ameliorate or slow down the disease process but very few tackle the disease in such a way as to cure it completely. This differs from the effects of antimicrobial agents in that in many cases they will completely cure the patient of their infection; and with their prophylactic use may prevent the infection from ever occurring.

The problem that faces us now is one that has existed all along - even before physicians or surgeons used the Beta-lactams, or any other antimicrobials, as therapeutic agents - namely bacterial resistance. Unfortunately many medical practitioners now take the abilities of antimicrobial agents for granted. Let us hope that the increasing problems of bacterial resistance to these agents *never* allow the clock to be turned back to the situation that prevailed in the "Septic" ward of the John Radcliffe Infirmary in February 1941.

In a useful contribution entitled "Observations on Spiralling Empiricism: Its Causes, Allure and Perils with particular reference to Antibiotic Therapy", Kim & Gallis (1989) wrote: "Fleming, Domagk and Florey could not have anticipated the proliferation of antibiotics that would follow their discoveries, nor would have

understood the profligate and injudicious use of these agents in modern therapeutics!"

Perhaps the real problem is a lack of basic understanding of the nature of the host - parasite relationship. "The search for essential, basic knowledge is tedious, difficult and time consuming, whereas the appeal of quick rewards is tough to resist. We are disheartened and diverted by the easy and quick marketing successes of practices that lack this essential, basic knowledge", Kornberg (1989).

Many have forgotten the earthy origins of the antimicrobial agents and that the mechanisms to overcome their effects have consequently evolved over a period of millions of years prior to their therapeutic use by man (Pollock,1967; Bassett, 1980). It is worthwhile examining more closely the complicated and fascinating interaction that has taken place between the host (man) and the parasite (bacteria) during the last fifty years or so since that first therapeutic use of antimicrobial agents.

HOST EVOLUTION : ADVANCES IN THERAPY AND THEIR IMPLICATIONS ON MICROBIOLOGICAL CONTROL

In the years since the Second World War many huge advances in most areas of medical practice have taken place. Perhaps the most remarkable have been developments in the technology which have made it possible to sustain, and indeed prolong, life and restore function for critically ill and injured patients. These advances, coupled with those in anaesthesia and blood transfusion have been the necessary basis for success of a number of the more aggressive forms of therapy e.g. radical surgery, cardiac surgery and organ transplantation.

EVOLUTION OF CRITICAL CARE

It was the polio epidemics of the early 1950's which led to the introduction of mechanical respiratory support (Bower *et al.*, 1950; Lassen, 1953; Ibsen, 1954). This was followed by the early respiratory intensive care units in Oxford, Toronto and Baltimore (Barber *et al.*, 1959; Safar *et al.*, 1961).

Since then, as new techniques have been developed, (see Table 1) critical care and specialist care units have evolved to support the postoperative care of surgical, cardiac, neurosurgical, trauma and burns patients as well as patients with acute respiratory, neurological, gastrointestinal, renal and metabolic crises. Further specialised units have appeared in the obstetric, paediatric and neonatal areas.

Critical care medicine has become synonymous with "cutting edge, high tech" medicine with : haemodynamic monitoring ; sophisticated forms of mechanical ventilatory support ; metabolic monitoring; fluid and electrolyte therapy ; total parenteral nutrition ; haemodialysis or continuous arteriovenous haemofiltration ; intracranial pressure monitoring with ventilatory and

pharmacological intervention for the prevention and treatment of cerebral oedema; innovative surgical therapies for trauma, burns, intra-abdominal catastrophies, intracranial haemorrhage and acute heart failure, plus a huge arsenal of potent drugs, particularly antimicrobials, Maki (1989).

Table 1. Evolution of Intensive Support

Technique or device	**Reference**
Resuscitation	
Internal cardiac massage	Igelsrud,1901 (quoted by Weil et al.,1989)
Open chest massage and defibrillation	Beck *et al.*,1947
Transthoracic pacing	Zoll,1952
External defibrillation	Zoll *et al*.,1956
Closed chest cardio-pulmonary resuscitation	Kouwenhoven *et al.*,1960
Respiratory support	
Intermittent positive pressure ventilation(IPPV)	Bower *et al.*,1950
Blood gas electrodes	Severinghaus & Bradley,1958
Blood gas laboratory	Udhoji *et al.*,1963
Cuffed endotracheal tubes	Pontoppidian *et al.*,1977

Table 1 (contd.)

Technique or device	Reference
Intravenous access and haemodynamic support	
Central venous catheters	Cournand & Ranges,1941
Right heart catheterisation	Richards,1945 Cournand *et al.*1945
Polyethylene intravenous catheters	Meyers,1945
Left heart catheterisation	Zimmerman *et al.*,1950
Subclavian lines	Aubaniac,1952
Flow directed pulmonary artery catheters	Swan *et al.*,1970
Metabolic support	
Intravenous feeding	Dudrick *et al.*,1968
"Fast" biochemistry	Weil *et al.*,1981
Renal support	
Haemodialysis	Kolff,1947
Percutaneous haemodialysis catheter	Shaldon *et al.*,1961

Table 1 (contd.)

Technique or device	Reference
Cardiac surgery	
Aortic valve replacements	Hufnagel & Harvey,1954
"Heart-lung machine"	Gibbon,1954
Coronary angiography	Sones *et al.*,1959
Mitral valve replacements	Starr & Edward,1961
Heart transplantation	Barnard,1967
Intra-aortic balloon pump	Kantrowitz *et al.*,1968
Aorto-coronary bypass	Favaloro,1968

This new technology has had two main effects in terms of the host-parasite relationship. Firstly these invasive devices (endotracheal tubes; urethral and intravascular catheters; intraventricular catheters and surgical wound drains) have negated one of the most important of the host defences i.e. the mechanical defences - the skin and mucous membranes.

Secondly these therapies allow progressively iller and iller patients to survive for longer periods often with multiple organ failure and consequently defects in their humoral and cellular defence mechanisms.

Therefore it is not surprising that these patients have a significantly higher incidence of hospital acquired (nosocomial) infection, Donowitz *et al.* (1982).

Critical care patients are particularly vulnerable to septicaemias (often associated with intravascular devices), pneumonias (often related to intubation and mechanical ventilation) and intra-abdominal infections (following trauma or surgery). Infection is the most common cause of death in patients who survive major trauma or full thickness burns and is the most common identified cause of multiple organ failure. (Fry *et al.*,1980; Pine *et al.*, 1983; Marshall & Dimick, 1983).

All these host factors interact with the parasite and, as we shall see, has had an effect on the evolution of the micro-organisms causing infections in these patients.

EVOLUTION OF THERAPY FOR HAEMATOLOGICAL MALIGNANCIES AND SOLID TUMOURS

The other group of patients who provide the clinical microbiologist with continuing problems of hospital acquired infections are those undergoing therapy for haematological malignancies and some solid tumours. It is now possible to cure some patients with 14 previously fatal malignancies, McCredie (1985).

1. Acute lymphocytic leukaemia
2. Acute non-lymphocytic leukaemia
3. Hodgkins disease
4. Diffuse histiocytic lymphoma
5. Nodular mixed lymphoma
6. Burkitt's lymphoma
7. Ewing's sarcoma

8. Neuroblastoma

9. Wilm's tumour

10. Testicular cancer

11. Ovarian cancer

12. Small cell carcinoma of the lung

13. Adenocarcinoma of the breast

14. Rhabdomyosarcoma in children

This has resulted from the use of antitumour drugs, ionising radiation, immunosuppressive agents and bone marrow transplantation. Chemotherapy alone over the last 35 years has probably cured between 15 - 25% of patients with acute myeloblastic leukaemia and up to 55% of good risk patients with acute lymphoblastic leukaemia (Powles, 1986). This means that most patients with acute leukaemia still die of their disease. Therefore alternative, more radical forms of therapy such as bone marrow transplantation (BMT) have evolved. BMT is used to "rescue" the patient from the intensive "supra-lethal" treatment necessary to kill all his tumour cells but which consequently kills his own bone marrow.

Successful BMT had to await several developments. The most important was the elucidation of the major histocompatibility loci (Epstein *et al.*, 1968) necessary for the rational choice of an appropriate donor. Although preliminary work had taken place ten or so years before (Kurnick *et al.*, 1958; Thomas *et al.*, 1957; Thomas *et al.*,1959; Uphoff, 1958) other developments were necessary including the support of patients through several weeks of pancytopenia. These included the development of increasingly powerful antimicrobial and antiviral

agents together with the haematological techniques allowing the harvesting of platelets and granulocytes from normal donors (Thomas *et al.*, 1975).

Therefore application of BMT as a treatment of human disease started in earnest in Seattle in the early 1970's (Thomas *et al.*, 1977). Subsequently these other treatments were aided by the use of longterm, tunnelled intravascular catheters (Hickman, 1979).

Therefore these patients have compromised mechanical defences not only because of the use of these intravascular catheters but also as the result of decreased mucosal integrity as a direct effect of the chemotherapy.

However, by far the more important effect of the therapy is on the host's cellular and humoral immune defences. In the case of the BMT patient profound neutropenia (<100 per μL) occurs in the early post transplant period. Septicaemia by Gram negative bacilli is the most commonly observed infection at this time (Winston *et al.*, 1979b).

Immunosuppressive chemotherapy

This exerts a profound, often complex, effect on host resistance to infection. High dose corticosteroid treatment significantly increases the risk of infection since it has multiple effects on the immune response including actions on neutrophils, lymphocytes and macrophages.

Cyclophosphamide and azathioprine increase the risk of infection primarily by causing neutropenia.These immunosuppressive drugs are widely used to treat immunologically mediated diseases such as systemic lupus erythematosus, polyarteritis and Goodpastures syndrome. Similarly corticosteroids are used in conditions such as rheumatoid arthritis, hepatitis and nephritis. But the patients at greatest risk from intercurrent infection "are patients

with haematological or other forms of malignancy and recipients of organ or bone marrow transplants", Cohen (1988).

Therefore it is clear that infection is an enormous problem in such patients, indeed in the mid 1970's infection was the commonest cause of death in patients with haematological malignancy (Chang *et al.*, 1976). This has however been significantly reduced with the early use of antimicrobials in febrile neutropenic patients (Pizzo *et al.*, 1982); the use of more aggressive diagnostic techniques (Burt *et al.*, 1981) and the general improvement in intensive therapy.

Therefore, there are two types of compromised hosts which have severe implications for microbiological control and antibacterial chemotherapy: firstly there are those patients with severely impaired mechanical defences with or without a degree of impaired immune function (dependant on the type and severity of their underlying condition) - **the intensive therapy patient.**

Secondly there are those patients with severely impaired immune function (often with profound neutropenia) with a degree of mechanical defence impairment - **the bone marrow transplant or oncology patient.**

EVOLUTION OF ANTIMICROBIAL AGENTS

The evolution that has taken place in antimicrobial chemotherapy is best seen in figure 1.

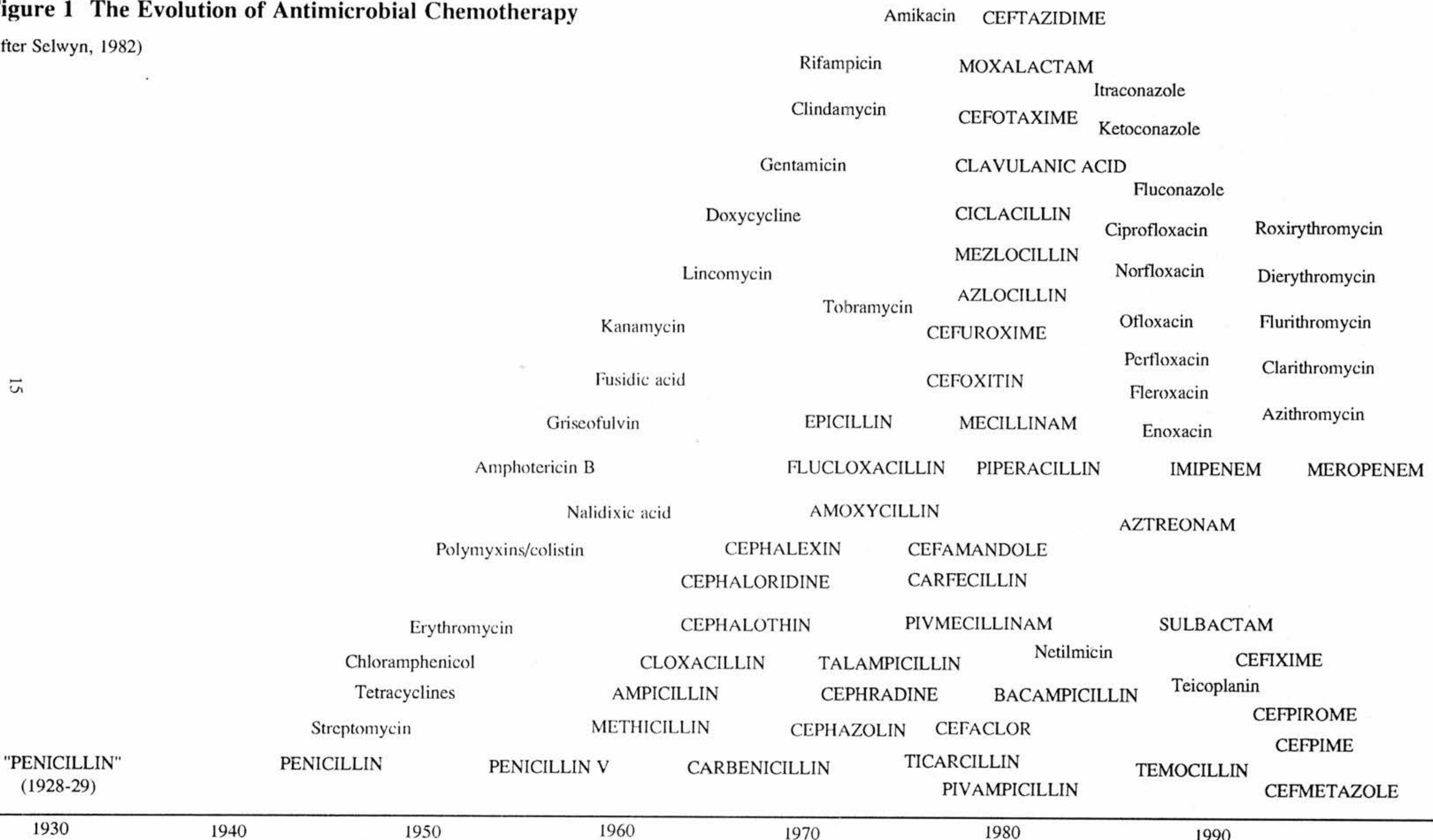
The evolution of the Beta-lactam drugs will be dealt with in more detail later (see fig.2).

Essentially the pharmaceutical companies have been driven to produce newer agents as often bacterial resistance mechanisms have rendered the original agents less effective. Therefore ever more powerful agents with often very broad

spectra of activity (e.g. imipenem and the quinolones) have been produced. Their evolution, of course, has been inextricably interwoven with the resistance mechanisms of the bacteria themselves.

Figure 1 The Evolution of Antimicrobial Chemotherapy

(after Selwyn, 1982)



PARASITE EVOLUTION

MECHANISMS OF RESISTANCE TO ANTIMICROBIAL AGENTS

The mechanisms of resistance to antimicrobial agents can be divided into 3 major types:

1. Alteration of the target
2. Inactivation of the drug (detoxifying enzyme)
3. Decreased uptake of the drug

They have been nicely summarised in a Table (Table 2) by Jacoby & Archer (1991)

Table 2 Major Mechanisms of Resistance to Antimicrobial Agents

TYPE OF RESISTANCE & ANTIMICROBIAL CLASS	SPECIFIC RESISTANCE MECHANISM
Altered target	
Aminoglycosides	Altered ribosomal protein
β-lactam antibiotics	Altered or new penicillin-binding proteins
Erythromycin & clindamycin	Ribosomal RNA methylation
Quinolones	Altered DNA gyrase
Rifampicin	Altered RNA polymerase

Table 2 (contd.)

TYPE OF RESISTANCE & ANTIMICROBIAL CLASS	SPECIFIC RESISTANCE MECHANISM
Altered target	
Sulphonamides	New drug-insensitive dihydrofolate synthase
Tetracycline	Ribosomal protection
Trimethoprim	New drug-insensitive dihydrofolate reductase
Vancomycin	Altered cell-wall stem peptide
Detoxifying enzyme	
Aminoglycosides	Acetyltransferase,nucleotidyl-transferase,phosphotransferase
β-lactam antibiotics	β-lactamase
Chloramphenicol	Acetyltransferase

Table 2 (contd.)

TYPE OF RESISTANCE & ANTIMICROBIAL CLASS	SPECIFIC RESISTANCE MECHANISM
Decreased uptake	
Diminished permeability	
β-lactams,chloramphenicol, quinolones,tetracycline trimethoprim	Alterations in outer membrane proteins
Active efflux	
Erythromycin	New transport system
Tetracycline	New transport system

(After Jacoby & Archer ,1991)

EVOLUTION OF BACTERIAL RESISTANCE MECHANISMS

Amyes (1989) stated: "Since the mid 1930's, clinically important bacteria have been subjected to successive onslaughts of antibacterial drugs. However, in response, the bacterial populations have overcome these attacks rapidly by the selection of resistant variants or by their ability to acquire novel genetic material.

Indeed, the development of new antibacterial drugs has been dominated by the bacterium's ability to resist the previous generation of drugs".

For instance when penicillin was first used in 1941, less than 1% of all strains of *Staphylococcus aureus* were already resistant to its action; however, by 1946 under the selective pressure of this antibiotic's extensive use, the proportion of penicillin resistant strains had risen to 14%. By 1947, 88% were resistant and at the present time more than 90% of all strains of *Staphylococcus aureus* are resistant,(Lewis, 1989).

It would seem that microbes have co-existed with antimicrobials for some time. Sneath (1962) revived some spores of *Bacillus licheniformis* which had been dormant in dried soil stuck to the roots of plant specimens preserved untouched in the British Museum since 1689. Pollock (1967) examined these strains and found them to produce a penicillinase.

Smith (1967) described a strain of *Escherichia coli* that had been stored in the 1930's which was found to carry a plasmid which conferred resistance to streptomycin and tetracycline.

Types of Resistance

Bacteria can be:

1. intrinsically resistant to an antimicrobial.

2. may undergo chromosomal mutation to become resistant e.g.streptomycin or rifampicin.

or

3. may become resistant via the acquisition of new genetic material.

It would seem that prior to the therapeutic use of antimicrobial agents resistant bacteria were rare but with the use of these agents both in medical and veterinary practice, the situation has dramatically changed.

Plasmids as transmissible agents of resistance (R plasmids) were discovered in Japan in 1959 (Watanabe, 1963) and by the late 1960's were accepted as the major cause of antimicrobial resistance in clinical isolates. Hedges and Jacob (1974) demonstrated that the plasmid-borne resistance genes were, in fact, more mobile that their plasmid hosts. Many were located on transposons capable of migrating between replicons within the same bacterial cell. This considerably improves their survival potential. Since the early discovery of transposons, almost all plasmid-borne genes have been found to be located on plasmids.

Jacoby (1985) pointed out the considerable advantages of having resistance genes carried on plasmids.

(i) Some chromosomal resistance mutations retard growth or diminish virulence whereas plasmid resistance does not.

(ii) A chromosomal mutation may revert, but the frequency at which a plasmid may be lost when it no longer confers a selective advantage is much higher.

(iii) Plasmid determined resistance can be amplified e.g. by gene duplication.

(iv) It can be carried on a segment of DNA that can transpose from one replicon to another allowing greater flexibility in resistance dissemination.

(v) Resistance to multiple antibiotics can be packaged on a single plasmid.

(vi) Plasmid determined resistance can be spread to new hosts often, into different species of bacteria.

Thus we can see the range of mechanisms at the disposal of microbes which allow them to adapt and evolve in the presence of antimicrobial agents.

This thesis will deal with Beta-lactam resistance manifested by Beta-lactamases; therefore, it is important to examine the close relationship between the development of new Beta-lactams and the evolution of the new Beta-lactamases. Wiedemann *et al*. (1989) divided this into 4 periods:

1. **1942-60 :** Development of penicillin. Increase of penicillin - resistant *Staphylococcus aureus* strains caused by plasmid-mediated penicillinases. Development of methicillin and oxacillin.

2. **1960-72** : Development of aminopenicillins e.g. ampicillin. Increase of ampicillin-resistant coliforms resulting from plasmid-encoded penicillinases like TEM-1.Development of first generation cephalosporins e.g.cephazolin.

3. **1972-80** : Development of second generation cephalosporins e.g. cefuroxime and cefoxitin. Selection of mutants that overproduce chromosomally-mediated cephalosporinases.Induction of chromosomally mediated enzymes.

4. **1980->** : Development of third generation cephalosporins e.g. cefotaxime and ceftazidime. Development of mutated plasmid-mediated Beta-lactamases such as TEM-3 to TEM-7 and SHV-2 and SHV-3, plasmid-coded broad spectrum Beta-lactamases.

EVOLUTION OF INFECTING ORGANISMS

The kinds of organisms that cause severe infections in hospitalized patients are clearly linked with :

1. The evolution in the type of patients themselves which is related to their host-defence mechanisms and therapies they undergo.

2. The use of antimicrobial agents (and their evolution into even more powerful agents).

3. The evolution of bacterial resistance mechanisms to these antimicrobial agents.

In the pre-antibiotic and early antibiotic era significant infections were caused by the virulent pyogenic cocci (such as *Staphylococcus aureus*, the pneumococcus and *Streptococcus pyogenes*). These infections often occurred in patients with reasonably complete host defence mechanisms (Abraham *et al.*, 1941; Garrod, 1979; McDermott & Rogers 1982).

With the availability of ampicillin and methicillin derivatives, infections tended, in the 1960's and 1970's to shift to Gram negative coliforms and organisms such as *Pseudomonas aeruginosa*. This change resulted from natural resistance to these compounds or the acquisition of a resistance mechanism. At the same time the development of modern therapies has led to patients who are more and more compromised both in terms of their immune and mechanical defence mechanisms.

Maki (1989) has stated that the intensive therapy unit has become a milieu within the hospital uniquely conducive to the occurrence of epidemic nosocomial infection, especially infection caused by antibiotic resistant pathogens.

"Intensive antimicrobial therapy, used in most intensive therapy units, grossly distorts the patient's microflora and fosters colonisation and ultimately infection". At present Gram negative organisms such as multiply resistant enterobacteriaceae e.g. *Serratia, Klebsiella, Enterobacter, Pseudomonas* or *Acinetobacter* are still significant problems; however, there is an increase in organisms such as methicillin–resistant *Staphylococcus aureus* (MRSA), coagulase negative staphylococci and enterococci. Yeasts such as *Candida albicans* may also cause problems.

Resistant nosocomial organisms from colonised and infected patients are transmitted between patients in the unit most frequently by the hands of the medical personnel and may be perpetuated by contaminated equipment such as bedpans, respiratory apparatus, pressure transducers and endoscopes.

Craven *et al.* (1988) studied the risk factors for nosocomial infection in ICU patients. They found that surgical ITU patients had a considerably higher incidence of nosocomial infection as compared to medical ITU patients. These infections were caused by enterococci, multiply resistant enterobacteriaceae, (*Klebsiella*, *Enterobacter*, and *Serratia* rather than *E.coli*) *Pseudomonas aeruginosa* and *Candida*. These pathogens are almost always selected out by antibiotic pressure.

Their surgical patients had significantly more antimicrobial therapy than their medical ITU patients. These workers (Craven *et al.*, 1988) also showed convincingly by multivariate analysis that invasive devices are at least as important in increasing the susceptibility to nosocomial infection as is the underlying disease. But that while invasive devices are powerful predictors of vulnerability to nosocomial infection, underlying diseases are much more important predictors of fatality.

A similar story emerges from oncology and bone marrow transplantation patients. Just as Donowitz *et al.* (1982) noted the shift of infecting organisms in the ITU from Gram negative to Gram positive and yeasts. Lowder *et al.* (1982) and Young (1985) have noted a similar shift to often less virulent but multiply resistant Gram positive organisms such *Staphylococcus epidermidis* and "JK" Corynebacteria. This shift was associated with the increasing use of indwelling central venous catheters. (Lowder *et al.*, 1982 ; Catovsky and Hoffbrand, 1989). Pneumococcal infections are also common in patients with splenectomy and post bone marrow transplantation (Winston *et al.*,1989a).

OVERALL PERSPECTIVE

In ending this section I will use a table (Table 3) from a recent review by Selwyn (1991). Like most of Selwyn's tables it has its good points and... its bad! It is useful for its historical nature - it shows the evolution of hospital-acquired (nosocomial) infections from the 18th century to the present day.

Table 3. The evolution of hospital acquired (nosocomial) infection

Date	Dominant hospital infections	Organism
->1800	Hospital fever (typhus)	*Rickettsia*
	Dysentery	*Shigella*
	The itch (scabies)	*Sarcoptes*
c.1750-1940	Puerperal fever	*Streptococcus pyogenes*
	Surgical fever	
	Erysipelas etc.	
	Surgical gangrene	Streptococci & anaerobes

Date	Dominant hospital infections	Organism
*c.*1940->	Staph. sepsis (skin, breast, wounds etc.	*Staphylococcus aureus* ('hospital staph.')
*c.*1955	'Opportunistic infections' (esp.'Gram negatives')	*Pseudomonas,* enterobacteria Yeasts & moulds Herpesvirus group *Pneumocystis* (protozoan) [1]
*c.*1965	Virus hepatitis	Hepatitis B virus ('Australia antigen')
1970s->	(Anaerobic infections) [2]	*Bacteroides* spp. etc.
	Legionnaires disease [3]	*Legionella* spp.
	Food poisoning [4]	*Salmonella* spp. etc.
1981->	HIV infection (AIDS) [5]	Retroviruses & opportunists inc.*Nocardia*, mycobacteria, *Cryptosporidium*

[1] But fungus-like on RNA sequencing.

[2] Increase more apparent than real.

[3] N.B.Stafford (1985).

[4] N.B.Stanley Royd, Wakefield (1984).

[5] Minimal risk of transmission in hospital.

(After Selwyn ,1991)

It is fine until "c.1955 - opportunistic infections (esp Gram-negatives)" where he correctly points out the importance of *Pseudomonas* and other multiresistant enterobacteria as nosocomial pathogens. He does mention the importance of yeasts and fungi but not the increasing problems with the Gram positive multiresistant bacteria such as the coagulase negative staphylococci (CNS), MRSA and JK diphtheroids (although he does mention them in the accompanying text). He does however discuss problems with other microorganisms such as Hepatitis B virus, the Herpes viruses and the "fungus-like" protozoan *Pneumocystis carinii* (though this organism is almost certainly endogenously acquired). It is particularly useful to mention the viruses and protozoa in order not to forget their increasing role in infections of the immunocompromised. This thesis will deal exclusively with the bacterial pathogens and it is timely to remember that they are not the only ones.

Where Selwyn is not so good is by describing as "dominant hospital infections" of the "1970s ...." the following epidemic infections that may be acquired in hospital: Legionnaires' disease (1985 outbreak in Stafford) and food poisoning (1984 outbreak of foodborne salmonellosis at Stanley Royd, Wakefield).

These infections are not the endemic problems that the clinical microbiologist sees day in and day out as the major causes of infection in his hospital and therefore should *not* be included.

He, under the same heading, also mentions anaerobic infections caused by *Bacteroides* spp. These infections do occur *in* hospitals - often as a result of surgery on, e.g., the large bowel - but they are relatively uncommon since the advent of prophylaxis and treatment with metronidazole* He himself states that they are "more apparent than real".

*(Anaerobic bacteria were the cause of only 140 of 2578 cases of nosocomially acquired bacteraemia, i.e. 5.6%, over a 20 year period in St Thomas's hospital, Eykyn *et al.,* 1990).

For good measure he adds HIV infection (AIDS) as a "dominant hospital infection" since 1981. This, as he himself states, is not often acquired by transmission within the hospital and it is therefore clearly not a "nosocomial" infection. It is a particular problem in specific areas of the UK: in some large cities but not all. However it is again useful in the context of this thesis to mention HIV infection since it has led to an evolution of infection. These immunocompromised patients suffer a variety of infections not only bacterial (e.g. salmonella, mycobacterial); but viral e.g. cytomegalovirus, herpesvirus; protozoal e.g. *Toxoplasma gondii,* cryptosporidiae, *Pneumocystis carinii;* and fungal e.g. *Cryptococcus neoformans* , *Candida albicans* .

But returning to the bacteria...

Table 4. Evolution of the Host-Parasite Relationship

PARASITE	HOST
SITUATION PRE-ANTIBIOTICS	
Highly Virulent	Good Defences
e.g. STAPH AUREUS	NON-COMPROMISED
CURRENT SITUATION	
Less Virulent	Poor Defences
with multiple antimicrobial resistance	(both mechanical and immune)
e.g. STAPH. EPIDERMIDIS	COMPROMISED

In terms of the host - parasite relationship clear changes have taken place over the last 50 years as each strand (of e.g. therapy and resistance) has evolved. The result of this is (for example) shown in Table 4 .

THE BETA-LACTAM ANTIBIOTICS

HISTORICAL PERSPECTIVE

"Purge me with hyssop, and I shall be clean"

Psalm 51,verse 7.

"Quinsye and Swelling of the Throte"

"For the quinsy and swelling vnder the eares. Take the musherom that groweth vpon an elder tree"

Boke of Chyldren

Thomas Phayre,1545.

Both these are quoted as examples of the use of moulds and fungi as empirical treatment of infections by Selwyn (1980).

The reference to hyssop may be relevant since the first description of *Penicillium notatum* was made following its isolation from a hyssop plant (Westling,1911).

In the case of Thomas Phayre, the fungus was taken in the form of a warm infusion and apparently had good results against diseases most usually caused by the penicillin-sensitive haemolytic streptococci.

For the medical historian, the history of the therapeutic use of moulds, the discovery of penicillin and the subsequent development and evolution of the Beta-lactam drugs is fascinating.

For an account of the early work on the antibacterial action of *Penicillium* species by workers such as Burdon-Sanderson, Lister, Roberts, Tyndall, Duchesne etc, I refer the reader to Selwyn (1980).

For an insight into the discovery of penicillin by Fleming and the early work on its isolation, purification and clinical use; I refer the reader to the two excellent biographies of Florey and Fleming by MacFarlane (1979 & 1984), a review by Abraham (1983) and the book chapter by Selwyn (1980).

THE EVOLUTION OF THE BETA-LACTAMS

The dramatic growth of the Beta-lactam drugs of the early 1950's can be seen in fig.2 (Rolinson, 1988).

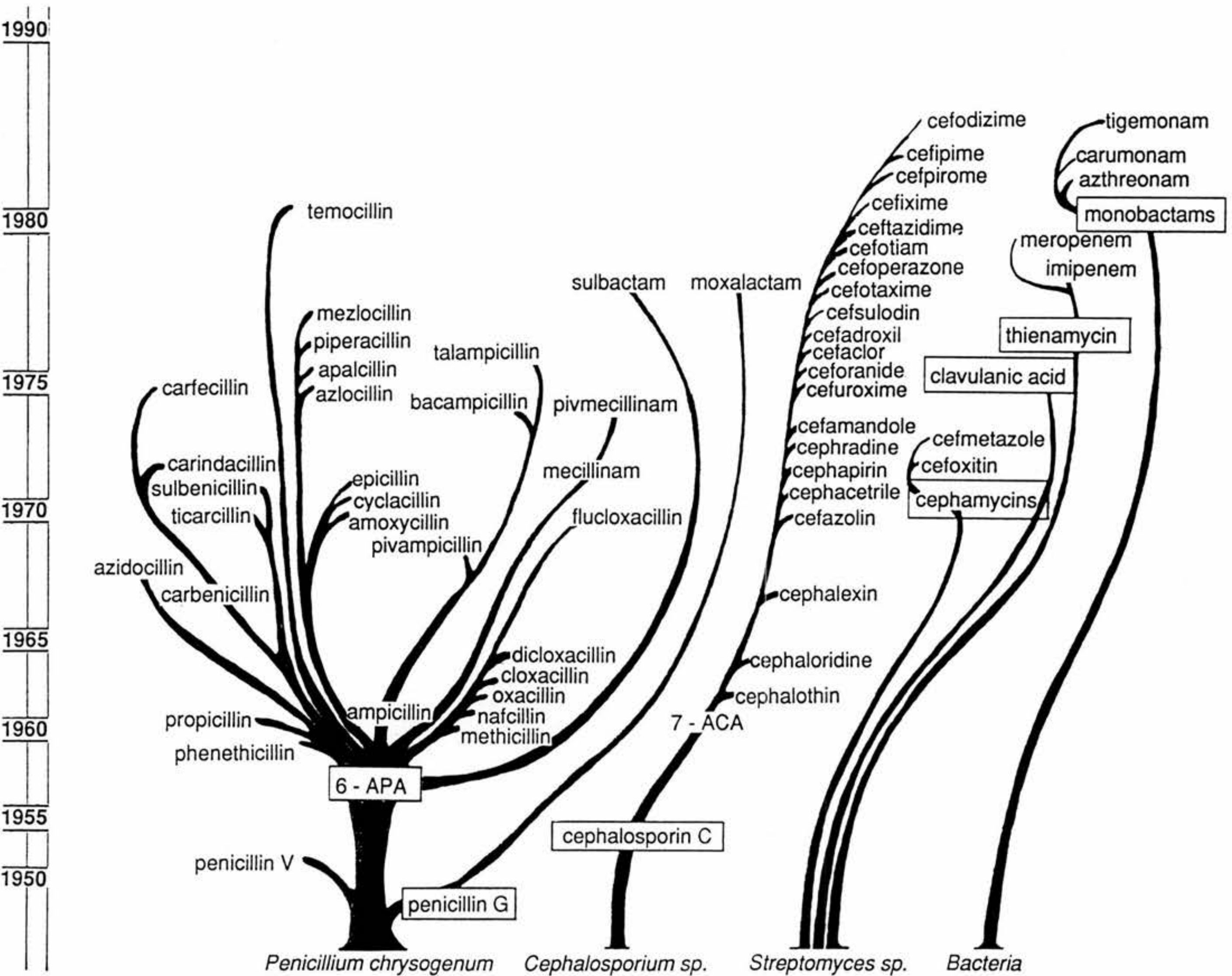
The rapid emergence of penicillin-resistant *Staphylococcus aureus* under the selective pressure of the use of penicillin in the late 1940's and the subsequent importance of the penicillinase-producing Gram negative bacteria provided the stimulus for the pharmaceutical companies to develop the Beta-lactam drugs.

During the 1950's two crucial occurrences provided the starting points for the dramatic surge in the clinical usefulness of the Beta-lactams as antimicrobial agents. First was the discovery of methods for obtaining 6-aminopenicillanic acid in quantity. Secondly the discovery of Cephalosporin C and its nucleus, 7-aminocephalosporanic acid (7-ACA) (See figs.3 & 4).

6 APA and the semisynthetic penicillins

In the late 1950's Beecham Research Laboratories developed the technology to produce large quantities of 6-APA (Batchelor *et al.*, 1959). This opened the way to the modification of the penicillin nucleus (6 APA) by the addition of novel side chains by chemical means through the 6-amino group.

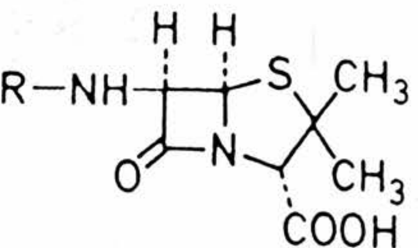
Figure 2



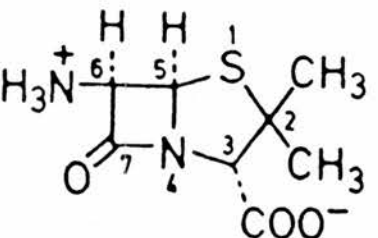
The evolutionary trees of the ß-lactam drugs (after Rolinson, 1988)

Figure 3

Structure of penicillins and 6-aminopenicillanic acid.



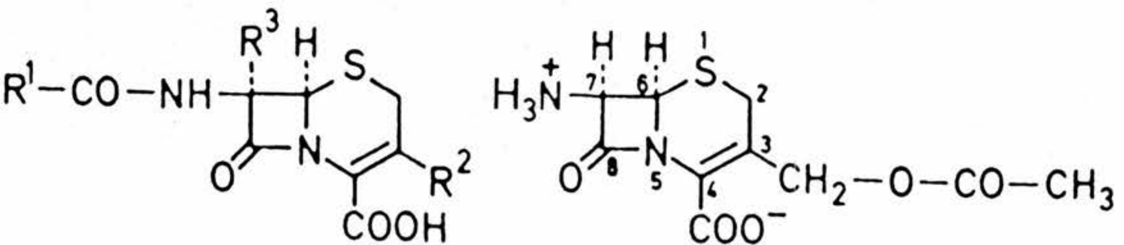
Penicillins



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

Figure 4

Structure of cephalosporins and aminocephalosporanic acid.



Cephalosporins Aminocephalosporanic acid (7-ACA)

[$C_{10}H_{12}N_2O_5S$ (272.28)]

Two significant derivatives of 6 APA were quickly to appear: methicillin and ampicillin. **Methicillin** - methicillin, cloxacillin and flucloxacillin were a major advance since they are stable to the Beta-lactamases produced by *Staphylococcus aureus.* They have remained the anti-staphylococcal drugs of choice, in most situations, until the recent problems with epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA). **Ampicillin** - with its wider spectrum of activity against Gram negative organisms coupled with its oral absorption led to it becoming one of the most widely used antibiotics. Derivatives such as the ureidopenicillins (e.g. piperacillin and azlocillin) have proved useful. As amoxycillin,and with the addition of a Beta-lactamase inhibitor(clavulanic acid) the spectrum of activity of ampicillin has been resuscitated (for the moment at least).

Intrinsically, penicillins lack sufficient flexibility because they possess only one position available for fundamental group development (C-6). Consequently, the penicillins have not enjoyed the variety nor the success of the cephalosporins - which will be discussed in the next section.

The discovery and development of the cephalosporins

Cephalosporin C was discovered by Newton & Abraham in 1953 (Newton & Abraham, 1955) while they were studying the antibiotics of a strain of *Cephalosporium acremonium* that had been isolated by Guiseppe Brotzu from the sea near a sewage outfall at Cagliari in Sardinia.

The importance of cephalosporin C was not only that it was effective against both penicillin-sensitive and penicillin-resistant staphylococci but also because it had a wide spectrum of antibacterial activity.

Unlike the penicillin nucleus, the cephalosporin nucleus contains several positions at which chemical modifications can be attempted (see fig.4) and therefore many thousands of cephalosporin antibiotics have been produced by various pharmaceutical companies world-wide (Williams & Williams, 1980).

For a fuller account of the early history and evolution of the penicillins and cephalosporins please see Abraham (1983) and Rolinson (1988).

Further important milestones in the Beta-lactam story include the discovery of:

1. cephamycins from *Streptomyces* (Daoust *et al.*, 1973)
2. carbapenems from *Streptomyces* (Kropp *et al.*, 1980)
3. clavams from *Streptomyces* (Reading & Cole, 1977)
4. monobactams from bacteria (*Nocardia*) (Aoki *et al.*,1976)

New Beta-lactam drugs have continued to be discovered and synthesized. Not only do they have a remarkable diversity of spectrum with a range from narrow (e.g. benzylpenicillin) to extremely broad (e.g. imipenem) but they all share a relative freedom from side effects.

The latest cephalosporin modifications have resulted in drugs with differing pharmacokinetic properties so that some drugs need only be given once or twice per day (e.g. ceftriaxone).

CLASSIFICATION OF THE BETA-LACTAMS

By the mid 1980's more than 50 different Beta-lactam drugs were in clinical use or at an advanced stage of evaluation. Many have similar names with either similar or differing properties. Rolinson (1986) therefore thought it useful

to publish a register of all Beta-lactams with their name, structure and both their antibacterial spectra and pharmacokinetic properties.

The easiest way to classify Beta-lactams is into chemically related groups. The basic Beta-lactam structure consists of a four membered ring fused to a second ring. Biochemical modifications have been made in attempts to increase penetration through bacterial cell walls, to decrease susceptibility to Beta-lactamases or to increase binding to PBP's (Donowitz & Mandell, 1988).

Penams : The four membered Beta-lactam ring (azetidinone ring) is fused with the sulphur containing thiazolidine ring. Examples are benzylpenicillin, ampicillin, flucloxacillin, the ureidopenicillins and temocillin.

Penems : The penem nucleus is the unsaturated analogue of the penam ring system.

Carbapenems : The carbapenems result from the replacement of the sulphur atom of the penem nucleus by a (CH_2) group. This type of Beta-lactam includes the thienamycin and olivanic acid families. Imipenem is an example. The small size and compact structure of these agents allow them to pass easily through Gram negative cell walls. Marked resistance to hydrolysis by Beta-lactamases is provided by the *trans* configuration of this group, which contrasts with the *cis* configuration of other Beta-lactams (Birnbaum *et al.*, 1985).

Clavams : The sulphur atom in the five membered ring is replaced by an oxygen.An example is clavulanic acid a potent suicide inhibitor of certain types of Beta-lactamase.

Cephems : A cephem is the bicyclic ring system obtained by the fusion of a azetidinone (Beta-lactam) ring with a dihydrothiazine ring. Numerous cephalosporins have been developed from this nucleus.

The cephamycins have a methoxy group at the C-7 position of the 7-ACA nucleus which increases Beta-lactamase stability (an example is cefoxitin).

The amino-thiazol substituted cephalosporins (e.g. ceftazidime, cefotaxime and cefuroxime) give a combination of increased activity and increased Beta-lactamase stability.

Oxacephems : The sulphur in the dihydrothiazine ring is substituted by an oxygen resulting in an increased spectrum against Gram negative species. The most notable member of this group is latamoxef (moxalactam).

Monobactams : The monobactams (azetidinone derivatives) are a novel group of compounds in which the bicyclic structure characteristic of other Beta-lactams is absent but the core configuration is that of a "naked" Beta-lactam ring. Since the 1-sulphonic acid group lowers the energy requirements for interaction of the Beta-lactam with PBPs, it is said to "activate" the molecule (Cimarusti & Sykes, 1984). Examples are aztreonam, carumonam and tigemonam.

Further details of the structure-activity relationship of the Beta-lactam drugs can be found in Dunn (1982), Hoover (1983) and Neu (1986a).

RESISTANCE TO THE BETA-LACTAM ANTIBIOTICS

Resistance to these antimicrobial agents can occur by at least three routes:

Inactivation : This is by far the most common cause of resistance - the production of a Beta-lactamase which hydrolyses the Beta-lactam to a biologically inert product.

Alterations in the permeability of the drug : In Gram negative bacteria resistance can also result from a decrease in the rate of penetration of the antibiotic through the outer membrane to the penicillin binding proteins (PBP's) in the cytoplasmic membrane. This resistance mechanism is unlikely to give high levels of resistance without the concomitant effect of limiting the uptake of nutrients and thereby seriously impairing the growth of the bacteria. This form of resistance has not been reported in Gram positive bacteria as they lack an outer membrane.

Alteration of the target : The third form of resistance to the Beta-lactam antibiotics is via alterations in the properties of the physiological targets of these antibiotics - the penicillin binding proteins.

"Each of these 3 factors are interdependant on the others", (Sanders & Wiedemann, 1988)

This thesis will concentrate on the resistance due to the Beta-lactamases, particularly in Gram negative bacteria. However the importance of the other two mechanisms of resistance should not be overlooked.

The role of permeability changes in resistance has been reviewed recently by Nikaido (1989) and the role of alterations in the penicillin binding proteins has been reviewed by Spratt (1989).

BETA-LACTAMASES

EARLY HISTORY

If the first serious therapeutic use of the Beta-lactam drugs took place on the 12 February 1941, "penicillinase" was born on 28 December 1940 (Hamilton-Miller, 1979). One may view the chronology of these milestones as a little ironic.

Abraham and Chain (1940) described the extraction of a substance from *B.coli (E.coli)* which destroyed the growth inhibiting property of penicillin. They concluded that this substance was an enzyme and named it "penicillinase".

They also observed the following:

1. The enzyme was found in a penicillin resistant Gram negative rod contaminating their *Penicillium* cultures.

2. They also found it in a culture of penicillin sensitive *Micrococcus lysodeikticus* and concluded that:

3. "The presence or absence of the enzyme in a bacterium may not be the sole factor determining its sensitivity to penicillin". They also postulated that:

4. Because various species of bacteria contain penicillinase, it "may have a function in their metabolism".

Only a few years later it was shown that penicillinase was responsible for clinical resistance in *Staphylococcus aureus* (Kirby, 1944; Gots, 1945). From then until the 1960's most attention focused on staphylococcal penicillinase as the

prevalence of penicillin resistant staphylococci quickly reached epidemic proportions in hospitals where penicillin was widely used.

It was shortly realised that Beta-lactamases from different sources had different properties. Brodersen (1947) showed that the enzyme from a "paracolon" organism has different properties from the Beta-lactamase of *Bacillus subtilis.* Abraham (1951) in the first major review of Beta- lactamases recognised that there was likely to be a wide variety of different Beta-lactamases. This had to remain unproven until the introduction of the new substrates - i.e. the semisynthetic penicillins - in the early 1960's. Smith and Hamilton-Miller (1963) pointed out "major qualitative differences" between the penicillinases of Gram positive origin and those of Gram negative origin. They also speculated that "each individual species of penicillinase-producing Gram negative bacteria may produce a 'species specific' penicillinase....."

This was to be (to some extent) confirmed by Matthew and Harris (1976) using the technique of isoelectric focusing.

As the first semisynthetic cephalosporins appeared it soon became obvious that although they were virtually unaffected by staphylococcal enzymes, they were rapidly destroyed by the Beta-lactamases from many Gram negative bacteria (Jago *et al.*, 1963; Hamilton-Miller *et al.*, 1965).

At the same time a discovery was made that was to have a profound effect on the spread of Beta-lactam resistance and was the spur to the production of the next generation of Beta-lactam drugs by the pharmaceutical houses. Datta and Kontomichalou (1965) first described resistance to Beta-lactam drugs mediated by R factors in Gram negative bacteria.This, of course, was invariably associated with specific Beta-lactamases.

Since then we have seen the concomitant evolution of the newer Beta-lactam antimicrobials (see later) and the "counter" evolution of the new Beta-lactamases. Classification schemes of these Beta-lactamases have necessarily evolved as these Beta-lactamases have been characterised. These will be described later.

ACTION OF BETA-LACTAMASE

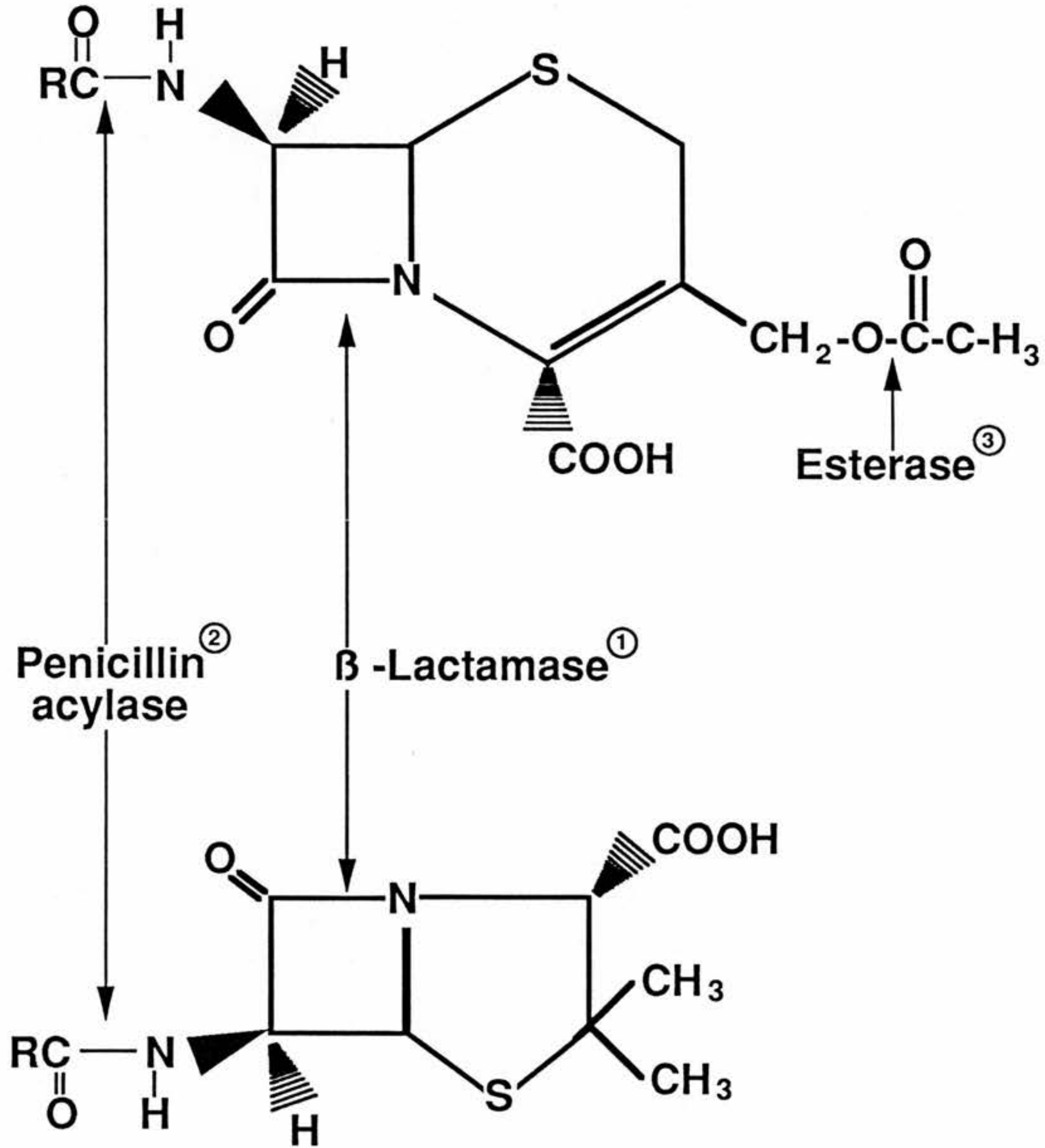
Beta-lactam drugs provide a substrate for a number of hydrolytic enzymes but the most important of these are the Beta-lactamases (Sykes, 1982).

Beta-lactamases hydrolyse the cyclic amide bond (see 1, fig.5) of susceptible Beta-lactams to give antibiotic inactive products. For penicillins these are relatively stable penicilloates but for cephalosporins are the relatively unstable cephalosporates.The pattern of fragmentation is dependent on the nature of the C-3 substituent.

The second type of enzymic degradation of Beta-lactams involves the removal of the acyl-side chains (see 2, fig.5) by amino acid acylases (often referred to as penicillin acylases or amidases). These enzymes are believed to have little role in antimicrobial resistance but are of immense commercial value in cleaving penicillins in the production of semisynthetic derivatives (Batchelor *et al.*, 1959).

Beta-lactamases (E) function by first binding the Beta-lactam substrate (S) in a noncovalent Michaelis complex (E.S). Then the enzyme and substrate may dissociate or commit themselves to the hydrolytic reaction by forming an acyl enzyme via an active site serine hydroxyl (E-S). Deacylation of this covalent complex with release of ring opened products (P) completes the mechanistic picture (see fig.6)(Bush & Sykes ,1986).

Figure 5



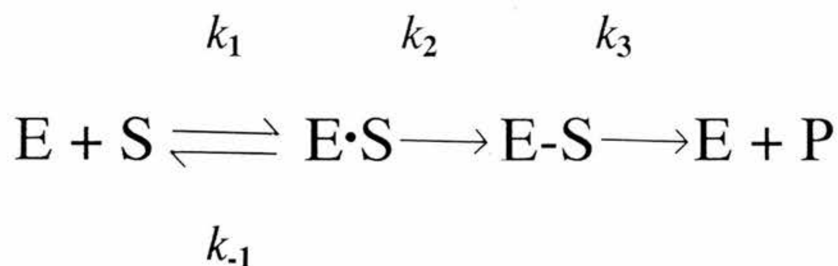
Interaction of cephalosporins (*top*) and penicillins (*bottom*) with hydrolysing enzymes. (After Sykes, 1982)

A third type of enzymic degradation involves the acetyl group of the cephalosporins containing the acetoxymethyl function at C-3 which is removed by esterases (see 3, fig.5). Such cleavage produces components of reduced antibacterial activity. The presence of esterases in mammalian tissue has however been exploited with inactive prodrug esters such as talampicillin,which is cleaved *in vivo* to release the active drug - ampicillin.

Mammalian kidney has been found to produce dehydropeptidases (Kropp *et al.*,1980) that specifically inactivate the carbapenems. This has necessitated the addition of a renal dipeptidase inhibitor (cilastatin) with imipenem in pharmaceutical preparations.

Figure 6

The mechanism of action of the β-lactamase


$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + P$$

NOMENCLATURE

For the years following the paper by Abraham and Chain (1940) "penicillinase" became the accepted term for this group of enzymes. In 1965 the Enzyme Commission gave penicillinase the official name "penicillin amide Beta-lactam hydrolase", E.C.3.5.2.6 (Enzyme Nomenclature, 1965). This name was very similar to that given to acylase (E.C.3.5.1.11) namely "penicillinamidohydrolase" (Hamilton-Miller, 1979).

With the rush of new substrates in the 1960's and the discovery that some Beta-lactamases hydrolysed cephalosporins, the term "cephalosporinase" was coined. Therefore in 1972 the Enzyme Commission introduced "cephalosporinase" as E.C.3.4.2.8. However they continued to confuse the situation by introducing the terms "Beta-lactamase I" for penicillinases and "Beta-lactamases II" for cephalosporinases. Thankfully few workers entertained the use of these terms (Hamilton-Miller, 1979). The Enzyme Commission finally grouped Beta-lactamases under one heading (E.C.3.5.2.6.) in 1984 (Enzyme Nomenclature, 1984).

THE CLASSIFICATION OF BETA-LACTAMASES

The method used to classify is that which ends up classifying in the most useful classification for the classifier. This is exactly what has taken place with the classification of the Beta-lactamases.

These classifiers can themselves be classified into 3 main groups:

1. Biochemists

2. Molecular biologists

3. Microbiologists

The molecular biologists themselves can be further subdivided into two: i.e. sequencers and crystallographers ; and perhaps a third group of geneticists. The microbiologists may have a tinge of the biochemist, the molecular biologist or the geneticist. Each one may have particular interests in the chromosomal or plasmid mediated Beta-lactamases or similarly may be more interested in Beta-lactamases from Gram positive than from Gram negative organisms. The above classification is of course far from complete and new subdivisions may be required in the future!

The properties that have been used to differentiate and classify Beta-lactamases are:

1. isoelectric point

2. molecular mass

3. relative activity towards different Beta-lactams (substrate profile)

4. interaction with inhibitors and inactivators

5. nature of the active site

6. amino acid sequence

7. three dimensional structure (Ambler, 1980)

and to this list we should add :

8. the genetic location of the Beta- lactamase genes i.e. plasmid or chromosomal

EARLY CLASSIFICATION SCHEMES

The first attempts at classification were made in the early 1960's, just after the introduction of ampicillin: Ayliffe (1963) described two "penicillinases" and Fleming *et al.* (1963) studied a Beta-lactamase mainly active against cephalosporins. These enzymes, and the most clinically important ones that followed, were produced by Gram negative bacteria. Classification schemes have, because of this clinical importance, essentially disregarded Gram positive Beta-lactamases. However, molecular biologists have been mainly interested in the Beta-lactamases from Gram positive bacteria which were either never a clinical problem (e.g. *Bacillus licheniformis*) or no longer the major clinical problem (e.g. *Staphylococcus aureus* with the advent of methicillin and its derivatives).

Sawai *et al.* (1968) divided Beta-lactamases on the basis of the enzyme's "substrate profile" i.e. its spectrum of hydrolytic activity against a range of Beta-lactam substrates.

Group 1 : comprised of typical cephalosporinases.

Group 2 : comprised of cephalosporinases that also had penicillinase activity (broad spectrum enzymes).

Group 3 : comprised of penicillinases.

Jack and Richmond (1970) produced a similar classification employing this biochemical parameter but divided the cephalosporinases into two categories i.e.:

Group 1 : comprised of the broad spectrum enzymes.

Group 2: comprised of the penicillinases.

Group 3: comprised of the cephalosporinases with little or no activity against penicillins.

Group 4: comprised of cephalosporinases with some hydrolytic activity against the penicillins.

With inclusion of additional parameters such as reaction against antisera; enzyme inhibition with pCMB and cloxacillin; and electrophoretic mobility, they identified at least 8 different Beta-lactamases types.

In 1973, Richmond and Sykes produced a more definite classification:

Class I: comprises the enzymes predominantly active against cephalosporins.

Class II: comprises the enzymes predominantly active against penicillins.

Class III: comprises the enzymes with approximately equal activity against penicillins and cephalosporins but that are sensitive to inhibition by cloxacillin and resistant to inhibition by pCMB.

Class IV: comprises the enzymes of similar substrate profile to the enzymes of Class III but that are resistant to the inhibition by cloxacillin and sensitive to the inhibition by pCMB. (Some enzymes in this class have the capacity to hydrolyse cloxacillin).

Class V: comprises the enzymes that have a "penicillinase" profile including activity against cloxacillin and that are resistant to pCMB.

Class I enzymes were also subdivided as in Table 5.

Table 5. Classification of chromosomal cephalosporinases

R&S class and type	Expression	Species	Other names
Ia	Inducible	*Enterobacter cloacae*	P99
Ib	Constitutive	*Escherichia coli*	AmpC β-lactamase
Ic	Inducible	*Proteus vulgaris*	Cefuroximase, cefotaximase
Id	Inducible	*Pseudomonas aeruginosa*	Sabath-Abraham enzyme

Taken from Sanders (1987)

Class I enzymes : are characteristically chromosomally mediated and are either constitutive or inducible. Typical examples are produced by strains of *E.coli, Enterobacter* spp., *Citrobacter* spp., indole positive *Proteus* spp., *Pseudomonas* spp. and *Serratia* spp.

Class II enzymes: these are chromosomally mediated with predominant penicillinase activity. They are rare but have been noted in *Proteus mirabilis* .

Class III enzymes: these are plasmid mediated TEM and SHV types. The TEM enzymes are the most commonly encountered plasmid mediated Beta-lactamases among clinically resistant isolates (to penicillins and cephalosporins) world-wide.

Class IV enzymes: the most important enzymes of this group are those produced by *Klebsiella* spp.

Class V enzymes: are a heterogeneous group of plasmid mediated Beta-lactamases and include the oxacillin hydrolysing enzymes such as OXA-I, OXA-2 and OXA-3 and the *Pseudomonas* "specific" enzymes (carbenicillinases) i.e. PSE-1,2,3,4.

Neu (1986b) added a **Class VI** - found in *Bacteriodes* spp. which hydrolyse cephalosporins better than penicillins and is inhibited by cloxacillin, carbenicillin, clavulanate but not cefoxitin.

Table 6 shows the early and continuing confusion resulting from multiple classification schemes (Table 4 of Richmond & Sykes ,1973)

Table 6.

Correlation of the Jack & Richmond (1970) Classification with the Scheme used in Richmond & Sykes (1973) and in Richmond *et al.* (1971)

Classification in Jack & Richmond (1970)		Classification in R&S (1973) and in Table 3 of Richmond *et al.* (1971)
Grouping in Fig 2	Type in Table 5	
I	1	IIIa
III	2	Ia
I	3	IVa
I	4	IVb
IV	5	Ib
II	6	IIb
II	7	IIa
I	8	IVc

Nevertheless this scheme has been widely used since, until an update was proposed by Bush (1988) and delineated by her (Bush, 1989 a,b,c).

It is interesting to note two statements made in this paper (Richmond & Sykes,1973): "Against this diffuse background, there have been a number of attempts to group the enzymes in various categories since there appears to be an instinctive feeling among workers in this field that the evolutionary pattern of a group of organisms will have given rise to a number of different categories of Beta-lactamase which should be reflected in their detailed properties, even if the evolutionary source of all the molecules is ultimately the same. This may, however, be pure illusion. In the last analysis the only information that gives any

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reliable indication as to the absolute similarity of the proteins is their polypeptide sequences, and we are still some way from obtaining this information for more than one of the enzymes concerned. But a number of arbitrary tests have been used to aid classification; the only rationale behind the choice of those used being that they do in fact give some sort of pattern within the whole range encountered so far". Perhaps the first allusion to the need for a more definitive classification based on molecular structure?

"One must admit, however, that the complexity of Beta-lactamase classification will soon require the full treatment by the formal techniques of numerical taxonomy".

Sykes and Matthew (1976) modified the classification of Gram negative Beta-lactamases by essentially subdividing them into: those normally mediated by the bacterial chromosome; or by a transmissible R plasmid; and by inclusion of the enzyme's isoelectric point (pI) as obtained by the newly applied technique of isoelectric focusing described by Matthew *et al.*(1975).The other parameters employed were:

1. Relative rate of hydrolysis (substrate profile)

2. Inhibition by pCMB and cloxacillin

3. Inducible or constitutive

4. Molecular weight

Five groups resulted i.e.

Chromosomally mediated

a) penicillinases

b) cephalosporinases

c) broad spectrum enzymes

R Plasmid mediated

a) those that *do not* hydrolyse methicillin or isoxazolyl Beta-lactams

b) those that *do* hydrolyse methicillin and isoxazolyl Beta-lactams

The importance of this paper is two fold. Firstly it splits Beta-lactamases into chromosomally mediated and plasmid mediated enzymes. At that time plasmid mediated Beta-lactamases were of considerable clinical importance as the mediators of Beta-lactam resistance and novel enzymes were about to appear rapidly. Secondly the use of analytical isoelectric focusing was:

i) going to be the most important method used to distinguish novel plasmid mediated Beta-lactamases (until the appearance of 3GC plasmid mediated Beta-lactamases with similar isoelectric points to previously known enzymes, in the mid 1980's).

ii) going to be the "splitters" dream but the "lumpers" nightmare in the classification of these enzymes because many new plasmid mediated enzymes required classification and it had shown that chromosomal Beta-lactamases are probably genus, species, and subspecies specific (Matthew & Harris, 1976).

As the plasmid mediated group of Beta-lactamases grew in terms of its size and importance it became clear that Group III and IV of the Richmond and Sykes classification provided insufficient criteria for such a large and diverse group of enzymes.

Several different classification and nomenclature schemes for plasmid mediated Beta-lactamases therefore were published. However,this then led to considerable confusion (Mederios, 1984). This is best exemplified by Table II of that paper (Table 7).

Table 7.

Nomenclature of plasmid-determined Beta-lactamases (Mederios, 1984)

Matthew	Mitsuhashi	Pitton	Labia & Phillipon	Richmond& Sykes
TEM-1	Type 1a	TEM-1,type1		IIIa
TEM-2	Type 1b			IIIa
SHV-1		TEM-1,type2		IV
HMS-1				
OXA-1	Type II			Va
OXA-2	Type III			Vb
OXA-3				V
PSE-1	Type IV		CARB-2	V
PSE-2				V
PSE-3			CARB-4	V
PSE-4			CARB-1	V

Meanwhile others who were more interested in chromosomally mediated Beta-lactamases were equally confused. Mitsuhashi and Inoue (1981) split chromosomal enzymes into two groups i.e. cephalosporinases and cefuroximases.

The Richmond & Sykes class Ia, Ib, Ic and Id enzymes were easily confused with the Mitsuhashi Ia and Ib plasmid mediated Beta-lactamases and the *Bacillus cereus* type I enzyme (Sanders 1987).

Sanders (1989) divided chromosomal Beta-lactamases into four major groups:

1. cephalosporinases
2. oxyiminocephalosporinases
3. penicillinases
4. broad spectrum Beta-lactamases

As can be seen all the above classification systems firstly rely heavily on biochemical "substrate profiles" coupled with the effects of inhibitors and secondly (particularly in the case of plasmid mediated Beta-lactamases) on the isoelectric points of the enzymes.

These biochemical classifications have important drawbacks:

1. Substrate profile

This is usually expressed as the rate of hydrolysis or V_{max} which is related, as a percentage, to that of a standard substrate e.g. penicillin , cephaloridine or in the case of weak enzymes , nitrocephin. This allows the enzymes to be categorised simply as cephalosporinases or penicillinases or more specifically as e.g. carbenicillinases. However this takes no account of the enzymes affinity for the substrate (K_m). Listing hydrolysis data without including the K_m value has been criticised on many occasions (Amyes, 1987; Bush & Sykes, 1986; Livermore, 1987). It has also been noted that individual rates of

hydrolysis for the same enzyme may vary widely between one laboratory and another.

2. Isoelectric point

Since its description in 1975 (Matthew *et al.*,1975) pI has "become one of the most critical assays used to verify the identity of Beta-lactamases (Bush, 1988). However with the recent explosion of "extended broad spectrum" (EBS) plasmid mediated Beta-lactamases, often with identical or similar pI's, it has become clear that the only conclusive method to identify and, therefore, classify them is by analysis of their amino acid sequence. This is exemplified by five recently described enzymes which have isoelectric points of 5.55 ± 0.05 (Quinn *et al.*, 1989)

3. Structural relationships

Biochemical classification systems do not consider the structural relationships between Beta-lactamases which are central to enabling meaningful structure-activity correlations. Such information will be of considerable assistance in the future design and synthesis of new Beta-lactam compounds (Baurenfeind, 1986). An understanding and comparison of the primary, secondary and tertiary structure of Beta-lactamases is crucial to the fascinating study into the evolution of these proteins.

Another more minor criticism of the previous classifications has been that they do not include Beta-lactamases produced by Gram positive species (Bush, 1988).

In summary the biochemical classifications of the Beta-lactamases can be viewed as minor variations (often confusing) of a central theme which has obvious drawbacks.

Therefore in 1980, Ambler proposed a scheme in which Beta-lactamases are classified by the molecular characteristics of the proteins, or the genes coding for them.

This, perhaps cynically, may be viewed as:

(i) finally clarifying the classification of Beta-lactamases.

or

(ii) confusing the situation even further.

Ambler (1980) himself apologised for imposing a new nomenclature. However, as I hope the following text will show, it was an important breakthrough and greatly aided our understanding of Beta-lactamases.

STRUCTURAL CLASSIFICATION FOR BETA LACTAMASES

The central protagonists in the Beta-lactamase story have always accepted that the knowledge of the primary, secondary and tertiary structures of these proteins would be crucial to their understanding of their evolution and function and would therefore provide their ultimate classification. (See previous quote from Richmond & Sykes, 1973).

The molecular biologists have always been in the vanguard of such thought. Pollock (1967) in the conclusion of his paper entitled "Origin and Function of Penicillinase: A problem in Biochemical Evolution" stated:

"All these scrappy and indecisive points, the indirect and circumstantial evidence, and the speculative and perhaps tenditious arguments may seem rather naive and primitive attempts for tackling so difficult and huge a problem as biochemical evolution, albeit of a single enzyme type. But here and there a few

indications and direction pointers have emerged, most of them leading towards a demand for greater information on the amino acid sequences of more and more enzymes".

Twenty-one years later Bush (1988) wrote: "Within the last decade a combination of efforts by protein biochemists and molecular biologists has made the subject of Beta-lactamase taxonomy more scientific and less empirical". A year later Bush (1989a) went on to state: "All major Beta-lactamases will eventually be sequenced, thus allowing meaningful correlations between structure and function to be made". But she went on, in true biochemical style, to say: "However, the biological activity associated with the enzyme should be emphasised as the most relevant attribute to a novel Beta-lactamase".

In 1980, Ambler on the basis of the amino acid sequences of *Staphylococcus aureus* PC1, *Bacillus licheniformis* 749/C, *Bacillus cereus* 569/H (Beta-lactamase type I) and *E. coli* plasmid mediated TEM-2, proposed that they all belonged to one homology group which he called Class A. On the basis of differences in mechanism and size, coupled with "apparent lack of sequence homology" with the metalloenzyme *B.cereus* Beta-lactamase type II he also proposed the Class B group. This was to be confirmed later by full sequencing (Hussain *et al.*,1985).

With the significant advances in molecular techniques of cloning; probing; amino acid and nucleotide sequencing that has taken place in the last decade - enough Beta-lactamase sequences have been elucidated to allow, at least, four (perhaps 5) distinct molecular groups of these enzymes to be characterised (See Table 8).

Class A

These enzymes all have a serine residue at their active sites and have molecular weights of around 29,000. They have significant amino acid homology and are either penicillinases or broad spectrum Beta-lactamases. Class A enzymes also show considerable homology with some D-alanine carboxypeptidases (PBP's) which will be discussed further later.

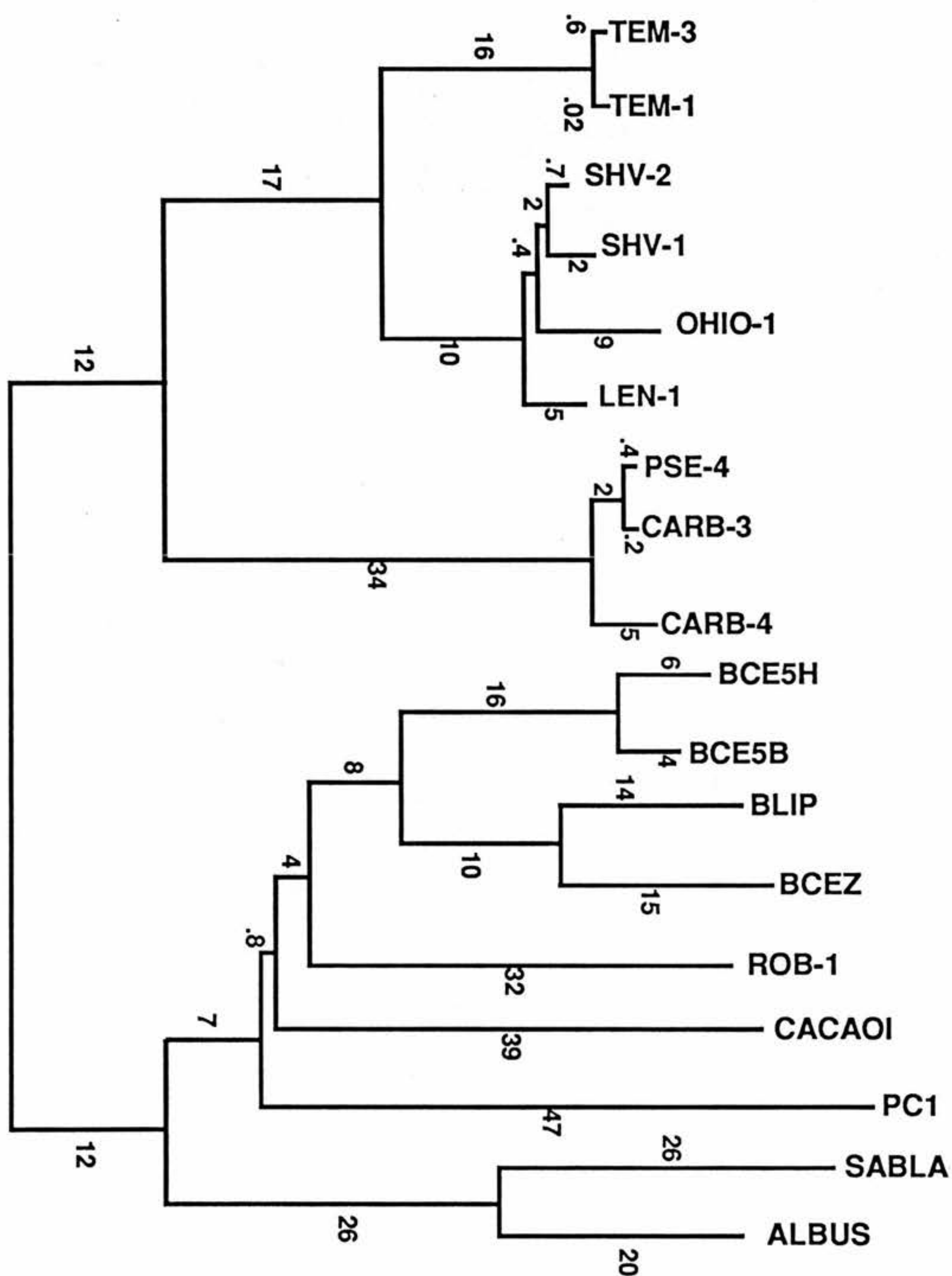
Based on the sequences of 18 Beta-lactamases of the Class A group, Huletsky *et al.* (1990) have contributed a phylogenic tree by the progressive alignment method (see fig. 7). Their data "suggested that the Beta-lactamases of Gram positive *Streptomyces, Staphylococcus* and *Bacillus* spp. appeared early in evolution, followed by the PSE and CARB enzymes of *Pseudomonas* species and, more recently by the SHV type and TEM type enzymes found in enteric bacteria".

Recently, Ambler *et al.* (1991) have proposed a standardised numbering system for the amino acid sequences of the Class A Beta-lactamases (ABL numbers - Class **A** **B**eta-**L**actamase). This should allow for a more meaningful comparison of homologous residues within different Class A enzyme sequences.

Class B (metalloenzymes)

This includes the metalloenzymes of *Bacillus cereus* II a cephalosporinase which requires a metal cofactor - normally ZnII (Davies & Abraham,1974). Recently, Thomson & Malamy (1990) and Rasmussen *et al.* (1990) have sequenced the Beta-lactamase genes of *Bacteriodes fragilis* (TAL2480/TAL3636).

Figure 7



Cladogram of Class A ß-lactamases (after Huletsky *et al.*, 1990).
The cladogram was constructed with the progressive method of Feng & Doolittle (1987).
Branch length values represent relative phylogenetic distances.

Although these enzymes show only 33% and 32% sequence similarity with the sequence of *Bacillus cereus* II, the Zn ligand binding and Cys residues are precisely conserved and the amino acids in the vicinity of these sites are highly conserved > 80% when the two proteins are compared.

The other metalloenzymes e.g. from *Flavobacterium odoratum* (Sato *et al.*, 1985); *Aeromonas sobria* and *Aeromonas hydrophila* (Iaconis & Sanders, 1990); *Legionella gormanii* (Fujii *et al.*, 1986); *Xanthomonas maltophilia* - L1 (Saino *et al.*, 1982 and Bicknell *et al.*,1985) and *Pseudomonas aerginosa*, GN17203 (Watanabe *et al.*, 1991) may yet be included in this group.

Class C

This group was proposed by Jaurin and Grundstrom (1981) though Ambler had speculated on its necessity (Ambler, 1980). They analysed the amp C Beta-lactamase of *E. coli* K12 and showed no significant homologies with Class A enzymes or with D-alanine carboxypeptidase. Confirmation of this group was shown by Knott-Hunziker *et al.*(1982a); Joris *et al.* (1986) and Lindberg & Normark (1986).

The molecular weights of these enzymes were found to be around 39,000 as opposed to those of Class A enzymes at around 29,000.

Recently it has been proposed that a novel plasmid-mediated Beta-lactamase (MIR-1), which confers a broad spectrum of resistance (including 3rd generation cephalosporins and cefoxitin but which is not inhibited by sulbactam or clavulanic acid) is a Class C Beta-lactamase (Papanicolaou *et al.*, 1990). Their evidence for this is that the provisional sequence of 150bp of the MIR-1 gene is 90% identical to the sequence of ampC from *Enterobacter cloacae* but only 71% identical to that of *E. coli.* The latter was used to explain why a probe for *E.coli*

ampC gene had failed to hybridise with MIR-1. TEM-1 and SHV-1 probes also failed to hybridise with MIR-1.

Although these workers felt that this was the first documented example of a plasmid-mediated "chromosomal" cephalosporinase it is likely that this is not the case. Woodford *et al.* (1990) previously described in *E. coli* a plasmid mediated enzyme resembling a chromosomally mediated Beta-lactamase of *Enterobacter cloacae* which conferred resistance against all penicillins, 1st, 2nd, 3rd generation cephalosporins and to clavulanic acid. This enzyme, named BIL-1, would seem to be a Class C Beta lactamase but as in the case of MIR-1, a full nucleotide sequence of the gene is awaited.

Nevertheless as Papanicolaou *et al.*(1990) stated "The presence of such a resistance determinant on a plasmid raises concern for rapid dissemination among Gram negative bacilli and the possible loss of effectiveness of the alpha-methoxy Beta-lactams".

Class D

OXA-1 and OXA-2 enzymes share greater than 48% homology but show no significant homology with the TEM enzymes nor with the Class A or Class C Beta-lactamases except at the region adjacent to the active site. No homology exists between the OXA and Class B Beta-lactamases. Ouellette *et al.*(1987) therefore proposed that the OXA-1 and OXA-2 be designated as Class D.

The nucleotide sequence of PSE-2 Beta-lactamase was determined by Huovinen *et al.*(1988) and was found to have distinct homology with the sequence of OXA-2 as previously deduced by Dale *et al.* (1985). He therefore suggested that, since neither of these enzymes exhibited structural similarities with TEM-1 or ampC Beta-lactamases, they should be put in a new Class D. Jacoby

(1988) demonstrated that the amino acid sequences of OXA-1, OXA-2 and PSE-1, PSE-2 showed greater homology between each other than with TEM-1. Levesque (1988) has also reported that the sequence of OXA-5 shows greater homology with the PSE and OXA enzymes than with TEM-1.

Although hybridisation studies have illustrated that PSE-4 shows homology with the PSE enzymes, the nucleotide sequence of this gene has shown a 50% homology with TEM-1, (Boissinot & Levesque, 1990). Consequently it has been placed in Class A.

?Class E

It has been suggested by Sanders (1989) that the L-1 metalloenzyme described in a strain of *Xanthomonas maltophilia* (Saino *et al.*,1982; Bicknell *et al.*, 1985) should be designated into a new Class E since its N-terminal amino acid sequence and biochemical properties show no similarity to these of *Bacillus cereus* II metalloenzyme of Class B (Bicknell *et al.*, 1985).

This is highly speculative and we will await the full nucleotide sequence of the gene encoding this enzyme before confirming this new class.

We await even more keenly the sequence of the gene encoding the plasmid mediated Beta- lactamase which confers imipenem resistance in a strain of *Pseudomonas aeruginosa* by Watanabe *et al.* (1991). It too is a metalloenzyme.

TABLE 8.

Classification of Beta-lactamases Based on Molecular Structure (Ambler 1980)

CLASS A Chromosomal

Beta-lactamase	**Reference**
Staphylococcus aureus PC1	Ambler (1975) Ambler (1980) Chang (1986) Wang & Novick (1987)
Actinomadura R39	Houba *et al*. (1989)
Bacillus licheniformis	Ambler (1980) Neugebauer *et al.* (1981)
Bacillus cereus type I	Ambler (1980) Sloma & Gross (1983) Wang *et al.* (1985) Madonna *et al.* (1987) Madgwick *et al.*(1987)
Bacillus cereus type III	Hussain *et al.* (1987)
Klebsiella pneumonia K1 (SC10436)	Joris *et al.* (1987)
Klebsiella pneumonia (LEN-1)	Arakawa *et al.* (1986)
Klebsiella aerogenes K1 (1082 E)	Emanuel *et al.* (1986)
Klebsiella oxytoca (E23004)	Arakawa *et al.* (1989)
Streptomyces albus G	De Meester *et al.* (1987) Dehottay *et al.* (1987)
Streptomyces aureofaciens	Reynes *et al.* (quoted by Huletsky *et al.* 1990)
Streptomyces badius	Forsman *et al*. (1990)

CLASS A Chromosomal (contd.)

Beta-lactamase	Reference
Streptomyces cacaoi (ULg)	De Meester *et al.* (1987) Lenzini *et al.* (1988)
Streptomyces cacaoi (blaU)	Forsman *et al.* (1990)
Streptomyces fradiae	Forsman *et al.* (1990)
Streptomyces lavendulae	Forsman *et al.* (1990)
Rhodopseudomonas capsulata	Campbell *et al.* (1989)

Beta-lactamase	Reference
CLASS A Plasmid mediated	
TEM-1	Sutcliffe (1978)
TEM-2	Ambler & Scott (1978) Barthelemy *et al.* (1985) Chen & Clowes (1987
TEM-3	Sougakoff *et al.* (1988)
TEM-4	Sougakoff *et al.* (1989)
TEM-5	Sougakoff *et al.* (1989)
TEM-6	Goussard *et al.* (1989) Mabilat & Courvalin (1990)
TEM-7	Collatz *et al.* (1989)
TEM-8	Mabilat *et al.* (1989) Mabilat & Courvalin (1990)
TEM-9	Mabilat *et al.* (1990)
TEM-10 to TEM-19	Mabilat & Courvalin (1990)
ROB-1	Juteau & Levesque (1990) Livrelli *et al.* (1991)
SHV-1	Barthelemy *et al.* (1988a) Mercier & Levesque (1990)
SHV-2	Barthelemy *et al.* (1988b) Huletsky *et al* (1990) Garbarg - Chenon *et al.* (1990)
SHV-3	Nicolas *et al.* (1989)
SHV-4	Barthelemy *et al.* (1988c)

Table 8 (contd.)

Beta-lactamase	**Reference**
CLASS A Plasmid mediated (contd.)	
SHV-5	Billot-Klein *et al.* (1990)
OHIO-1	Shaes *et al.* (1990)
PSE-4	Boissinot & Levesque (1990)
CARB-3	Lachapelle & Levesque (unpublished data)(quoted by Huletsky *et al.* 1990)
CARB-4	Bejaoui & Levesque (unpublished data) (quoted by Huletsky *et al.* (1990)
CLASS B Chromosomal	
Bacillus cereus type II	Ambler (1980) Hussain *et al.* (1985)
Bacteroides fragilis (TAL 3636)	Rasmussen *et al.* (1990)
Bacteroides fragilis (TAL 2480)	Thompson & Malamy (1990)
CLASS C Chromosomal	
Escherichia coli (K12)	Knott-Hunziker *et al.* (1982b)
Pseudomonas aeruginosa (1822 S/H)	Knott-Hunziker *et al.* (1982b)
Citrobacter freundii (0S60)	Lindberg & Normark (1986)
Enterobacter cloacae (P99)	Joris *et al.* (1984) Galleni *et al.* (1988)
Serratia marcescens (SC8247)	Joris *et al.* (1986)

Table 8 (contd.)

Beta-lactamase	Reference
CLASS C Plasmid mediated	
MIR-1	Papanicolaou *et al.* (1990)
BIL-1	Woodford *et al.* (1990)
CLASS D Plasmid mediated	
OXA-1	Ouellette *et al.* (1987)
OXA-2	Dale *et al.* (1985)
OXA-5	Levesque (1988)
PSE-1	Jacoby (1988)
PSE-2	Houvinen *et al.* (1988)
CLASS E Chromosomal	
Xanthomonas maltophilia L1	Bicknell *et al.* (1985) (suggested by Sanders, 1989)

THE SUPERFAMILY OF ACTIVE SERINE ENZYMES:

THE PENICILLIN-RECOGNISING ENZYMES

Gene sequencing has also been used to elucidate the structure of the DD-peptidases/PBP's (Duez *et al.*, 1987; Broome - Smith *et al.*, 1983; Todd *et al.*, 1986; Broome - Smith *et al.*, 1985; Asoh *et al.*,1986 ; Nakamura *et al.*, 1983).

Thus these can be compared with the gene sequences known for Beta-lactamases together with the secondary and tertiary structures and the active site environment that has been determined for some of these proteins (Kelly *et al.*,1986; Samraoui *et al.*, 1986; Knox *et al.*, 1976; Herzberg & Moult, 1987; Dideberg *et al.*, 1987; Kelly *et al.*, 1987).

The active-site-serine Beta-lactamases are defensive enzymes; they hydrolyse the Beta-lactams into inactive metabolites. The active-site-serine DD peptidases which are involved in bacterial cell wall metabolism, catalyse the attack of the C-terminal D-alanyl-D-alanine peptide bond in peptidoglycan precursors. They are inactivated by the Beta-lactam antibiotics whose endocyclic amide linkage is equivalent to the scissile peptide bond in the peptidoglycan precursors.

Therefore these two groups of enzymes not only bind similar ligands but they also operate by a common acyl enzyme mechanism.

Crucial to this mechanism is the transfer of the electrophilic group R-C=O of the scissile (peptide, amide) bond to the hydroxyl group of the active-site-serine residue. The ester-linked acyl-enzymes formed by the reaction with the DD peptidases are usually very long lived. In contrast those found in the reaction between the Beta-lactams and the Beta-lactamases are usually very short lived.

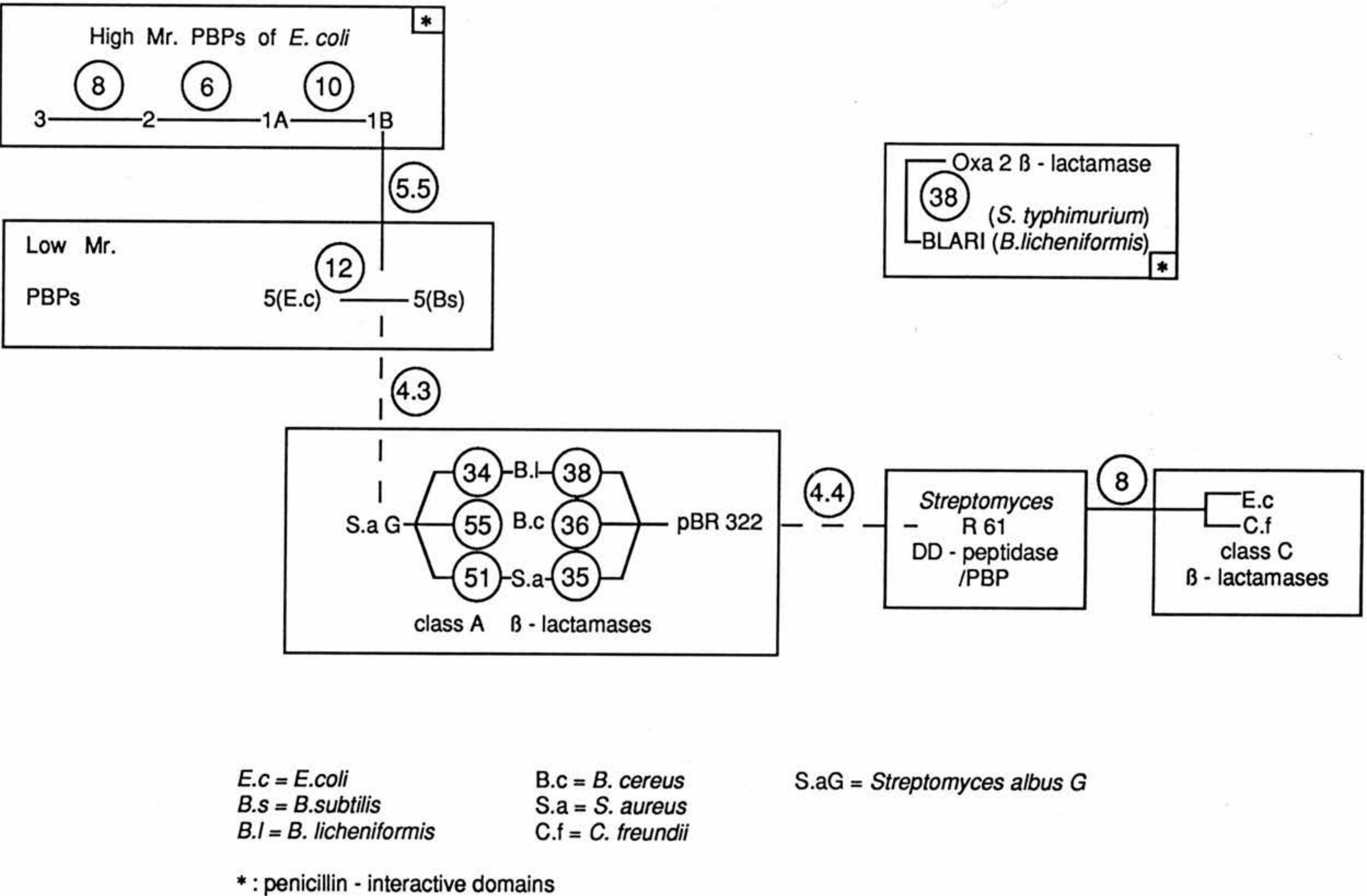
Consequently the Beta-lactam antibiotics are substrates of the Beta-lactamases and inactivators of the DD peptidases which therefore behave as penicillin-binding proteins (PPBs) (Cartwright & Waley, 1983; Ghuysen *et al.*, 1984; Frere & Joris, 1985).

Joris *et al.* (1988) employed the *Streptomyces* R61 DD peptidase/penicillin binding protein as a reference and compared the Beta-lactamases of Classes A and C; the OXA-2 (as an example of Class D); the low M_r DD peptidases/PBP's (PBP5 of *E.coli* and *B.subtilis*) and the penicillin binding domains of the high M_r PBP's of *E.coli* (PBP1A, PBP1B, PBP2 and PBP3) by homology searches and amino acid alignments.They produced a tentative family tree of the penicillin - recognising enzymes (see fig.8).

They stated: "Though the evolutionary distance may vary considerably, all these penicillin-interactive proteins and domains appear to be members of a single superfamily of active-site-serine enzymes distinct from the classical trypsin or subtilin families. The amino acid alignments reveal several conserved boxes that consist of strict identities or homologous amino acids".

Boxes II, III, IV, V, and VI occupy critical positions in the 3D structure of *Staphylococcus aureus* and *Streptomyces albus* G Beta-lactamases. These workers found that amino acid replacements in each of these boxes affect or abolish the activity of: several Beta-lactamases, low M_r PBP5 and high M_r PBP3 of *E.coli*.

Figure 8



Family tree of penicillin - interactive proteins (after Joris *etal.*, 1988). A standard deviation unit (SDU), values are circled, of 5 or higher indicates a satisfactory significant homology.

EVOLUTION AND ORIGIN OF BETA-LACTAMASES

Pollock (1967) described experiments he carried out on the spores of *Bacillus licheniformis* which had "lain dormant in the dried soil stuck to the roots of some plant specimens preserved untouched in the British Museum since 1689".

Sneath (1962) had revived the organisms and Pollock examined the penicillinases produced by them. They all fell "clearly" into one of the two groups of penicillinases that existed in the 1960's, both in their substrate profiles and their immunological reactions. Pollock took this to indicate that no evolutionary change had occurred in 270 years - an equivalent of 100 million years of human generation time. In other words long enough "for evolution to have occured if cell generations were the main operative factor".

In the summary of the paper he concluded: "Consideration of the evolutionary origin of penicillinase remains highly speculative, though it is clear that the various forms of the enzyme, as we now know then, pre-existed the therapeutic penicillin era, the only effect of which, in this context, has been to increase by natural selection the proportion of bacteria producing them.

Search for a possible penicillinase-ancestor is focused on the only other known class of protein that must, on *a priori* reasoning, be capable of specific combination with penicillin - that is, one or other of the enzymes involved in biosynthesis of the mucopeptide of the bacterial cell wall, a reaction known to be specifically inhibited by this antibiotic".

However, Pollock's work was inconclusive and 20 years later, Joris *et al.* (1988) have provided some evidence to support this hypothesis. (See previous section).

Ghuysen(1988) reiterated that all the active-site-serine, penicillin-interactive proteins are related in an evolutionary sense and form a superfamily of enzymes. This he felt was an example of divergent evolution. Depending on the evolutionary distance, they had acquired: different amino acid sequences; distinct functionalities; and specificities. Yet they had conserved the same pattern of polypeptide scaffolding. Divergent evolution means, of course, that these enzymes have a common ancestor. He made the following points to back up this theory:

1. The DD peptidases/PBP's are important or even essential bacterial enzymes, whereas the Beta-lactamases are dispensable unless Beta-lactam antibiotics are present in the environment.

2. *Streptomyces* are soil bacteria and one may assume that they were among the first bacteria to be exposed to Beta-lactam molecules.

3. *Streptomyces* are the only known bacteria that spontaneously excrete some low molecular weight DD-peptidases/PBP's during growth.

4. The exocellular DD-peptidase/PBP of *Streptomyces* R61 occupies an important position in the family tree of interactive penicillin proteins where it serves as a bridge between the Beta-lactamases of Class A and the Beta-lactamases Class C (see fig.8).

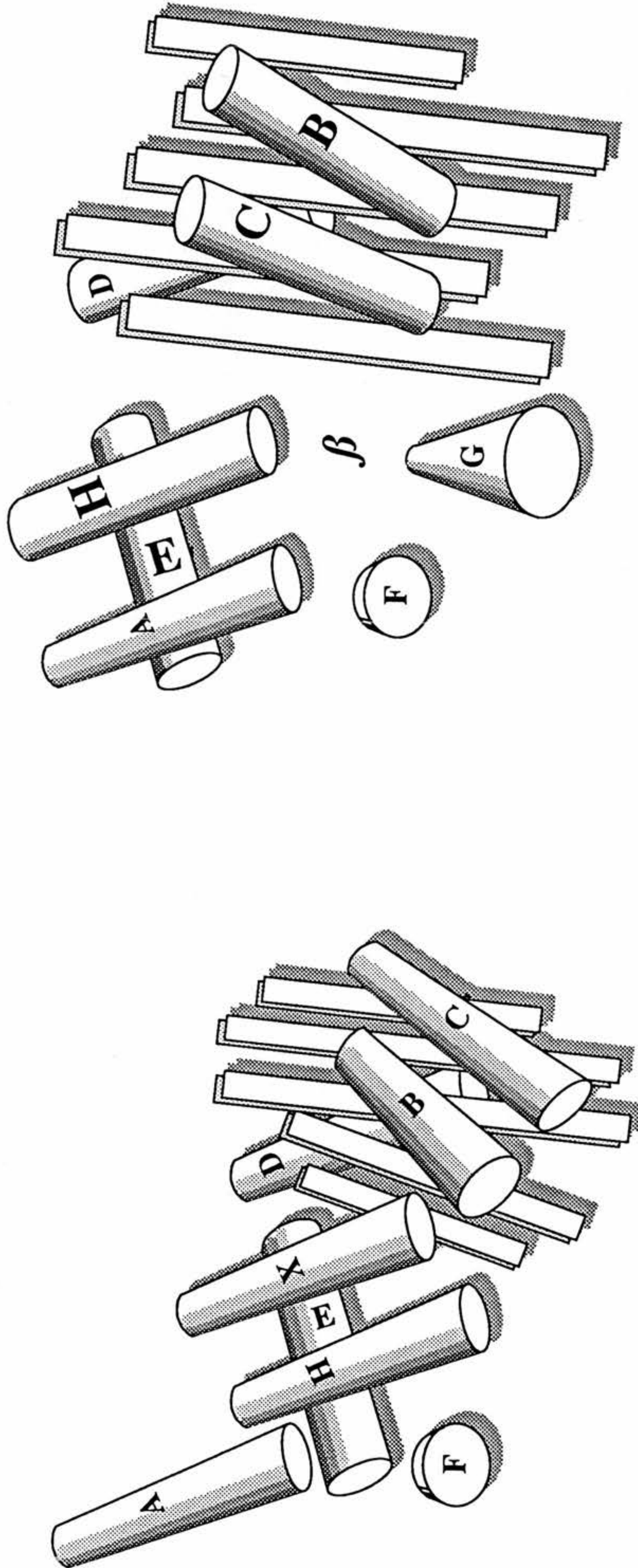
Ghuysen therefore concluded:"On this basis, one may propose a possible mechanism for the emergence of Beta-lactamases. The primary response of soil *Streptomyces* to exposure to Beta-lactam compounds produced by other micro-organisms was to develop an excretion mechanism permitting release of a membrane bound PBP into the environment and immobilisation of the Beta-lactam molecules in the form of stable acyl enzymes. Further improvements of

this detoxication mechanism was the conversion of this water soluble Beta-lactam-binding enzyme into a Beta-lactam-hydrolysing enzyme by remodelling of the active site".

This is certainly an attractive and very plausible theory for the origin of Beta-lactamases. The importance of comparing secondary and tertiary structures rather than just primary amino acid structure when looking at the evolution of proteins was stressed by Kelly *et al.* (1986). "Ancestral origin is difficult or impossible to establish with certainty. Amino acid sequences change rather rapidly on the genetic time scale, and they are therefore intrinsically less informational than the more time-stable three-dimensional foldings that are maintained in accordance with external entropic and energetic limitations. The DD peptidase (*Streptomyces* R61) and Beta-lactamase (*Bacillus licheniformis*) are from different species and lack close relatedness in their overall primary structures. Yet they share the same basic catalytic mechanism, they recognise the same Beta-lactam compounds, they contain several conserved amino acids in their active sites, and they exhibit clear similarity in their 3 dimensional structures (see fig 9). The analogous positioning of secondary structure elements even well away from the active site, suggests that these enzymes may be another example of divergent evolution where structural changes required for their distinctive functions appeared only in the relatively compact catalytic area".

Richmond (1988) widened this hypothesis. He noted that many organisms have been shown to produce very small amounts of monobactams and he wondered whether these molecules were widely distributed in bacteria as "endogenous regulators of peptidoglycan biosynthesis". Presumably he is also implying, since he feels that peptidoglycan biosynthesis is an extremely complicated and organised system "where things have to be switched on precisely and switched off precisely..." that the regulatory effect of the Beta-lactams is itself

Figure 9



Secondary structure elements in *B. licheniformis* ß - lactamase (left) and *Streptomyces* R61 DD -peptidase (right).
Cylinders are helices and ribbons are ß-strands.
The known site of ß-lactam binding in the DD-peptidase is indicated by ß (after Kelly *et al.*, 1986).

regulated by the Beta-lactamases (However there is no experimental evidence to suggest that this hypothesis is correct).

Therefore perhaps our use of Beta-lactams therapeutically, is analogous to our use of the morphine-like drugs which are very similar to the naturally occurring "painkillers" - the endorphins.

The subsequent evolution from a common ancestor is best seen in the unrooted phylogenetic study of Class A Beta-lactamases by Huletsky *et al.*, (1990) (see fig. 7).

They observed two major groups of proteins, the Beta-lactamases of Gram positive bacteria, including BCE5H, BCE5B, BLIP, BCEZ, CACAOI, PCI, SABLA and ALBUS that presumably appeared early in evolution, followed by the Beta-lactamases of Gram negative bacteria such as CARB-3, CARB-4, PSE-4, LEN-1, OHIO-1, SHV-1, SHV-2, TEM-1, and TEM-3. The Gram negative bacteria were further subdivided into two main groups: the PSE and CARB enzymes of *Pseudomonas* spp. followed by the SHV and TEM type enzymes.

In the Gram positive group of bacteria they noted SABLA and ALBUS Beta-lactamases from *Streptomyces* spp. at the bottom of the phylogenetic tree followed by the well known PCI enzyme of *Staphylococcus aureus*. Surprisingly close to the PCI enzyme branch was the plasmid mediated ROB-1 Beta-lactamase from *Haemophilus influenzae* a Gram negative rod. Presumably this had been acquired by horizontal gene transfer from a Gram positive organism. This was followed by the Beta-lactamase from *Streptomyces cacaoi*. Finally, at the top of the Gram positive group branch of the tree they found the Beta-lactamases of *Bacillus* spp. indicating a more recent derivation of these enzymes.

In the Gram negative group the PSE and CARB enzymes presumably branched off early, starting with CARB-4. Next they noted the recent divergence of the SHV family. LEN-1, the chromosomal Beta-lactamase from *Klebsiella* spp. appeared first followed by OHIO-1. SHV-1 and SHV-2 were separated by a very short evolutionary distance indicating that SHV-2 is a very recent derivative of SHV-1. The more recent Class A Beta-lactamases found in the cledogram are the TEM enzymes commonly found in enteric bacteria that are highly related to SHV-type Beta-lactamases. A very short evolutionary distance also separated TEM-1 from TEM-3 because of only 3 amino acid substitutions.

These data support the hypothesis proposed in the 1960's by Naomi Datta that the TEM-1 Beta-lactamase evolved from the chromosomal Beta-lactamase of *Klebsiella pneumoniae*. This was also proposed by Arakawa *et al.* (1986) and Arakawa *et al.* (1989).

Interestingly the structural subgroupings delineated by Huletsky *et al.* (1990) seem to be directly related to the biochemical properties of these enzymes. The SHV and TEM enzymes are members of the broad spectrum enzymes of Class 2b and 2b'; PSE and CARB enzymes are Class 2c; and the Beta-lactamases of the Gram positive bacteria are penicillinase enzymes of Class 2a of Bush's classification system (Bush, 1989 a,b,c).

THE BIOCHEMISTS STRIKE BACK : THE BUSH CLASSIFICATION

In 1986, Bush and Sykes published a much needed set of guidlines in the hope of standardising the study of Beta-lactamases. They felt this was necessary because of the plethora of new Beta-lactamases and the, correctly perceived, need for a more detailed knowledge of their kinetic parameters rather than the simple substrate profiles that had sufficed in the past. The hope was that with standardised methodology more meaningful "structure - activity relationships and mechanistic interpretations" could take place.

The requirements included:

1. ideally a homogeneous enzyme preparation (95% pure)
   OR at least
2. a single Beta-lactamase activity on IEF
3. a specific activity (μmol substrate hydrolysed per minute per mg of protein) for partially pure enzyme, OR a K_{cat} value for a homogenous enzyme with a standard substrate
4. hydrolysis data - K_m and V_{max} values to cephaloridine and penicillin or nitrocephin (if only weak activity is detected)
5. inhibition studies - with well characterised inhibitors such as : clavulanic acid , sulbactam , cloxacillin and aztreonam; EDTA , pCMB , and boronic acids - carried out under defined conditions
6. induction studies - using antibiotic levels likely to be encountered extracellularly.

The logical step was then to create a new classification for the Beta-lactamases employing the data derived from such a standard methodology and incorporating it with the structural data (if available) from the same enzymes.

After all, in structure - activity relationships there must be data relating to the biochemical activity of the enzyme.

Therefore Bush (1988) proposed a scheme which incorporated both biochemical and molecular aspects and divided the enzymes into 3 classes,

Subsequently Bush (1989a,b,c) expanded this classification into 4 groups to include most Beta-lactamases known at that time. The parameters employed were:

(i) genetic location - chromosomal or plasmid

(ii) relative rates of hydrolysis

(iii) inhibition studies

(iv) molecular mass

(v) isoelectric point

(vi) molecular class (if known)

The general outline of this scheme is shown in Table 9.

Table 9. General classification scheme for bacterial β-lactamases

Group	Subtitle	Preferred substrates	Inhibited by:		Representative enzyme(s)
			CA[a]	EDTA	
1	CEP-N	cephalosporins	No	No	Chromosomal enzymes from Gram negative bacteria
2a	PEN-Y	penicillins	Yes	No	Gram positive penicillinases
2b	BDS-Y	cephs & pens	Yes	No	TEM-1,TEM-2
2b'	EBS-Y	cephs,cefotaxime & penicillins	Yes	No	TEM-3,TEM-5
2c	CAR-Y	pens,carbenicillin	Yes	No	PSE-1,PSE-3,PSE-4
2d	CLX-Y	pens,cloxacillin	Yes[b]	No	OXA-1,PSE-2
2e	CEP-Y	cephalosporins	Yes	No	*Proteus vulgaris*
3	MET-N	variable	No	Yes	*Xanthomonas maltophilia* L1, *Bacillus cereus* II
4	PEN-N	penicillins	No	?[c]	*Pseudomonas cepacia*

[a] 10μM clavulanic acid ; [b] Inhibition by clavulanic acid may occur at higher concentrations for some members of the group. ; [c] Variable. (Taken from Bush , 1989a).

It is clear that this scheme will serve to differentiate the Beta-lactamases that have been described to date and will provide the necessary framework for the classification of additional enzymes as they become described. It will also serve as the corollary to the structural information elucidated from the Ambler classification of these enzymes.

THE PAYNE-AMYES CLASSIFICATION OF THE PLASMID-MEDIATED EXTENDED-SPECTRUM BETA-LACTAMASES

These authors provide us with the most recent subclassification of beta-lactamases (Payne & Amyes, 1991) They feel that since there are now, at least, 27 reports of transferable genes encoding resistance to the broad spectrum cephalosporins - there needs to be a classification scheme. They acknowledge that a structurally based scheme is important, but some way off as all the genes need to be sequenced. Therefore they have classified these enzymes into 4 groups based on their relative efficiencies of hydrolysis to cefotaxime and ceftazidime.

Group 1

Enzymes that hydrolyse cefotaxime and ceftazidime with poor efficiency, cefotaxime > ceftazidime, paradoxically since all the group 1 enzymes confer greater resistance to ceftazidime than cefotaxime. This may be explained by their different permeability characteristics (Nikaido, 1990).

Group 2

3GC enzymes that hydrolyse ceftazidime more efficiently than cefotaxime.

Group 3

3a. TEM - derived

3b. SHV - derived

3c. unknown derivation

Most enzymes in this group confer a greater resistance to ceftazidime.

Group 4

This group contains Beta-lactamases that confer resistance to all generations of cephalosporins and clavulanic acid eg BIL-1 (Woodford *et al.*, 1990) and MIR-1 (Papanicolaou *et al.,* 1990).

It would seem likely that there should now be a **Group 5** to include the plasmid mediated imipenemase described by Watanabe *et al.* (1991).

NEW FORMS OF RESISTANCE TO THE BROAD SPECTRUM BETA-LACTAM ANTIBIOTICS IN GRAM NEGATIVE BACTERIA

I have previously briefly alluded to the evolution of this form of bacterial resistance (Weidemann *et al.*, 1989).

The broad spectrum cephalosporins, carbapenems and monobactams were developed essentially to deal with the Beta-lactamase producing Gram negatives that were resistant to ampillicin and the ureidopenicillins (Jacoby & Archer, 1991). Bacteria have met this challenge in several ways.

Chromosomally-mediated resistance to the broad spectrum Beta-lactamases

By the mid 1980's clinical microbiology laboratories began to report resistance to these new extended spectrum Beta-lactams in Gram negative bacteria. This was due to hyperproduction of the chromosomal class I (AmpC) Beta-lactamases in these species. Hyperproduction of these enzymes can occur in one of two ways.

(i) In species such as *Enterobacter*, *Pseudomonas*, *Serratia*, *Citrobacter*, indole positive *Proteus* and *Providencia* hyperproduction can arise transiently as a result of **induction**. (Sanders & Sanders, 1988).

(ii) In the same species hyperproduction can occur on a permanent basis via mutation of the regulatory gene - the production of **stably derepressed mutants (SDM).**

SDM can be produced at a frequency of between 10^{-5} and 10^{-10} depending on the species, strain and selection conditions (Phillipon, 1987; Weidemann, 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 generation of cephalosporins (Curtis *et al.*,

1986). The importance of inducible Beta-lactamases and SDM is reviewed by Sanders & Sanders (1988).

Livermore (1987) has characterised the Beta-lactam drugs into either **labile** or **stable** substrates and **strong** or **weak** inducers of the chromosomal Beta-lactamases. This is shown in Table 10.

Table 10. Chromosomal cephalosporinases : expression and resistance

Compound	Behaviour	Susceptibility			Selection pressure for SDM
		I	SDM	B	
1st gen.cephs. cefoxitin ben.pen. ampicillin	LABILE & STRONG INDUCERS	R	R	S	**WEAK**
cefuroxime 3rd gen.cephs ureido.pens.	LABILE & WEAK INDUCERS	S	R	S	**STRONG**

S = sensitive

R = resistant

B = basal β–lactamase production

I = inducible β-lactamase production

SDM = β-lactamase stably derepressed production

(After Livermore ,1987)

Livermore's thesis being ,that it is the **labile** and **weak** inducers that provide **strong** selection pressure for SDM. Since they are resistant, these mutants have an advantage over the inducible cells when challenged with labile, weak inducers e.g. the newer cephalosporins. They easily overrun the population both ***in*** *vitro* and *in vivo* and once selected they continue to manufacture large quantities of enzyme even when the inducer is absent.

However, the turnover numbers (k_{cat}) for cefuroxime, most third-generation cephalosporins, monobactams and carboxypenicillins are extremely low, therefore they are "stable" poor substrates - so why are the organisms producing these chromosomal Beta-lactamases resistant?

The resistance is explained by two broad models. The arguments raged through the early 1980's as to which of these theories was correct.

"Trapping" or "sponge" theory

Then & Angehrn (1982) and Gutmann & Williamson (1983) proposed that the low K_m of these enzymes (i.e. their high affinity for the 3GC substrates) "trapped" and inactivated these antibiotics in non-covalent E.S complexes or covalent E-S complexes (see fig. 6). Covalent "trapping" is often suggested in the cases of aztreonam and moxalactam (though the debate still rages even in the case of these drugs: Hewinson *et al.*, 1989; Livermore, 1989). Non-covalent "trapping" is viewed as reversible while covalent "trapping" is irreversible.

Efficient-hydrolysis model

It is now felt that the major factor in 3GC resistance *is* Beta-lactamase hydrolysis of these "stable" substrates. Vu and Nikaido (1985) proposed that the assays of *in vitro* hydrolysis were misleading. Firstly the enzyme concentrations are 100 - 10,000 - fold lower than those in the periplasm resulting in the significance of slow hydrolysis being underestimated and secondly the high

enzyme-antibiotic affinity ensures that the drug turnover remains fully efficient even at very low drug concentrations.

Clearly slow entry of the drug into the bacterial cell is a fundamental assumption of both models. It is suggested therefore that the Beta-lactamase molecules are able to prevent the periplasmic drug concentration rising to toxic levels since the rate of drug influx through the outer membranes is extremely slow. If such a barrier did not exist the enzyme would rapidly be overwhelmed.

Livermore (personal communication) perhaps sums this up well with the analogy of a few soldiers guarding a narrow pass against a much larger army. Which is the most important - the soldiers or the narrowness of the pass?

Clearly an understanding of the genetic control of the chromosomal Beta-lactamases is required in order to circumvent the clinical problems that result from induction and the selection of SDM. This is as yet poorly understood and I refer the reader to the following : Kobayashi *et al.*,1982; Lindberg *et al.*, 1988; Korfmann & Sanders, 1989; Oliva *et al.*,1989; Everett *et al.*,1990; Normark *et al.*, 1990.

Plasmid-mediated resistance to the broad spectrum Beta-lactamases (extended broad spectrum, EBS)

In 1979, Matthew *et al.* described the properties of 11 plasmid mediated Beta-lactamases which were easily differentiated by their isoelectric points on IEF. In epidemiological studies it became clear that TEM-1 was the commonest plasmid-encoded Beta-lactamase (60% of all plasmid mediated Beta-lactam resistance in *E.coli*) (Simpson *et al.*, 1980; Roy *et al.*, 1983; Medeiros, 1984). SHV-1 is also frequent in *Klebsiella pneumoniae* and PSE-1 is common in *Pseudomonas aeruginosa* (Medeiros, 1989)

The success of these enzymes seems firstly to be due to the fact that the resistance genes are located on transposons and secondly because they are

extremely efficient at hydrolysing penicillins and many first generation cephalosporins (but not second or third generation) (Payne & Amyes, 1991).

The first report of transferable cefotaxime resistance came in 1983 from West Germany (Knothe *et al.,* 1983) in three strains of *Klebsiella pneumoniae* and one of *Serratia marcescens.* This enzyme was found to be a modification of the ubiquitous klebsiella enzyme SHV-1 and was called SHV-2 (Kliebe *et al.,* 1985). Subsequently, Payne *et al.*(1989 & 1990) have shown that the earliest EBS enzyme originated from an English strain in 1982. Since their discovery in 1983 there has been a large number of publications from every continent describing "novel" EBS Beta-lactamases.

Nearly all of these new enzymes appear to be modifications of either the TEM or SHV groups of Beta-lactamases (Philippon *et al.*,1989). See Table.11.

Therefore the bacteria have evolved not in this case by mutation of the gene controlling the production of the Beta-lactamase but by mutation of the structural gene itself.

Many have shown that EBS can be easily obtained spontaneously from TEM-1/2 or SHV-1 *in vitro* (See Payne & Amyes, 1991).

There now has been at least 27 reports of transferable genes encoding 3GC resistance. One particularly worrying aspect of these new resistances has been that the organisms were resistant not only to Beta-lactams but also other classes of antimicrobials e.g. aminoglycosides, tetracyclines, sulphonamides, quinolones, chloramphenicol and trimethoprim. (Sirot *et al*., 1988).

Table 11. Differences in amino acid substitutions among sequenced broad spectrum β-lactamases and related enzymes. *Italicized* amino acids indicate differences from the first enzyme in the series

	Amino acid substitution [a]						
Enzyme	***37***	***102***	***162***	***235***	***236***	***237***	***261***
TEM-1	Gln	Glu	Arg	Ala	Gly	Glu	Thr
TEM-2	*Lys*	Glu	Arg	Ala	Gly	Glu	Thr
TEM-3	*Lys*	*Lys*	Arg	Ala	*Ser*	Glu	Thr
TEM-4 [b]	Gln	*Lys*	Arg	Ala	*Ser*	Glu	*Met*
TEM-5	Gln	Glu	*Ser*	*Thr*	Gly	*Lys*	Thr
TEM-7	*Lys*	Glu	*Ser*	Ala	Gly	Glu	Thr
SHV-1	Gln	Asp	Arg	Ala	Gly	Glu	Leu
SHV-2	Gln	Asp	Arg	Ala	*Ser*	Glu	Leu

[a] Amino acid positions are numbered according to Sutcliffe (1978).

[b] Also, substitution of Phe at position 19 for Leu.

After Philippon, Labia & Jacoby (1989)

Other factors that are important about EBS Beta-lactamases are:

(i) they are well inhibited by existing Beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Bush & Singer, 1989; Gutmann *et al.*, 1989).

(ii) they have greater affinities than their parent enzymes for both the inhibitors and substrates but their total activities are several orders of

magnitude lower than that of the older TEM enzymes when measured against penicillin and cephaloridine. Their k_{cat} values against the 3GC's as substrates are also low. Thus, although they have a wider spectrum of hydrolytic activity they are "functionally lazy offspring of the classical Beta-lactamases", Bush (1989d).

(iii) the majority of these new Beta-lactamases have been reported to be produced by less than ten clinical isolates. This suggests that they are not spreading as one particular "epidemic" enzyme i.e. no one particular enzyme has been able to predominate and spread in the manner of TEM-1 (Payne & Amyes, 1991). Only one enzyme (TEM-3) has been responsible for nearly 500 clinical isolates in French hospitals (Sirot *et al.*, 1988) but with no worldwide or even countrywide pandemic.

Therefore it has been suggested that these enzymes at present are more of an inconvenience than a major threat (Payne & Amyes, 1991). However as they themselves said: "these are still early days and our controlled use of later cephalosporins will probably determine whether we can keep these enzymes as a minor inconvenience rather than let them emerge as a major threat".

Only the future will reveal whether other recently described novel transferable broad spectrum Beta-lactam resistance - such as BIL-1 (Woodford *et al.*, 1990), MIR-1 (Papanicolaou *et al.*, 1990) and the plasmid mediated imipenemase of Watanabe *et al.*, (1991) - will pose an even greater threat and perhaps return us to the "septic" ward of the John Radcliffe Infirmary of February 1941.

ACINETOBACTER

IDENTIFICATION

Acinetobacters are short, plump Gram negative rods that are often coccoid in the stationary phase. The cells commonly occur in pairs or chains and they can be difficult to destain. Although they have no flagella and are considered to be non-motile, "twitching" and "gliding" motility has been described on semi-solid media (Barker & Maxted, 1975; Henrichsen, 1975). Many strains are capsulated. All acinetobacter are non-fastidious strict aerobes which are catalase positive and oxidase negative. Most will grow on simple media with a single carbon and energy source between 20 and 30°C. The GC content is 38-45 mol%. (Towner *et al.,* 1991).

TAXONOMY

The only word that can accurately be used to describe the taxonomy of *Acinetobacter* is **confused**. Only recently, has some sense finally prevailed with the development of a reasonable taxonomic scheme (Bouvet & Grimont, 1986 ; Grimont & Bouvet, 1991).

Historical perspective

Excellent reviews by Baumann *et al.* (1968), Henriksen (1973 & 1976) and Juni (1978) provide us with much information on the long and varied history of these organisms.

In 1911, Beijerinck isolated an organism from the soil, this he named *Micrococcus calco-aceticus*, but he did not describe it. This organism was stored, however, and subsequently re-examined and found to be very similar to organisms that in the intervening years were described as: ***Bacterium anitratum***, *Moraxella glucidolytica* and *Neisseria winogradski* (Henriksen, 1973).

Henriksen (1937) described *Alcaligenes haemolysans,*a similar but strongly haemolytic organism, which differed from the above by not producing acid from sugars.

In 1939, De Bord introduced a new "tribe" name for the *Neisseria* - like bacteria - *Mimeae,* since they "mimicked" *Neisseria.* The type species was named *Mima polymorpha* and De Bord (1942) subsequently described two other genera - *Herellea vaginicola* and *Colloides anoxydana. Mima polymorpha* was not described in significant detail for the Code of Nomenclature, and Andureau (1940) described more closely a similar organism under the name of *Moraxella lwoffii. H. vaginicola* has subsequently been confused with *B. anitratum*, however, Henriksen (1973) feels that they differ markedly and were *not* likely to be the same organism. Its name *(H.vaginicola*) was subsequently rejected (Judicial Commission, 1971).

Colloides anoxydana was found to be biochemically identical to *Citrobacter* therefore this name too suffered the same fate as De Bord's other two proposed species.

Schaub & Hauber (1948) described an organism which they called *Bacterium anitratum,* Piechaud *et al.* (1951) described a similar organism as *Moraxella lwoffii* var *glucidolytica* (after, elevated to *M. glucidolytica*). Brisou and Morichau- Beauchant (1952) showed that these two organisms were identical.

Lemoigne *et al.* (1952) further confused the issue by describing an identical organism as *Neisseria winogradski* .

In 1954, Brisou proposed the inclusion of *B.anitratum* in the genus *Achromobacter* for the non-motile species of *Acinetobacter.* At this time it included both the oxidase-positive (Moraxella) and the oxidase negative strains.

Shortly after this *Acinetobacter* flirted briefly with several other bacterial genera. Lautrop (1961) suggested transfer of *B.anitratum* to the genus *Cytophaga*

but this was based solely on, supposed, "gliding and creeping motion" of the cells of *B.anitratum*. Lautrop (1965) himself changed his mind shortly after.

Seeliger *et al.* (1968) assumed that the organism, previously very briefly described by von Lingelsheim (1906) as *Diplococcus mucosus*, was identical to *B.anitratum/M.glucocidolytica.* They consequently proposed transfer of these species into a new genus *Lingelsheima*.

Henricksen (1973) disagreed strongly stating that the original von Lingelsheim strains had not survived to allow their proper characterisation and that their original description was too vague.

Delineation of the genus

The genus *Acinetobacter* was again exhumed by Baumann *et al.* (1968) who showed by nutritional studies that the oxidase-negative strains clearly differed from the oxidase-positive strains within the genus *Moraxella*.

In 1971 the subcommittee of the Taxonomy of *Moraxella* and Allied Bacteria proposed that the genus *Acinetobacter* should include only the oxidase-negative strains. It was classified in the family *Neisseriaceae* and originally as only one species : *A.calcoaceticus.*

The genus was well delineated by the transformation studies of Juni (1972). DNA from oxidase-negative strains of *Moraxella* (*Acinetobacter* spp) was found to transform a competent strain (strain BD413 *trpE* 27) of that group, but DNA from oxidase-positive strains failed to transform this strain.

Acinetobacter strains were therefore seen as a natural group distinct from the genera *Moraxella* and *Neisseria*. Grimont and Bouvet (1991) gave the current definition of the genus Acinetobacter (which was adapted from Juni, 1984) as: "strictly aerobic, non motile, oxidase negative coccobacilli; Gram-negative, but sometimes difficult to destain; grows well on complex media between 20 and 30°C without growth factor requirements; nitrates are rarely reduced; extracted

DNA is able to transform strain BD413 *trpE* 27; the G and C content of the DNA is between 39 and 47mol/% ".

Van Landschoot *et al.*(1986) described the genus *Acinetobacter* as a discrete phylogenetic branch in superfamily II of the Proteobacteria. The closest genera being: *Moraxella, Pseudomonas* (fluorescent group), and *Xanthomonas* (Woese *et al.*, 1985 and Van Landschoot *et al.*, 1986).

Recently, Rossau *et al.* (1991) have proposed a new bacterial family: the *Moraxellaceae* fam. nov. to accommodate the genera: *Moraxella, Acinetobacter* and *Psychrobacter*. Thus finally removing these genera from the family *Neisseriaceae*. Henricksen (1976) felt this split was necessary but perhaps felt that *Acinetobacter* should form a family of its own: **the Acinetobacteriaceae!**

Delineation of species within the genus

Only two species (*A.calcoaceticus* and *A.lwoffii* are on the Approved Lists of Bacterial Names (Skerman *et al.*,1980) and only one species (*A.calcoaceticus*) is described in Bergey's Manual of Systematic Bacteriology (Juni, 1984). However the studies of Baumann *et al.* (1968) and Johnson *et al.* (1970) have shown that the genus is clearly heterogeneous both biochemically and genetically.

Using DNA hybridisation techniques, Bouvet and Grimont (1986) identified 12 hybridisation (genomic) groups. Ten genomic species could be identified by phenotypic tests (including carbon source utilisation).

Subsequent work (using genetic transformation, DNA hybridisation and RNA sequence comparisons) has resulted in the identification of 17 genomic species and 19 biotypes. The major genomic species comprises *A.baumannii* (9 biotypes), *Acinetobacter* sp. 3 (6 biotypes) , *A.haemolyticus, A.johnsonii, A.junii, A.lwoffii,* (emended) *A.calcoaceticus* (emended) *Acinetobacter* sp. 6 and *Acinetobacter* sp. 11 (Grimont & Bouvet, 1991).

Work carried out simultaneously by others (Tjernberg & Ursing, 1989; Nishimura *et al.* 1988b) have corroborated the groupings outlined by Grimont & Bouvet (1986) and Bouvet & Grimont (1991). Phenotypic group 5 has subsequently been named as *Acinetobacter radioresistans* (Nishimura *et al.*, 1988a)

Thus, hopefully, a satisfactory classification of the genus *Acinetobacter* is now at the disposal of microbiologists (Towner *et al.*, 1991).

THE ECOLOGICAL SIGNIFICANCE OF THE NEW TAXONOMIC CHANGES

The whole question of the role of *Acinetobacter* in human infection and the epidemiology of these infections has clearly been hampered by the taxonomic confusion. In conjunction with the new changes described, Grimont and Bouvet (1991) have outlined the different habitats of the new species.

(i) *A.calcoaceticus sensu stricto*

This organism is seldom associated with human infection and its normal habitat is in the soil - where it was originally described by Beijerinck (1911).

(ii) *A.baumannii*

Has so far only been isolated from humans. It is the most commonly isolated *Acinetobacter* in cases of nosocomial infection (Bergogne- Berezin & Joly - Guillou, 1991a). Bouvet & Grimont (1987) found that it comprised of 244 of 291 isolates from 264 cases (84%). The authors made a very important point when they stated that "no habitat other than man is known for this species. Thus, its presence on inanimate objects in the hospital environment can be interpreted as contamination from an infected patient". (Grimont & Bouvet, 1991).

(iii) *Acinetobacter* sp 3

Unlike the French studies this species formed the largest group of clinical isolates studied by Tjernberg & Ursing (1989). It has been found in both soil and clinical samples.

(iv) *A.haemolyticus*

Is only occasionally isolated from patients and the hospital environment.

(v) *A.junii*

Is found both in the environment and in clinical samples.

(vi) *A.johnsonii*

This species can be found in the environment (soil, activated sludge), animal products (chicken, raw milk), the skin of normal people and only rarely from clinical specimens. It is commonly found on the skin (hands) of nurses but also on the skin of non-hospital workers (Bouvet & Grimont, 1987). Since this species is unable to grow at 37°C its isolation from clinical specimens should be treated with suspicion since hand-borne contamination is most likely. Grimont & Bouvet (1991) pointed out that "In an epidemiological investigation, the presence of *Acinetobacter* strains on the hands of personnel is meaningless unless the identification procedure differentiates *A.baumannii* from *A.johnsonii* and the other species".

vii) *A.lwoffii*

Has been isolated from human cases (3-6% of total isolates), from the hands of non infected personnel, eviserated chickens and activated sludge (Bouvet & Grimont, 1987; Duncan *et al.,* 1988).

viii) *A.radioresistens*

Originally found in association with cotton it has also been isolated from patients (Tjernberg & Ursing, 1989).

Concluding, Grimont & Bouvet (1991) made the following important statement: "Accurate identification to the species level of a clinical isolate belonging to the genus *Acinetobacter* is essential since:

(i) identification is a primary epidemiological marker

(ii) habitats may differ between species

(iii) the antibiotic susceptibility of *A.baumannii* compared with other species is quite different (Freney *et al.* 1989)".

CLINICAL PROBLEMS POSED BY ACINETOBACTER

These have been well reviewed by Bergogne - Berezin *et al.* (1987). *Acinetobacter* spp. is a good example of the new group of organisms, previously thought to be of low grade pathogenicity, which have become increasingly important as causes of nosocomial infection, especially in the intensive therapy situation.

The significant problems posed by this species are partly related to its high rates of resistance to most antimicrobial agents. Towner *et al.* (1991) describe Acinetobacters as exhibiting "one of the most impressive patterns of antibiotic resistance found in nosocomial bacteria". Acinetobacter are often resistant to most members of the Beta-lactam and aminoglycoside classes. Bergogne- Berezin & Joly- Guillou (1991b) described resistance of 84% to the Beta-lactams, 64% to the aminoglycosides, 65% to co-trimoxazole and 63% to perfloxacin. The latter resistance to the fluoroquinolones was particularly worrying since they were initially sensitive. They mentioned that in many cases the only effective treatment was imipenem but resistance to even this powerful agent is appearing. In this series 5.5% of their isolates were resistant to all available anitmicrobial agents (Bergogne- Berezin & Joly - Guillou, 1991).

Major Infections caused by Acinetobacter

i) Nosocomial pneumonia (Cunha *et al.,* 1980)

ii) Nosocomial urinary tract infections (Hoffmann *et al.,* 1982)

iii) Others include : meningitis; skin and wound infections; burn wound infections; and bacteriuria (Bergogne - Berezin *et al.,* 1987)

HOSPITAL EPIDEMIOLOGY OF ACINETOBACTER INFECTION

This has suffered from the taxonomic problems and the lack of reliable typing schemes. Noble (1991) has recently reviewed this subject.

Normal carriage

Acinetobacter spp. (presumably *A.baumannii*) are normal inhabitants of the human skin and are found in at least 25% of the normal population in the groins, axillae, toewebs and antecubetal fossae, (Taplin *et al.*,1963 ; Noble,1991). This fact has always led to a debate as to whether isolation of these organisms from blood or wound swabs etc. was indicative of infection or mere contamination.

Role of contaminated equipment and fomites in Acinetobacter infections

A recurrent theme of Acinetobacter nosocomial infection is contaminated equipment (see Table 12).

Table 12. Contaminated equipment and fluids in Acinetobacter infections.

Equipment	Reference
Resuscitators in a baby ITU	Stone & Das (1986)
Wright respirometers	Cunha *et al.* (1980)
Cold water room humidifiers	Smith & Massanari (1977)
Water reservoir in mist tents	Snydman *et al.* (1977)
Bed mattresses	Sheretz & Sullivan (1985)
CAPD bags	Abrutyn *et al.*(1978)
Intrathecal methotrexate / needles	Kelkar *et al.* (1989)
Parenteral nutrition fluid	Ng *et al.* (1989)
Intra-arterial pressure transducers	This thesis

Acinetobacters are known to survive drying better than most other Gram negative bacteria - once dried they may persist for at least 7 days. (Buxton *et al.,* 1978). Therefore airborne spread with contamination of equipment and other patients may play an important role in the natural history of nosocomial infections caused by Acinetobacter. However, as with other nosocomial pathogens, transmission occurs most frequently, directly by the hands of the medical/nursing personnel and indirectly via contaminated equipment.

TYPING SCHEMES FOR ACINETOBACTER

These have recently been comprehensively reviewed by Bouvet (1991). (See Table 13).

Table 13. Typing schemes for Acinetobacter.

Typing scheme	**References**
Biotyping	Towner & Chopade (1987)
	Buisson *et al.* (1990)
Phage typing	Vieu *et al.* (1979)
	Vieu *et al.* (1980)
	Giammanco *et al.* (1989)
Serotyping	Das & Ayliffe (1984)
	Traub (1989)
Cell envelope protein patterns	Alexander *et al.* (1984)
	Dijkshoorn *et al.* (1987 a, b &1989)
	Crombach *et al.* (1989)
	Bouvet *et al.* (1990)
Plasmid profile & plasmid	Gerner-Smidt (1989)
fingerprinting	Villa *et al.* (1989)

Table 13 (contd.)

Typing scheme	References
Antimicrobial resistance patterns	Castle *et al.* (1978)
	French *et al.* (1980)
	Gerner-Smidt *et al.* (1985)
	Buisson *et al.* (1990)
Low-frequency-cleavage restriction endonuclease analysis	Allardet-Servent *et al.* (1989)
Electrophoretic analysis of isoenzymes	Picard *et al.* (1989)

He emphasised that the initial step in the typing of Acinetobacter should be the establishment of which particular species of this genus are "of clinical and epidemiological relevance". Therefore the first step in typing should be the identification of the organism to the species level. He felt that identification to the species level and biotyping using physiological and nutritional properties were "easy and accurate methods for screening strains". He also felt that the investigation of small outbreaks within specific ward areas caused by *A.baumannii* could easily be carried out by using, in addition, cell envelope protein profile typing. But the latter technique was perhaps too laborious for large scale epidemiological studies.

He concluded that: analysis following treatment with low-frequency-cleavage restriction endonucleases, electrophoretic analysis of isoenzymes, or restriction endonuclease analysis of rRNA genes - currently being evaluated - may prove extremely useful epidemiological tools in the future.

THE EVOLUTION OF ANTIMICROBIAL DRUG RESISTANCE IN ACINETOBACTER

I do not intend to discuss this in detail here, since it has been extensively and comprehensively reviewed (including some of the work presented in this thesis) by Professeur E. Bergogne- Berezin (Bergogne- Berezin & Joly- Guillou, 1985; Joly- Guillou *et al.*, 1987 ; Bergogne- Berezin & Joly Guillou, 1991a).

She concluded that although much work had been carried out on the resistance mechanisms of *Acinetobacter* spp., a great deal remains to be investigated. This includes the resistance that has emerged to the fluorquinolones and imipenem. "Problems of outer membrane permeability are suspected, but not yet demonstrated" (Bergogne-Berezin & Joly -Guillou, 1991a).

The role of *Acinetobacter* spp. as a reservoir of resistance genes within hospitals also requires careful study in the future.

This thesis will look particularly at the nature and role of the (presumed) chromosomal Beta-lactamases of the genus *Acinetobacter*.

MATERIALS AND METHODS

BACTERIAL STRAINS

The majority of the clinical isolates of Gram negative aerobic bacilli studied in this thesis were collected from the patients in the Royal Infirmary of Edinburgh (RIE) during the period of January 1986 to December 1987.

The following *Acinetobacter* spp. were obtained from the blood culture collection of the RIE Bacteriology laboratory 75, 5B86, 6B101, 6B230, 6B253, 6B299, 6B324, 6B410 and 7B52. Strains H246, 247, 248, 284, 285 and 286 were kindly donated by the Bacteriology Laboratory of the City Hospital, Edinburgh. Strains H201 through to H243 were kindly donated by the Bacteriology Department of the University of Dundee, Ninewells Hospital.

All strains were maintained at -70°C in nutrient broth with 10% glycerol v/v. Identification of all strains was carried out with the API 20E or API 2ONE system (API System, S.A. France). Details of the individual strains are given in the appendix. The standard bacterial strains used in this thesis are listed in Table 14 Details of the strains producing standard beta-lactamases are shown in Table 15.

Table 14. Standard Bacterial Strains

Bacterial strain	Markers	Reference
NCTC 10418 *E.coli*		
NCTC 10662 *Ps.aeruginosa*		
BRL JT39 *E.coli*		
J62-2 *E.coli*	*pro⁻ his⁻ trp⁻ lac⁻*	Bachmann (1972)

Table 15.Standard β-lactamase producing strains

Bacterial strain	β-lactamase produced	Plasmid	Original reference
E.coli J53-2 2316E	TEM-1	R6K	Hedges *et al.* (1974)
E.coli J53-2 2137E	TEM-2	RP4	Hedges *et al.* (1974)
E.coli J53-2 2141E	SHV-1	R1010-6	Petrocheilou *et al.* (1977)
E.coli J53-2	OXA-1	R455	Dale & Smith (1974)
E.coli J53-2	OXA-2	R46-T^S	Dale & Smith (1974)
Ent.cloacae	P99		Fleming *et al.* (1963)
Kl.pneumoniae 1082E	K1		Marshall *et al.* (1972)
Kl pneumoniae	K14		Matthew & Harris (1976)

INFORMATION STORAGE AND RETRIEVAL

Hood isolates (prefixed by H)

Information concerning each bacterial strain was stored in the database program of dBASE III (Ashton-Tate, Milton Keynes). This system was used for the identification and removal from the survey of repeat isolates from individual patients.

Cefuroxime resistance survey

A hospital wide survey was performed of the incidence of cefuroxime resistance within the population of non-pseudomonal aerobic Gram negative bacilli reported from the clinical bacteriology laboratory, RIE from April 1986 until March 1991. This was obtained from the computer records held in archive disks of a Digital Equipment Corporation (DEC) PDP 11/44 computer which ran as Digital Standard Mumps (DSM) version 4.1. In-house programs were written by Karen Wilson (CSU Lothian Health Board) and A. Bruce Harris (MLSO 3 Dept of Bacteriology) to retrieve the appropriate data. Hard copy was produced to ensure all patient and isolate duplicates were removed.

Antimicrobial usage

This hospital-wide survey of all antimicrobial usage in RIE for the financial year April 1989 to March 1990 was obtained from Mr M. Grieve Principal Pharmacist RIE.

Patient throughput

This hospital wide survey of patient admissions and discharges was obtained from Mr N Pettinger, RIE.

MATERIALS

Antimicrobial Agents

The antimicrobial agents and their suppliers are listed in Table 16.

Table 16. Antimicrobial agents

Compound	**Supplier**
Ampicillin	SmithKline Beecham Pharmaceuticals
Aztreonam	E.R.Squibb &Sons
Benzylpenicillin	Glaxo Laboratories Ltd.
Carbenicillin	SmithKline Beecham Pharmaceuticals
Cefotaxime	Roussel Laboratories Ltd.
Cefoxitin	Merck Sharp &Dohme Ltd.
Ceftazidime	Glaxo Group Research Ltd.
Ceftriaxone	Roche Products Ltd.
Cefuroxime	Glaxo Laboratories Ltd.
Cephaloridine	Glaxo Group Research Ltd.
Cephradine	E.R.Squibb &Sons
Ciprofloxacin	Bayer (UK) Ltd.
Clavulanic acid	SmithKline Beecham Pharmaceuticals
Gentamicin	Nicholas Laboratories
Imipenem	Merck Sharp &Dohme Ltd.
Netilmicin	Schering Plough Ltd
Nitrocephin	Glaxo Group Research Ltd.
Rifampicin	Le Petite

Buffers

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

Media

Sterilisation of Media

All growth media, both agar and broth, were sterilised by autoclaving at 15lbs/in^2 for 15 minutes.

Complex media

The following complex media were used: Nutrient broth No 2 (CM67), Diagnostic Sensitivity Test Agar (DSTA) (CM261), MacConkey agar (CM7) (Oxoid, Basingstoke, Hants).

Minimal medium (DM agar)

Double strength minimal salts medium was prepared as described by Davis and Mingioli (1950) (Table 17).Fifty ml of double strength DM was then supplemented with appropriate amounts of the required amino acid stock solutions to achieve the final concentrations as shown in Table 18 .

Table 17. Preparation of double strength Davis & 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

After appropriate quantities of antibiotics were added 2.5ml of 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 No 1 (1g of agar/40ml of distilled water) and, after gentle mixing, the plates were poured.

Table 18. Amino acid solutions

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

METHODS

Antibacterial drug susceptibilities

Minimum Inhibitory Concentration (MIC) Determination on Solid Media

Serial dilutions of an overnight, nutrient broth culture was made in single strength DM to 10^{-4} . Approximately 1 μl of this was inoculated onto the surface of DSTA plates, containing the appropriate concentrations of antimicrobial, employing a Denley A 400 Multipoint inoculator. The inoculum suspension contained approximately 10^{5} colony forming units per ml (cfu/ml).

Each plate was also inoculated with two control organisms *E. coli* NCTC 10418 and *Ps. aeruginosa* NCTC 10662. A DSTA plate containing no antimicrobial agents was also inoculated as a positive control. Plates were incubated for 18h at 37°C. The MIC was expressed as the first concentration inhibiting all visible growth.

Conjugation experiments

In the 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 and Gould (1984). This mixture was then incubated at 37 ^{O}C for 6h.

E. coli J62-2 transconjugants of the clinical *Acinetobacter* spp. isolated were selected for on DM agar supplemented by histidine (50mg/L), tryptophan (50mg/L) and proline (50mg/L) and containing rifampicin (25µg/ml) and aztreonam (1µg/ml).

Beta-lactamase Preparation

Large scale preparation of crude cell extracts

Cells were grown in 4.5ml of nutrient broth overnight at 37°C and used to inoculate a 1 L culture of nutrient broth and this was shaken (200 osc/mm) at 37°C for 18h. Cells were harvested at 5000g for 15 min at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments) and the pellet was washed in 500ml of 25mmol/L sodium phosphate buffer (pH 7.0) and re-centrifuged at 5000g for 15 min. The pellet was then resuspended in 3ml of sodium phosphate buffer (pH 7.0) and the cells were disrupted by ultrasonication with constant cooling (8µM, 1min x 3, MSE Soniprep 150, MSE Instruments,Crawley).

The cell debris was removed by centrifugation at 38,000g for 15 min at 4°C (Sorvall).The supernatant, crude Beta-lactamase preparation, could then be stored at -20 ^{O}C until required.

Small scale preparation of crude cell extracts

Small quantities of Beta-lactamases were prepared by the method described by Livermore *et al.* (1984).Cultures were grown on 2ml nutrient agar slopes which were incubated for 18h at 37°C. A cell suspension was obtained by the addition of 1ml of sodium phosphate buffer (pH 7.0, 25 mM) to the agar surface coupled with gentle agitation. The cells were then disrupted at 4°C by ultrasonication (8μM, 30s, Soniprep). The cell lysate was cleared employing a MSE Microcentaur centrifuge (11,500g, 10min, 4°C). The cleared lysate was stored at -20 ^{O}C until required.

Assessment of Beta lactamase activity of Beta lactamase preparations

Thirty μL of the beta-lactamase extract was mixed with 100 μL of a nitrocephin solution (50 mg/L) in a microtitre tray. The time taken in seconds for the mixture to change from yellow to red was taken as an indication of the Beta-lactamase activity of the enzyme preparation. The amount of Beta-lactamase added to an IEF gel in (μL) was equal to the time taken (in seconds) for this colour change to take place. In each gel up to 100μL was employed as the sample volume.

Beta-lactamase Enzyme Induction

The method employed was that described by Minami *et al.* (1980). Cefoxitin, a well documented inducer of Beta-lactamase production, was added to the growing culture at one quarter of the MIC value for that particular strain.Beta-lactamase enzymes were extracted as previously described for large-scale preparation of crude enzyme extracts.

Conventional analytical isoelectric focusing of Beta-lactamases

Gel preparation

Beta-lactamases were identified (if possible) by analytical isoelectric focusing as described by Matthews *et al.* (1975). The extracts were focused on a thin layer polyacrylamide gel containing carrier ampholines (pH 3.5 - 10.6, LKB). The composition of the gel is shown in Table 19 .

Table 19. Composition of a conventional IEF gel

Material	Supplier	Volume employed (ml)	Final concentration
5% 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 300ml distilled water.	BDH	9.0	acrylamide: 75g/L bisacrylamide: 2g/L
Distilled water.		25.0	
Riboflavin (20mg/L).	Sigma	4.0	2mg/L

* TEMED : tetramethyl-ethylenediamine

Running conditions

Samples of the enzymes (up to 100μL) were applied, about 2cm from the anode, onto the gel surface. The amount added in (μL) being equal to the time taken (in seconds) for a mixture of 30μL of Beta-lactamase preparation and 100μL of nitrocephin solution (50mg/L) to change from yellow to red.

Isoelectric focusing was performed at $4^{O}C$ at 1W (constant), 550V (limiting) and 20 mA (limiting) for 18h. Five μL of isoelectric point (pI) standard markers (BDH) were also spotted on the gel in order to quantify the pI gradient

Staining

Polyacrylamide gels were stained by repeatedly overlaying the surface with a sheet of Whatman No 54 paper which had been dipped in nitrocephin solution (500mg/L). The focused bands of Beta-lactamase activity appeared red on a yellow background.

Photography

Serial photographs of the IEF gels were taken after treatment with the nitrocephin solution. Kodak Ortholith film (35mm) with transmitted hight and a Wratten 58 green filter was used initially. Subsequently a polaroid film system with a green filter was employed.

SDS-free PAGE

The apparatus and discontinuous buffer system employed was essentially that described by Laemmli (1970) except that it did not contained SDS.

Buffers

(i) electrode buffer:

0.025M Tris, 0.192M Glycine

(ii) Separating gel buffer: (double strength)

0.75M Tris/HCl, pH 8.8

(iii) Stacking gel buffer: (double strength)

0.25M Tris/HCl, pH 6.8

(iv) Sample buffer: (double strength)

0.0625M Tris.HCl, pH 6.8

2%, β-mercaptoethanol

20% glycerol

0.002% bromophenol blue

Gel preparation

A separating gel of 12% acrylamide with a stacking gel of 4% acrylamide were employed. The gel mixtures were prepared as in Tables 20 and 21.

Table 20. Composition of discontinuous buffer separating gel

Solutions	**Volume (ml) per gel**
Distilled water	5.2
0.75M Tris/HCl,pH 8.8	17.5
40% acrylamide (stock) *	10.5
Ammonium persulphate (15mg/ml)	1.75
TEMED	0.05

Table 21. Preparation of stacking gel

Solution	Volume (ml) per gel
Distilled water	3.5
0.25M Tris/HCl pH 6.8	5.0
40% acrylamide (stock) *	1.0
Ammonium persulphate (15mg/ml)	0.5
TEMED	0.02

40% acrylamide stock solution *

40g acrylamide and 1.08g methylbisacrylamide dissolved in a total volume of 100ml of distilled water.

Sample preparation

An equal volume of sample was added to double strength sample buffer which contained:

0.0625M Tris/HCl pH 6.8

2%, β-mercaptoethanol

20% glycerol

0.002% bromophenol blue

Running conditions

See appropriate results section.

Molecular size (M_r) Estimation by gel filtration

This was carried out by employing the method of Andrews (1964).

Preparation of G-75 and Sephacryl S-300 columns

The media (Sephadex G-75 or Sephacryl S-300, Pharmacia) was swollen with the appropriate volume of 25mM sodium phosphate buffer (pH 7.0) at 100°C for 1h. The slurry was allowed to cool and then carefully poured into a Pharmacia gel filtration column (2cm^2 x 90cm). When the column was full, the top was connected and the flow commenced in an upward direction with a LKB peristaltic pump. The gel was then allowed to equilibrate for 48h with 25mM sodium phosphate buffer (pH 7.0). Following this equilibration period a flow rate of 15ml/h was attained by adjusting the speed of the peristaltic pump.

Calibration of G-75 and Sephacryl S-300 columns

(i) G-75

Prior to the estimation of a Beta-lactamase of unknown M_r it was calibrated with proteins of known molecular size (see Table 22).

Table 22. Protein standards (10mg in 1ml buffer)applied to G-75

Protein standard	Molecular size (Kd)
Cytochrome C	12.4
chymotrypsinogen	25.5
ovalbumin	45.0

After the application of the standard proteins, 100 fractions were collected by an Ultrorack fraction collector(LKB). The fraction size was set to

collect 2ml of eluate. The absorbance of each fraction was measured at 280nm to give an estimate of its protein concentration. The position of the peaks of the protein markers was then established by plotting the OD of the fractions against the fraction number (Waddell, 1956). A standard curve of M_r versus fraction number could then be drawn.

(ii) Sephacryl S-300

This column was calibrated with the following protein standards (see Table 23).

Table 23. Protein standards (10mg in 1ml buffer) applied to S-300

Protein standard	Molecular size (Kd)
ovalbumin	45.0
alcohol dehydrogenase	150.0
apoferritin	443.0

Determination of the Mr of Beta-lactamases

1ml of crude enzyme preparation, prepared as described previously, was applied to the column. The settings of the peristaltic pump and fraction collector were maintained at the same parameters as those employed during the calibration procedure. One hundred fractions of 2ml volumes were eluted from the column after the application of the sample.

The Beta-lactamase peak was first identified by each fraction for activity with the chromogenic cephalosporin, nitrocephin. Thirty µL of each fraction were added to 100µL of nitrocephin solution (50mg/L) in a microtitre plate. Those which showed a rapid colour change (<120s) from yellow to red indicated the presence of beta-lactamase activity.

These fractions were then further assayed by the spectrophotometric method O'Callaghan *et al.*(1972) to determine the peak beta-lactamase activity. If the activity appeared in the void volume of the G-75 column another 1ml of crude preparation was added to a similar column containing Sephacryl S-300 and the peak fractions determined as before. The M_r was then determined from the standard curve.

Fractions containing significant Beta-lactamase activity were pooled and used for the spectrophotometric analyses described in the next section and were also used to load the isoelectric focusing gels.

Spectrophotometric Assay of Beta-lactamase Activity

The basic method employed for spectrophotometric assay was that of O'Callaghan *et al.*(1972). For all spectrophotometric assays either a Pye Unicam SP1800 uv/vis spectrophotometer or a Perkin Elmer Lambda 2 Spectrometer was employed. Both instruments had themostatically controlled cell carriers and all measurements were performed at 37 ^{o}C.

Penicillin and cephalosporin substrates were prepared at $10^{-2}M$ and $10^{-3}M$ respectively in 25mM sodium phosphate buffer (pH 7.0).

Test and blank cuvettes were prepared and equilibrated at 37°C. The test cuvette contained 0.3ml substrate and 2.6ml sodium phosphate buffer (25mM, pH 7.0). The blank cuvette contained 2.9ml buffer. Beta-lactamase (0.1ml) was added to each cuvette and monitoring of the decrease in optical density (OD) at the appropriate λ max, was begun immediately. Kinetic measurements were performed on Beta-lactamases which had been partially purified by gel filtration.

The initial linear part of the reaction curve was used to obtain a value for change in OD minute $^{-1}$.

The rate (R) of the reaction was calculated according to the equation

$$R = \frac{\Delta OD \times N \times (\text{enzyme dilution})}{OD_1 \times \text{time}}$$

where R = μ moles of substrate hydrolysed minute $^{-1}$ ml $^{-1}$ enzyme

ΔOD = change in optical density

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

OD_1 = optical density of intact substrate.

Specific Activities

Rates for individual Beta-lactamases were calculated at 10^{-2}M for penicillins and 10^{-3}M for cephalosporins. Protein concentrations were determined by the method of Waddell (1956) and specific activities were expressed as pico-moles of substrate hydrolysed per minute per mg of protein (ρ moles/ min/mg).

If no activity was detectable at a concentration of 10^{-3}M against the following substrates: aztreonam, ceftazidime. cefotaxime and cefuroxime, they were repeated at a concentration of 10^{-2}M. This was carried out employing the following method. Each test cuvette contained 2.6ml sodium phosphate buffer (25mM, pH 7.0), 0.3ml of substrate (10^{-1}M) and 0.1ml of test Beta-lactamase. Each blank cuvette contained 2.9ml of buffer and 0.1ml of test Beta-lactamase. Each reaction mixture was diluted 1:100 prior to measurement of the OD at the appropriate wavelength. The reaction mixtures were then incubated at 37°C in a waterbath and the OD of 1:100 dilutions made at intervals up to 18h later.

Determination of Michaelis Menton Kinetics

The K_m and V_{max} values were obtained by measuring the rate of hydrolysis at limiting substrate concentrations and plotting the reciprocal of the substrate concentration against the reciprocal of the rate by the Lineweaver - Burk (1934) method.

The V_{max} values were normalised with respect to nitrocephin i.e.,

$$\text{relative } V_{max} = \frac{V_{max} \text{ (per } \mu L \text{ enzyme) of substrate}}{V_{max} \text{ (per } \mu L \text{ enzyme) of nitrocephin}} \times 100$$

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 described above (O'Callaghan *et al.*1972). Firstly, the rate of hydrolysis of nitrocephin (at 10^{-4}M) by the Beta-lactamase was measured. This procedure was then repeated in the presence of 10^{-8}M of the potential inhibitor and then with increasing concentrations of this inhibitor until inhibition approached 100%.

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

Inhibition Studies with Clavulanic acid

In these studies the enzyme and clavulanic acid were pre-incubated together for 5 minutes prior to the addition of the nitrocephin substrate.

Method for the preparation of the cell membrane fraction of *Acinetobacter baumannii* (Strain H68)

This was carried out by the methods described previously by Hills and Fewson (1983) and Allison *et al*. (1985 a and b).

Growth of bacteria

A loopful of stock culture was used to inoculate 50ml of complex media. the composition of which is shown in Table 24. This culture was incubated, statically at 30°C overnight. The resultant culture was then used to inoculate 400ml of complex media which was incubated overnight at 30°C with shaking (120 rev/min). This in turn was used to inoculate 4L of complex media in a 10L

fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury, Glos., UK). The fermenter was operated at 30°C with an aeration rate of 4L of sterile air/min with stirring set at of 2.5 (approx 350 rev/min). After 18h growth the bacteria were harvested.

Table 24. Composition of Complex media

Ingredient	Manufacturer	Quantity dissolved in 1L(g)
Oxoid nutrient broth no.1	Oxoid	26
Lactic acid	BDH	1.5
L-glutamate HCl	SAS	0.9
KH_2PO_4	BDH (Analar)	2.0
$(NH_4)_2SO_4$	BDH (Analar)	1.0
$MgSO_47H_2O$	BDH (Analar)	0.4

The pH was adjusted to 7.0 using 5M NaOH, then made up to 1L with distilled water. It was sterilised by autoclaving at 15lbs/in^2 for 15 min. Polypropylene glycol was added to a concentration of 0.005% when the above media was used in the fermenter.

Harvesting

The cells were harvested by centrifugation (MSE 18) at 6000g for 20 min at 4°C, washed by resuspension in ice-cold phosphate buffer (pH 7.5, 40mM) and recentrifuged at 12,000g for 30 min at 4 ^{o}C.

Disruption

The cells were disrupted either by passage through a French pressure cell or by ultrasonication.

i) French pressure cell

20g of wet cells were resuspended in 20ml of ice-cold phosphate buffer and disrupted by four passages through a cooled French pressure cell (95MPa). The broken cell suspension was centrifuged (MSE18) at 12,000g for 30 min at 4 ^{o}C and resuspended in ice-cold phosphate buffer.

ii) Ultrasonication

The cells were resuspended in 25ml of ice-cold phosphate buffer to give an A_{500} of 100-200. This suspension was disrupted (with cooling) by 5 cycles of ultrasonication each of 30sec at 80W. The broken cell suspension was treated by centrifugation of 12,000g for 30 min at 4 ^{o}C.

The resulting preparations produced by both methods were stored overnight at 4 ^{o}C.

Preparation of washed cell membranes

The suspensions obtained from the previous steps were centrifuged (Beckman L5-65) at 113,000g for 150 min at 4°C ,then the precipitate was resuspended in ice-cold phosphate buffer and recentrifuged. This procedure was

repeated once more (giving 3 high-speed spins in total) and the resultant pellet was regarded as washed cell membranes.

Estimation of Beta-lactamase activity

The Beta-lactamase activity at various points of the purification procedure was assayed by the spectrophotometric method previously described, employing nitrocephin as the substrate.

Protein assay

Protein concentrations were measured by the procedure of Lowry *et al.* (1951).

RESULTS

SCREEN FOR CEFUROXIME RESISTANCE

During the two year period between January 1986 and December 1987 inclusive, 400 strains of Gram negative aerobic bacilli, that were oxidase-negative, and showed resistance on disc testing by the Stokes' Method to cefuroxime (30μg) were collected. All strains were identified by AP1 20E or AP1 2ONE. These strains were given "H" numbers and are listed in Appendix I. Three hundred and fifty one strains originated from the RIE, 43 from the Bacteriology Laboratory, Ninewells Hospital, Dundee and 6 from the Bacteriology Laboratory, City Hospital, Edinburgh.

The original aims were threefold. Firstly to compare the cefuroxime resistance in these strains to their sensitivity/resistance to eleven other antimicrobial agents by carrying out MIC estimations.

Secondly to elucidate the role of Beta-lactamases in the resistance of these strains to cefuroxime.

Thirdly to attempt to relate the cefuroxime resistance to particular ward areas of the RIE.

MIC ESTIMATIONS

These were carried out on all 400 strains. The antimicrobials were:

Ampicillin	as an example of a common penicillin
Cephradine	as an example of a first generation cephalosporin
Cefuroxime	as an example of a second generation cephalosporin
Cefoxitin	as an example of a second generation cephalosporin
Cefotaxime	as an example of a third generation cephalosporin
Ceftazidime	as an example of a third generation cephalosporin
Ceftriaxone	as an example of a third generation cephalosporin
Aztreonam	as an example of a monobactam

Imipenem	as an example of a carbapenem
Ciprofloxacin	as an example of a quinolone
Gentamicin	as an example of an aminoglycoside
Netilmicin	as an example of an aminoglycoside

The MIC_{50}, MIC_{90}, MIC range and the percentage resistance (employing break points as described by the Working Party of the BSAC, 1991) to each of the 12 antimicrobials is seen in Table 25.

The following should be noted:

i) The overall resistance to cefuroxime was around 68.5% and cefoxitin resistance was very similar, at 69%.

ii) Resistance to ampicillin and cephradine was almost complete at 96% and 92%, respectively.

iii) The third generation cephalosporins cefotaxime and ceftriaxone had similar resistance figures of 57.8% and 54%, respectively, whereas the 22.3% resistance of ceftazidine was significantly better in this population.

iv) Surprisingly perhaps aztreonam had a lower rate of resistance than the 3rd generation cephalosporins -19%.

v) This was comparable with the resistance of 21% and 17.8% to the aminoglycosides gentamicin and netilmicin, respectively.

vi) As one would expect the resistance to the two newest drugs: (neither on the market at the time) imipenem and ciprofloxacin were the lowest at 2.8% and 3%, respectively.

Table 25

MIC values estimated for 400 strains of Gram negative, oxidase-negative, aerobic bacilli
(% resistance calculated with breakpoints suggested by the Working party of the BSAC, 1991)

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	>256	>256	64	64	1.0	2.0	2.0	1.0	1.0	<0.06	0.5	0.5
MIC_{90}	>256	>256	>256	256	32	32	16	8.0	4.0	0.5	32	8.0
RANGE	<0.12- >256	2->256	<0.12- >256	<0.12- >256	<0.12- >256	<0.12- 128	<0.12- 128	<0.12- 64	0.12- >256	<0.06- 16	<0.12- >256	<0.12- >256
%R	96	92	68.5	69	19	57.8	54	22.3	2.8	3	21	17.8
B/P	8	8	16	16	8	1	1	2	4	1	4	4

%R = percentage resistance
B/P = break point (mg/L)
MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

CEFUROXIME-RESISTANT STRAINS (MIC $\geq$ 32mg/L)

Out of the 400 initial strains a group of 200 strains was delineated. These were all clearly resistant to cefuroxime (MIC$\geq$32mg/L) and excluded any strains that had originated from outside the RIE and any strains of *Pseudomonas* or *Xanthomonas* (see Appendix II).

Their MIC_{50}, MIC_{90}, MIC range and percentage resistance (to a specific breakpoint) is given to the twelve antimicrobials in Table 26.

Therefore as one might have expected the resistance to ampicillin and cephradine in this population of cefuroxime resistance strains was almost 100%. Cefoxitin, the other second generation cephalosporin, was much the same as cefuroxime resistance at 84%. Aztreonam, cefotaxime and ceftriaxone all had significantly better levels of resistance at 25.5%, 24% and 29% respectively. The resistance to ceftazidime was slightly greater, at 35% reflecting the removal of ceftazidime-sensitive strains of *Pseudomonas* spp. from the original 400 strains.

Imipenem and ciprofloxacin, again, came out best with resistance of only 1% and 2% of strains, respectively.

The aminoglycosides showed similar resistance rates (to each other) of 26.5% for gentamicin and 19.5% for netilmicin. Table 27 shows the proportion of different bacterial species within this group of 200 strains.

The resistances to the 12 antimicrobials in the five major species are shown in Tables 28 to 32.

Table 26

MIC values estimated for the 200 strains with clear resistance to cefuroxime

(% resistance calculated with breakpoints suggested by the Working party of the BSAC, 1991)

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	>256	>256	128	64	4.0	8.0	4.0	2.0	1.0	<0.06	0.5	0.5
MIC_{90}	>256	>256	>256	256	64	32	16	8.0	4.0	0.5	32	8.0
RANGE	8->256	32- >256	32- >256	0.5- >256	<0.12- >256	<0.12- 128	<0.12- 128	<0.12- 64	0.25-8	<0.06-4	<0.12- 128	<0.12- 128
%R	99.5	100	100	84	25.5	24	29	35	1	2	26.5	19.5
B/P	8	8	16	16	8	1	1	2	4	1	4	4

%R = percentage resistance
B/P = break point(mg/L)
MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

Table 27 Proportion of different bacterial species within the 200 cefuroxime - resistant strains.

Species	Number
Enterobacter spp.	100
Acinetobacter spp.	43
Serratia spp.	24
Klebsiella spp.	11
Citrobacter spp.	9
Escherichia coli	3
Morganella spp.	4
Proteus / Providencia	3
Hafnia alvei	2
Erwinia spp.	1
Total	200

Table 28

MIC values estimated for the 100 strains of *Enterobacter* spp.

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	>256	>256	256	128	2.0	8.0	4.0	2.0	1.0	<0.06	0.25	0.5
MIC_{90}	>256	>256	>256	>256	8.0	32	16	8.0	2.0	0.12	16	8.0
RANGE	256->256	32->256	32->256	4->256	<0.12-16	0.25-128	<0.12-128	<0.12-32	0.5-8.0	<0.06-4.0	<0.12-64	<0.12-16
%R	100	100	100	86	4	85	80	41	1	1	36	26
B/P	8	8	16	16	8	1	1	2	4	1	4	4

%R = percentage resistance
B/P = break point (mg/L)
MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

Table 29
MIC values estimated for the 43 strains of *Acinetobacter* spp.

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	64	256	64	64	64	16	16	2.0	0.5	0.5	1.0	2.0
MIC_{90}	128	>256	128	128	128	32	32	8	1.0	1.0	32	8
RANGE	32- >256	128- >256	32- >256	16- >256	0.25- >256	1.0-64	1.0-64	<0.12- 64	0.5-1.0	0.12- 4.0	<0.12- 128	0.25- 128
%R	100	100	100	97.7	97.7	97.7	97.7	41.9	0	7	14	11.6
B/P	8	8	16	16	8	1	1	2	4	1	4	4

%R = percentage resistance
B/P = break point (mg/L)
MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

Table 30

MIC values estimated for the 24 strains of *Serratia* spp.

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	128	>256	256	16	<0.12	0.25	<0.12	<0.12	1.0	0.12	0.5	2.0
MIC_{90}	>256	>256	>256	64	0.5	4.0	2.0	0.5	4.0	1.0	1.0	2.0
RANGE	8->256	64->256	32->256	8->256	<0.12-32	<0.12-16	<0.12-16	<0.12-2.0	0.5-4.0	0.12-1.0	0.5-1.0	0.25-4.0
%R	95.8	100	100	50	4.2	29.2	37.5	0	0	0	4.2	0
B/P	8	8	16	16	8	1	1	2	4	1	4	4

%R = percentage resistance
B/P = break point (mg/L)
MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

Table 31

MIC values estimated for the 11 strains of *Klebsiella* spp.(numbers insufficient to give percentage resistance).

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	>256	64	64	1.0	4.0	0.25	1.0	<0.12	1.0	<0.06	16	4.0
MIC_{90}	>256	>256	128	64	32	16	16	4.0	1.0	0.25	64	16
RANGE	64- >256	64- >256	32- >256	0.5-64	0.25-32	<0.12- 64	0.25-32	<0.12- 32	0.25- 1.0	<0.06- 0.5	0.25-64	0.5-16

MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin

CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

Table 32

MIC values estimated for the 9 strains of *Citrobacter* spp.(numbers insufficient to give percentage resistance).

	AMP	CED	CXM	CFX	AZ	CTX	CTR	CAZ	IMI	CIP	GENT	NET
MIC_{50}	>256	>256	64	128	4.0	4.0	4.0	4.0	1.0	<0.06	0.25	0.5
MIC_{90}	>256	>256	256	256	8.0	8.0	16	16	2.0	<0.06	0.5	8.0
RANGE	>256	64->256	64->256	4->256	<0.12-16	<0.12-8.0	<0.12-16	<0.12-32	0.5-2.0	<0.06-0.12	0.25-32	0.25-8.0

MIC = minimum inhibitory concentration (mg/L)

AMP = ampicillin
CED = cephradine
CXM = cefuroxime
CTR = ceftriaxone
CAZ = ceftazidime
GENT = gentamicin
CFX = cefoxitin
AZ = aztreonam
CTX = cefotaxime
IMI = imipenem
CIP = ciprofloxacin
NET = netilmicin

The following are worth noting:

1. All species were essentially resistant to **ampicillin**, **cephradine** and **cefuroxime**.

2. **Cefoxitin**: resistance to this antibiotic varied from 97.7% in *Acinetobacter*, 86% in *Enterobacters* to 50% in *Serratias*.

3. **Aztreonam** : in *Enterobacters* and *Serratias* this was a useful drug with only 4% and 4.2% resistance respectively. However in *Acinetobacter* there was resistance in 97.7% of the strains tested.

4. **Cefotaxime and Ceftriaxone** : in *Enterobacters* resistance was 85% and 80%. In *Acinetobacters* both were 97.7% (as with aztreonam). There was a lower resistance in *Serratias* at 29.2% and 37.5%, though this was significantly higher than aztreonam (4.2%).

5. There was generally a lower resistance to **ceftazidime** than to cefotaxime and ceftriaxone, 41% in *Enterobacters* ; 41.9% in *Acinetobacters* and 0% in*Serratias*. This was better than aztreonam in *Acinetobacter* and *Serratia* but worse than aztreonam in *Enterobacters*.

6. **Imipenem** was by far the most active Beta-lactam with only 1% resistance in *Enterobacters* ; none in either *Acinetobacters* or in *Serratias*. All the Klebsiellas and Citrobacters had MIC's within the sensitive range too.

BETA-LACTAMASES PRODUCED BY THE CEFUROXIME-RESISTANT POPULATION

The Beta-lactamases produced by the 200 strains were identified by analytical isoelectric focusing (IEF) of small-scale crude enzyme extracts.

Comparisons with standard marker enzymes enabled identification of the Beta lactamases as to whether they were of plasmid-mediated or probable chromosomal origin. The Beta-lactamases detected in these strains are listed in Appendix III.

Distribution of Beta-lactamases

The distribution of these enzymes within different bacterial species is shown in Table 33.

Enterobacter spp.

Forty-eight strains produced an enzyme of typically chromosomal origin. Four strains produced the plasmid-mediated enzyme TEM-1. Forty-eight strains produced a typically chromosomal enzyme and TEM-1. The 96 chromosomal enzymes produced by the *Enterobacters* are for convenience described as either having a pI the same as P99, >P99 or <P99. In fact at least 5 distinct pI's for *Enterobacter* chromosomal enzymes were seen. In two strains, two typically chromosomal enzymes were seen (P99 and >P99). This has previously been noted by Medeiros (1987). Fifty-two *Enterobacter* strains produced the TEM-1 plasmid mediated Beta-lactamase.

Acinetobacter spp.

All 43 strains produced a typically chromosomal enzyme. No plasmid mediated enzymes were detected. Only one strain (H126) produced a

Table 33 Results of the analytical isoelectric focusing of the 200 cefuroxime-resistant strains - β-lactamases produced.

Species	Chromosomal β-lactamase alone		Plasmid β-lactamase alone		Chromosomal & plasmid β-lactamases	
	β-lactamase	*number*	*β-lactamase*	*number*	*β-lactamases*	*number*
Enterobacter spp.	P99	23	TEM-1	4	P99+TEM-1	41
	>P99[1]	23			>P99+TEM-1	5
	<P99[2]	1			P99+>P99 +TEM-1	2
	P99+>P99	1				
Acinetobacter spp.	chromosomal	43	0	0	0	0
Serratia spp.	chromosomal	23	TEM-1	1	0	0
Klebsiella spp.	K14	7	TEM-1	1	K1+TEM-1	1
	other chromosomal	1			other chromosomal	1

[1] pI of chromosomal β–lactamase higher than P99

[2] pI of chromosomal β–lactamase lower than P99

Table 33 (contd.)

Results of the analytical isoelectric focusing of the 200 cefuroxime-resistant strains - β-lactamases produced.

Species	Chromosomal β-lactamase alone		Plasmid β-lactamase alone		Chromosomal & plasmid β-lactamases	
	β-lactamase	*number*	*β-lactamase*	*number*	*β-lactamase*	*number*
Citrobacter spp.	chromosomal	7	TEM-1	2	0	0
Morganella spp.	chromosomal	3	0	0	chromosomal +TEM-1	1
Proteus/ Providencia	chromosomal	3	0	0	0	0
E.coli	chromosomal	3	0	0	0	0
Hafnia alvei	0	0	0	0	chromosomal +TEM-1	1
					chromosomal +SHV-1	1
Erwinia spp.	chromosomal	1	0	0	0	0

Beta-lactamase on isoelectric focusing with a discernable pI. The rest produced a blur of activity near the cathode.

Serratia spp.

Twenty-three strains produced a typical chromosomal enzyme and only one strain produced the plasmid mediated TEM-1, alone.

Klebsiella spp.

Of the 11 strains, eight produced a typical chromosomal enzyme while two produced a typical chromosomal enzyme and TEM-1. Only one produced TEM-1 alone.

Citrobacter spp.

Seven produced a typical chromosomal enzyme and two produced the TEM-1 Beta-lactamase alone.

Morganella spp.

Three produced chromosomal enzymes alone while one produced a chromosomal enzyme and the TEM-1 Beta-lactamase.

Proteus/Providencia spp.

All three produced chromosomal enzymes alone.

Escherichia coli

All three produced chromosomal enzymes alone.

Hafnia alvei

One strain produced a chromosomal enzyme and the TEM-1 Beta-lactamase. One strain produced a chromosomal enzyme and the SHV-1 Beta-lactamase.

Erwinia spp.

One strain produced a chromosomal enzyme alone.

Therefore in summary 192 strains produced a typical chromosomal Beta-lactamase. Of these 192 strains, 139 produced a typical chromosomal enzyme alone; 52 produced a typical chromosomal enzyme plus the TEM-1 Beta-lactamase and one produced a typical chromosomal enzyme and the SHV-1 Beta-lactamase. Eight strains produced a TEM-1 Beta-lactamase alone.

It is therefore most likely that the resistance to cefuroxime in most of these strains resulted from the presence of a "typical" chromosomal Beta-lactamase. However conjugation experiments should be performed on all these strains to at least partially corroborate this statement .

TRANSFERABLE THIRD GENERATION CEPHALOSPORIN RESISTANCE

In collaboration with Dr D J Payne 54 of 79 strains (from the original collection of 400 strains - excluding *Acinetobacter* spp) which had MIC's to ceftazidime, cefotaxime or ceftriaxone of $\geq$4mg/L were investigated for the presence of transferable 3GC resistance. The Beta-lactamases produced by these 54 strains were examined by analytical isoelectric focusing and they were tested for transferable 3GC resistance by conjugation experiments as described in the Materials and Methods.

There was no evidence that any of the 54 strains conferred transferable 3GC resistance to either ceftazidime or cefotaxime. There was no evidence of any of the recently reported TEM or SHV derived Beta-lactamases which confer 3GC resistance in these strains.

Consequently the 3GC resistance conferred by the strains in this survey most probably resulted from the production of "typical" chromosomal cephalosporinases.

CEFUROXIME RESISTANCE WITHIN DIFFERENT AREAS OF THE ROYAL INFIRMARY OF EDINBURGH

One of the original aims of this thesis was to attempt to relate the resistance levels of cefuroxime to particular units within the RIE. Anecdotal evidence suggested that resistance to cephalosporins was higher in certain ward areas and it seemed lower in areas where aminoglycosides were still used frequently. None of these "observations" were based in hard scientific evidence.

Therefore initially it was thought that the results from the survey of 400 strains of "supposed" cefuroxime resistance might shed some light on this problem. However it became quite clear that these 400 strains (subsequently more clearly defined as 200 strains with cefuroxime resistance of $\geq$ 32mg/L) could not be used to investigate the relationship between ward area and cefuroxime resistance, for two main reasons.

Firstly the numbers were too small to attain statistical significance and secondly the 200 strains did not show a true reflection of the numbers of resistant organisms in any given area. This was due to sampling error. Of the three main laboratory groupings within the department, only one area consistently kept cefuroxime-resistant strains that could be included in the study. This was revealed by running a computer program over a period of 1 year (1986-1987) which picked out the number of cefuroxime resistant strains (duplicates excluded) that were reported by the laboratory during that period by ward area. This was expressed as a percentage resistance.

Another program was devised which gave the number of organisms - duplicates excluded - that were reported as cefuroxime-sensitive. The organisms included in this computer search were Gram negative, aerobic, oxidase-negative bacilli escluding *Pseudomonas* spp.

This initial computer search not only showed the sampling error within the original 400 (and 200) survey strains but also provided the first clear evidence of differences in resistance between areas within the RIE.

It was therefore decided to look at both cefuroxime and gentamicin resistances within 18 distinct patient areas of the RIE over a longer time period. These ward areas could also be grouped under three major headings - namely the Surgical hospital, the Medical hospital and the Intensive therapy areas.

The following should be noted:

(i) the isolates were collected between 1 April 1986 and 31 March 1989

(ii) all patient duplicates were removed

(iii) cefuroxime and gentamicin sensitivities were estimated by laboratory staff using the modified Stokes' method (Pearson & Whitehead, 1974). The cefuroxime disc contained 30 μg and gentamicin contained 10 μg of each drug, respectively

(iv) strains were designated as either **Sensitive** or **Resistant**

(v) any group of resistant isolates that appeared to be of the same resistance phenotype and part of an epidemiologically distinct outbreak were counted as a single isolate

(vi) for logistical reasons it was not possible to check by hand the numbers of sensitive isolates for duplicate patients missed by the computer search. Therefore the numbers of sensitive isolates *may be* an overestimate. However this meant that the final figures of gentamicin and cefuroxime resistance is (if anything) an *underestimate*

Table 34 shows the resistance figures for the initial 3 year period (1986-1989) for both cefuroxime and gentamicin. These are shown both as an overall resistance

rate for the hospital and also broken down into rates for the Surgical, Medical and Intensive care areas.

Table 34 Cefuroxime and gentamicin resistance rates within the general hospital population isolated between 1986-1989.

Area	Proportion resistant	Percentage resistance(%)
CEFUROXIME RESISTANCE		
Surgical hospital	511/4600	11.1
Medical hospital	289/2577	11.1
ITU areas	270/976	27.7
Overall	1070/8153	13.1
GENTAMICIN RESISTANCE		
Surgical hospital	88/4635	1.9
Medical hospital	78/2512	3.1
ITU areas	40/894	4.5
Overall	206/8041	2.6

The overall resistance to cefuroxime in the hospital was 13.1% (1070/8153); in the Surgical hospital 11.1% (511/4600); in the Medical hospital 11.1% (289/2577) and in the Intensive therapy areas 27.7% (270/976).

The resistances within the Surgical and Medical hospitals are almost identical but within the ITU's it is more than twice these rates.

The overall gentamicin resistance in the hospital was 2.6% (206/8041); in the Surgical hospital 1.9% (88/4635); in the Medical hospital 3.1% (78/2512) and in the Intensive therapy areas 4.5% (40/894). Therefore the gentamicin resistances were very much the same in each area, if slightly higher within the ITU areas.

However the rate of gentamicin resistance was, in all areas, significantly lower than the cefuroxime resistance.

To estimate the validity of these resistance patterns obtained by the Stokes' method, we decided to compare them with the resistance patterns in the blood culture isolates, the same organism types and from the same ward areas.

The blood culture isolates had their MIC's to cefuroxime and gentamicin estimated and those with MIC's of $\geq$ 32mg/L for cefuroxime and $\geq$ 4mg/L for gentamicin were considered resistant to these agents. This work was carried out by Mr R Paton. Table 35 shows a comparison of the resistance rates between the blood culture and general isolates (1986-1989) by hospital area for both cefuroxime and gentamicin.

The resistance to cefuroxime within the two groups of isolates (general and blood culture) is remarkably similar.

The resistance to gentamicin within the two groups of isolates was also similar, particularly within the ITU area.

Within the Medical and Surgical hospitals the blood culture isolates were a little more resistant than in the general population of isolates but within the ITU areas the resistance in the blood culture and general isolates were similar.

Table 35 Comparison of the resistance rates between the general and blood culture isolates (1986-1989) by hospital area for both cefuroxime and gentamicin

Area	General isolates	Blood culture isolates
CEFUROXIME	Percentage resistant(%)	Percentage resistant(%)
RESISTANCE	n=8153	n=460
Surgical hospital	11.1	10.7
Medical hospital	11.1	11.2
ITU areas	27.7	21.5
Overall	13.1	12.8
GENTAMICIN	Percentage resistant(%)	Percentage resistant(%)
RESISTANCE	n=8014	n=460
Surgical hospital	1.9	3.3
Medical hospital	3.1	5.4
ITU areas	4.5	3.8
Overall	2.6	4.6

Therefore it seems that the cefuroxime and gentamicin resistance figures obtained by Stokes' method are valid.

Tables 36-38 show the cefuroxime resistance within the general isolates from 18 separate ward areas (units) over the 3 year period. Tables 39-41 show, in a similar manner, the gentamicin resistance in the same units over the same time period.

These resistances were related, as far as was possible, with what, as clinical microbiologists, we believed to have been the pattern of antibacterial usage within each unit.

This was based essentially on our knowledge of the particular antibiotic policies that we had discussed with each individual unit. This was certainly the case with the prophylactic regimes for the Surgical hospital and the initial blind therapy employed by the haematologists within their population of neutropenic patients. However we had no hard data on the actual usage within these areas over that time period. Therefore the statements in Tables 36-41 listed under "Antimicrobial use" should be viewed with some circumspection.

In trying to relate the gentamicin resistance to a particular area without the aid of the actual aminoglycoside use, it was decided that it could at least be related indirectly to that use. This was attempted by running another computer search which counted the number of different patients within each unit who had at least one aminoglycoside assay (duplicates and multiples excluded).

These are enumerated in Tables 39-41 and are also expressed as a percentage of the total aminoglycoside assays over the 3 year time period.

Cefuroxime resistance within the surgical hospital

The overall rate of cefuroxime resistance was 11.1% but the range varied from 4.7% to 20.9%. The general units 1 (14%) and 2 (12.7%) had similar rates of resistance whereas general unit 3 (7%) was significantly lower.

Table 36

SURGICAL HOSPITAL

Cefuroxime resistance 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	CEFUROXIME	RESISTANCE
			proportion	*percentage*
1.	General	cefotaxime prophylaxis & treatment	17/507	14.0
2.	General	cefotaxime prophylaxis & treatment	71/559	12.7
3.	General	gentamicin prophylaxis & treatment	22/315	7.0
4.	Vascular	cefuroxime prophylaxis	75/358	20.9
5	Urology	heavy aminoglycoside & β-lactam use	131/824	15.9
6.	Orthopaedics	high cefuroxime use	76/645	11.8
7.	Gynaecology	low antimicrobial use	65/1392	4.7

Table 37

MEDICAL HOSPITAL

Cefuroxime resistance 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	CEFUROXIME RESISTANCE *proportion*	*percentage*
8.	Renal/CAPD	heavy use of antimicrobials	38/205	18.5
9.	General	low use of antimicrobials	32/383	8.4
10.	General/ Haematology	netilmicin plus azlocillin in neutropenic cases	70/573	12.2
11.	General	low use of antimicrobials	39/461	8.5
12.	General	low use of antimicrobials	22/288	7.6
13.	General/ Haematology	netilmicin plus azlocillin in neutropenic cases	88/667	13.2

Table 38
ITU AREAS
Cefuroxime resistance 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	CEFUROXIME *proportion*	RESISTANCE *percentage*
14.	Cardiothoracic	cefuroxime prophylaxis	88/252	34.9
15.	General	heavy cefuroxime use	75/197	38.1
16.	Head injury	heavy antimicrobial use	34/138	24.6
17.	Renal	aminoglycosides predominantly	30/135	22.2
18.	SCBU	aminoglycosides predominantly	43/254	16.9

It was known that units 1 and 2 preferred cefotaxime for prophylaxis whereas unit 3 preferred an aminoglycoside. However the number of patients having aminoglycoside assays carried out in unit 1 (75) and unit 3 (68) was not appreciably different.

The vascular surgery unit (4) had a much higher rate of cefuroxime resistance at 20.9%. It was known that every vascular patient was given cefuroxime prophylactically.

Unit 5 (urology) was perceived as a unit likely to have resistant Gram negative aerobes and to be a heavy user of antimicrobials. Therefore it was not surprising that the cefuroxime resistance was higher than in the general surgical units at 15.9%.

Gynaecology (unit 6) had the lowest rate of resistance at 4.7%. This unit contained patients who mostly had simple uncomplicated operations and it had the highest bed occupancy rate for the hospital.

Orthopaedics (unit 7) with a resistance rate of 11.7% was perhaps lower than expected since cefuroxime was one of the orthopaedic surgeons favourite drugs because of its antistaphylococcal activity coupled with a broader cover than flucloxacillin.

Cefuroxime resistance within the medical hospital

Overall resistance was 11.1% with a range from 7.6% to 18.5%.

The actual antimicrobial usage within the general medical units (9, 11 and 12) was particularly sketchy, however it was known that (at that time) within units 10 and 13 (general / haematology) there was considerable use of netilmicin and azlocillin. This combination formed the blind treatment of choice for pyrexial neutropenic patients.

Unit 8, the renal ward which included patients with infections related to chronic ambulatory peritoneal dialysis (CAPD), was known to heavily use antimicrobials. This unit had the highest rate of resistance at 18.5%.

The general medical units 9 (8.4%), 11 (8.5%) and 12 (7.6%) had similar rates of resistance to cefuroxime.

Interestingly the general/haematology units (10 and 13) had higher rates of resistance at 12.2 and 13.2% respectively.

Cefuroxime resistance within the ITU areas

This area had an overall resistance rate of 27.7% with a range from 16.9% to 38.0%.

As expected the cefuroxime resistance within these patients was significantly higher than in the rest of the hospital.

The following was known about antimicrobial use in these units.

In the cardiothoracic ITU (14) all patients received cefuroxime as prophylaxis prior to and during surgery and this was continued until all invasive lines were removed.

In the general ITU (unit 15) the illest patients in the hospital were treated. Cefuroxime was, at times, the commonest drug (in the widest sense) prescribed in this area.

In the head injury unit (16) the most injudicious antimicrobial therapy within the hospital took place. There was also a subset of severely injured patients on assisted ventilation.

The renal ITU (17) and special care baby unit (18) were the two biggest individual users of the aminoglycosides.

Cefuroxime resistance within the ITU areas seemed to follow the perceived pattern of prescribing. Resistance was highest in the general ITU (15)

at 38% with 34.9% resistance in the cardiothoracic ITU (14) and 24.6% in the Head injury unit (16).

Resistance was much lower in the two units where aminoglycoside use was higher i.e. 22% (Renal ITU, 17) and 16.9% (SCBU, 18).

Gentamicin resistance within the surgical hospital

The overall resistance to gentamicin was 1.9% with a range of 0.7% to 5.0%.

The resistance was highest in the urology unit (5) at 5% which had the third highest number of patients (149) on aminoglycosides in the hospital.

The rates of resistance were similar in general surgical unit 2 (1.6%), 3 (1.4%), vascular unit 4 (1.4%) and orthopaedics (1.2%).

General surgical unit 1 had a higher rate of resistance at 2.6% than the other general surgical units but it had a similar number of patients with aminoglycoside assays as unit 3 (75 and 68 respectively) which was thought to use aminoglycosides more than the others.

Gynaecology had the lowest rate of resistance at 0.7%.

Gentamicin resistance within the medical hospital

The overall gentamicin resistance in this area was 2.6% with a range from 1.0% to 6.1%.

The general medical units 11 and 12 had similar rates of resistance at 1.9% and 1.0% respectively. This differed from a rate of 3.1% in the other general medical unit 9. This led to a reassessment of the original data since the difference appeared to be inexplicably large. The resistant patient numbers were scrutinised revealing that in the male ward of this unit, during the summer of 1986, there had been 7 who had been colonised/infected (predominantly catheter urines) with the same gentamicin resistant *Enterobacter cloacae*. A readjustment

Table 39
SURGICAL HOSPITAL
Gentamicin resistance and gentamicin assays 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	GENTAMICIN RESISTANCE		GENTAMICIN ASSAYS	
			proportion	*percentage*	*number*	*% of total*
1.	General	cefotaxime prophylaxis & treatment	12/455	2.6	75	4.1
2.	General	cefotaxime prophylaxis & treatment	9/569	1.6	41	2.2
3.	General	gentamicin prophylaxis & treatment	5/359	1.4	68	3.7
4.	Vascular	cefuroxime prophylaxis	5/358	1.4	63	3.4
5.	Urology	heavy aminoglycoside & β-lactam use	39/775	5.0	149	8.0
6.	Orthopaedics	high cefuroxime use	8/653	1.2	23	1.2
7.	Gynaecology	low antimicrobial use	10/1466	0.7	15	0.8

Table 40

MEDICAL HOSPITAL

Gentamicin resistance and gentamicin assays 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	GENTAMICIN RESISTANCE		GENTAMICIN ASSAYS	
			proportion	*percentage*	*number*	*% of total*
8.	Renal/CAPD	heavy use of antimicrobials	12/197	6.1	117	6.3
9.	General	low use of antimicrobials	13/422	3.1	34	1.8
10.	General/ Haematology	netilmicin plus azlocillin in neutropenic cases	16/523	3.1	53	2.9
11.	General	low use of antimicrobials	9/481	1.9	48	2.6
12.	General	low use of antimicrobials	3/304	1.0	30	1.6
13.	General/ Haematology	netilmicin plus azlocillin in neutropenic cases	25/585	4.3	164	8.9

Table41

ITU AREAS

Gentamicin resistance and gentamicin assays 1986-1989 by unit and related to presumed antimicrobial use.

Unit	Type	Antimicrobial use	GENTAMICIN RESISTANCE		GENTAMICIN ASSAYS	
			proportion	*percentage*	*number*	*% of total*
14.	Cardiothoracic	cefuroxime prophylaxis	9/197	4.6	80	4.3
15.	General	heavy cefuroxime use	10/172	5.8	94	5.1
16.	Head injury	heavy antimicrobial use	2/138	1.5	30	1.6
17.	Renal	aminoglycosides predominantly	8/119	6.7	110	5.9
18.	SCBU	aminoglycosides predominantly	11/268	4.1	638	34.4

of the figures for unit 9 gave a more realistic gentamicin resistance rate of 1.7% - very similar to the rates of the other medical units.

The general medical/haematology units (10 and 12) had significantly greater resistance rates to gentamicin then the general units, at 3.1% and 4.3% respectively. Unit 13 had, at least, 164 patients during this time period on aminoglycosides.

The highest rate of gentamicin was in the Renal/CAPD unit (8) which had an estimated 117 patients on aminoglycosides and a rate of 6.1%.

Gentamicin resistance within the ITU Areas

The overall resistance to gentamicin was 4.5% with a range of 1.5% to 6.7%.

The highest resistance was found in the renal ITU (17) at 6.7% where 110 patients had aminoglycoside therapy (this undoubtedly represented in the region of 30% of their patients).

The general ITU had a resistance of 5.8% with 94 patients having received aminoglycosides. Again this represented about 25% of their patients.

The most interesting figure however was a rate of 4.1% in the SCBU where over the 3 year period at least 638 babies had aminoglycoside assays. This unit, on average, has 400-500 admissions per annum, therefore about 40% - 50% of all babies received aminoglycosides in this unit. So they were the largest users (in patient terms) of aminoglycosides in the hospital, yet their resistance rate was low.

The above data clearly give much to discuss with the wide range of cefuroxime resistance within various units of the hospital (which was not so marked with the resistance to gentamicin) and the generally higher rate of resistance to cefuroxime than to gentamicin. While most of the differences could

be explained by anecdotal evidence it was clear that an attempt to relate the differences to the actual antimicrobial use within these areas was important.

It was also felt important to continue to calculate the cefuroxime and gentamicin resistances within these wards over a longer time period, not only to achieve a larger series but to ascertain whether the resistances were changing over time Therefore the series was extended from 1 April 1986 - 31 March 1989 to 1 April 1989 to 31 March 1991.

By the financial year April 1989 to March 1990 the pharmacy department of the RIE had developed software which enabled the downloading of data which revealed the actual antimicrobial usage in each ward area. Although these data in themselves were important it was also clearly necessary to relate them to bed occupancy within each area, over the same time period. These data were obtained from the RIE bedstate figures.

The percentage resistance to cefuroxime and gentamicin are shown in Tables 42-44 for each of the 17 units over both time periods (the antimicrobial usage figures for urology were not available).

Table 45-47 show the total number of patients admitted to each unit, the total amount of Beta-lactams used per patient, the total amount of 2nd and 3rd generation cephalosporins used per patient, the total amount of cefuroxime used per patient and the total cost of all antimicrobial agents per patient. This is related to the cefuroxime resistance over the time period April 1989 to March 1990.

Table 48-50 shows the usage of the aminoglycosides in each of the units related to patient admissions and the gentamicin resistance over the period April 1989 to March 1991.

Table 42 **Cefuroxime and gentamicin resistance by unit and over 3 time periods.**

SURGICAL HOSPITAL		CEFUROXIME RESISTANCE *Proportion resistant (%R)*			GENTAMICIN RESISTANCE *Proportion resistant (%R)*		
Unit	**Type**	**1986-89**	**1989-91**	**1986-91**	**1986-89**	**1989-91**	**1986-91**
1.	General	71/507	86/419	157/926	12/455	2/382	14/837
		(14.0)	(20.5)	(17.0)	(2.6)	(0.5)	(1.7)
2.	General	71/559	65/431	136/990	9/569	5/393	14/962
		(12.7)	(15.4)	(13.7)	(1.6)	(1.3)	(1.5)
3.	General	22/315	18/232	40/547	5/359	4/202	9/561
		(7.0)	(7.7)	(7.3)	(1.4)	(2.0)	(1.6)
4.	Vascular	75/358	78/299	153/657	5/358	2/289	7/647
		(20.9)	(26.0)	(23.3)	(1.4)	(0.7)	(1.1)
5.	Urology	131/824	135/734	266/1558	39/775	18/706	57/1481
		(15.9)	(18.4)	(17.1)	(5.0)	(2.5)	(3.8)
6.	Orthopaedics	76/645	76/407	152/1052	8/653	2/329	10/982
		(11.8)	(18.7)	(14.5)	(1.2)	(0.6)	(1.0)
7.	Gynaecology	65/1392	35/757	100/2149	10/1466	5/686	15/2152
		(4.7)	(4.6)	(4.7)	(0.7)	(0.7)	(0.7)
	All	511/4600	493/3279	1004/7879	88/4635	38/2987	126/7622
		(11.1)	(15.0)	(12.7)	(1.9)	(1.3)	(1.7)

Table 43
Cefuroxime and gentamicin resistance by unit and over 3 time periods.

MEDICAL HOSPITAL		CEFUROXIME RESISTANCE *Proportion resistant (%R)*			GENTAMICIN RESISTANCE *Proportion resistant (%R)*		
Unit	**Type**	**1986-89**	**1989-91**	**1986-91**	**1986-89**	**1989-91**	**1986-91**
8.	Renal/CAPD	38/205	25/104	63/309	12/197	2/102	14/299
		(18.5)	(24.0)	(20.4)	(6.1)	(2.0)	(4.7)
9.	General	32/383	10/248	42/631	7/416	2/231	9/647
		(8.4)	(4.0)	(6.7)	(1.7)	(0.9)	(1.4)
10.	General/	70/573	43/307	113/880	16/523	5/239	21/762
	Haematology	(12.2)	(14.0)	(12.8)	(3.1)	(2.1)	(2.8)
11.	General	39/461	37/252	76/713	9/481	4/220	13/701
		(8.5)	(14.7)	(10.7)	(1.9)	(1.8)	(1.9)
12.	General	22/288	21/234	43/522	3/304	2/197	5/501
		(7.6)	(9.0)	(8.2)	(1.0)	(1.0)	(1.0)
13.	General/	88/667	75/517	163/1184	25/585	6/380	31/965
	Haematology	(13.2)	(14.5)	(13.8)	(4.3)	(1.6)	(3.2)
	All	289/2577	211/1662	500/4239	72/2506	21/1369	93/3875
		(11.2)	(12.7)	(11.8)	(2.9)	(1.5)	(2.4)

Table 44
Cefuroxime and gentamicin resistance by unit and over 3 time periods.

ITU AREAS		CEFUROXIME RESISTANCE *Proportion resistant (%R)*			GENTAMICIN RESISTANCE *Proportion resistant (%R)*		
Unit	**Type**	**1986-89**	**1989-91**	**1986-91**	**1986-89**	**1989-91**	**1986-91**
14.	Cardiothoracic	88/252	100/207	188/459	9/197	7/157	16/354
		(34.9)	(48.3)	(41.0)	(4.6)	(4.5)	(4.5)
15.	General	75/197	121/268	196/465	10/172	4/183	14/355
		(38.1)	(45.1)	(42.2)	(5.8)	(2.2)	(3.9)
16.	Head injury	34/138	97/227	131/365	2/138	6/167	8/305
		(24.6)	(42.7)	(35.9)	(1.5)	(3.6)	(2.6)
17.	Renal	30/135	17/69	47/204	8/119	3/51	11/170
		(22.2)	(24.6)	(23.0)	(6.7)	(5.9)	(6.5)
18.	SCBU	43/254	48/228	91/482	11/268	5/202	16/470
		(16.9)	(21.0)	(18.9)	(4.1)	(2.5)	(3.4)
	All	270/976	383/999	653/1975	40/894	25/760	65/1654
		(27.7)	(38.3)	(33.1)	(4.5)	(3.3)	(3.9)

Table 45
SURGICAL HOSPITAL
β–lactam use related to unit, patient throughput and cefuroxime resistance.

Unit	Type	Total number of patients	Total β-lactam use/unit(g)	Total β-lactam/ patient (g)	2/3 cephs./ patient (g)	cefuroxime/ patient (g)	Total antimicrobial costs/patient (£)	Cefuroxime resistance 1989-91(%)
1.	General	1512	6164.0	4.0	2.5	0.6	14.46	20.5
2.	General	1737	3201.0	1.8	1.0	0.9	6.90	15.4
3.	General	1557	4407.0	2.8	1.0	0.7	7.00	7.7
4.	Vascular	969	5758.0	5.9	2.3	2.2	12.34	26.0
6.	Orthopaedics	3443	10390.0	3.0	1.8	1.7	6.86	18.7
7.	Gynaecology	3842	6112.6	1.6	0.2	0.2	2.90	4.6

Table 46

MEDICAL HOSPITAL

β–lactam use related to unit, patient throughput and cefuroxime resistance.

Unit	Type	Total number of patients	Total β-lactam use/unit(g)	Total β-lactam/ patient (g)	2/3 cephs./ patient (g)	cefuroxime/ patient (g)	Total antimicrobial costs/patient (£)	Cefuroxime resistance 1989-91(%)
8.	Renal/CAPD	376	1950.0	5.2	1.1	0.5	23.29	24.0
9.	General	1650	6327.0	3.8	0.6	0.3	6.09	4.0
10.	General/ Haematology	1561	7193.0	4.6	1.4	0.2	20.06	14.0
11.	General	1148	4862.0	4.2	0.5	0.4	8.37	14.7
12.	General	1485	3716.0	2.5	1.1	0.2	9.34	9.0
13.	General/ Haematology	1774	7642.0	4.3	1.1	0.3	24.17	14.5

Table 47
ITU AREAS
β–lactam use related to unit, patient throughput and cefuroxime resistance.

Unit	Type	Total number of patients	Total β-lactam use/unit(g)	Total β-lactam/ patient (g)	2/3 cephs./ patient (g)	cefuroxime/ patient (g)	Total antimicrobial costs/patient (£)	Cefuroxime resistance 1989-91(%)
14.	Cardiothoracic	799	2889.0	3.6	2.4	2.2	13.80	48.3
15.	General	109	2164.0	19.9	12.6	7.8	88.04	45.1
16.	Head injury	950	3065.0	3.2	1.1	0.5	8.98	42.7
17.	Renal	85	1175.0	13.8	6.2	2.3	69.92	24.6
18.	SCBU	429	507.0	1.2	n/a	n/a	n/a	21.0

Table 48 Aminoglycoside use related to unit, patient throughput and gentamicin resistance, within the Surgical hospital (1989-1990).

Unit	Type	Total aminog.used (g)	Total no of patients	Aminog./ patient (g)	Gentamicin resistance (%)
1.	General	95.9	1512	0.06	0.5
2.	General	27.1	1737	0.02	1.3
3.	General	80.6	1557	0.05	2.0
4.	Vascular	16.6	969	0.02	0.7
6.	Orthopaedics	1.4	3443	0.0004	0.6
7.	Gynaecology	6.1	3842	0.002	0.7

Table 49 Aminoglycoside use related to unit, patient throughput and gentamicin resistance, within the Medical hospital (1989-1990).

Unit	Type	Total aminog.used (g)	Total no of patients	Aminog./ patient (g)	Gentamicin resistance (%)
8.	Renal/CAPD	43.5	376	0.12	2.0
9.	General	12.7	1561	0.008	0.9
10.	General/ Haematology	74.3	1650	0.04	2.1
11.	General	9.9	1148	0.009	1.8
12.	General	17.7	1485	0.01	1.0
13.	General/ Haematology	133.0	1774	0.07	1.6

Table 50 Aminoglycoside use related to unit, patient throughput and gentamicin resistance, within the ITU areas (1989-1990).

Unit	Type	Total aminog.used (g)	Total no of patients	Aminog./ patient (g)	Gentamicin resistance (%)
14.	Cardiothoracic	8.3	799	0.01	4.5
15.	General	23.8	109	0.2	2.2
16.	Head injury	30.9	950	0.03	3.6
17.	Renal	9.7	85	0.1	5.9
18.	SCBU	17.6	429	0.04	2.5

CEFUROXIME RESISTANCE OVER 5 YEARS

Surgical hospital

1. The overall resistance rose between 1986-89 and 1989-91 from 11.1% to 15.0%
2. Of the seven units studied the resistance rose in five and remained similar in two (unit 3, general surgery and unit 7, gynaecology)
3. The rise in resistance was most marked in orthopaedics (6), general surgery (unit 1) and vascular (unit 4)

Medical hospital

1. The overall resistance rose from 11.2% (1986-89) to 12.7% (1989-91)
2. Of the six units studied the resistance rose in five and fell in one (general medical, unit 9)
3. The rise in resistance was most marked in Renal/CAPD (unit 8) and general medical unit 11

ITU areas

1. The overall resistance rose from 27.7% in 1986-89 to 38.3% in 1989-91
2. It rose in all five units studied
3. The rise was most marked in the Head injury unit (16), the cardiothoracic ITU (14) and the general ITU (15)

GENTAMICIN RESISTANCE OVER 5 YEARS

Surgical hospital

1. Overall resistance to gentamicin fell from 1.9% in 1986-89 to 1.3% in 1989-91
2. The resistance fell in five of the seven units studied.

Medical hospital

1. Overall resistance fell from 2.9% in 1986-89 to 1.5% in 1989-91
2. It fell in five of the six units and remained the same in the other

ITU

1. Overall resistance to gentamicin fell from 4.5% in 1986-89 to 3.3% in 1989-91
2. It fell in all the units studied apart from the Head injury unit (16)

STATISTICAL ANALYSES OF THE RESISTANCE RATES

The analyses were carried out by Dr A Bowman of the Department of Statistics, University of Glasgow.

The resistance rates were plotted for each unit for each drug over the two time scales. This was carried out on a logit scale:

logit (r) = log (r/(1-r))

where r is the rate = number resistant/number tested

The plots are shown in figure 10 where :

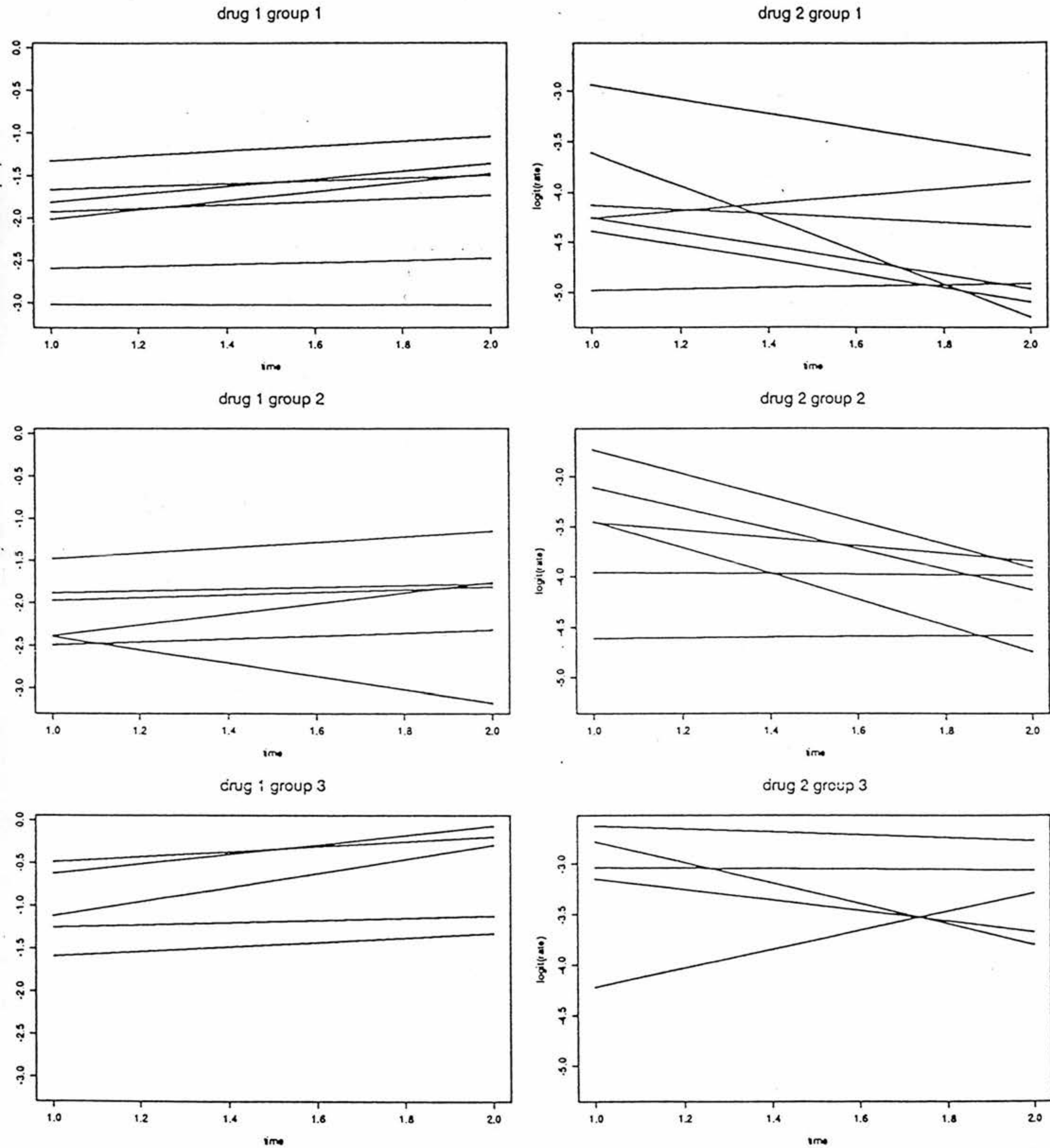
drug 1	=	cefuroxime
drug 2	=	gentamicin
group 1	=	Surgical hospital
group 2	=	Medical hospital
group 3	=	ITU areas

Interpretation

Resistance rates over the two time periods

Differences between the two times were assessed by taking the difference across time for each ward and checking whether these differences were significantly different from zero. The p-values are shown in Table 51

Figure 10



Plots (employing a logit scale)showing the statistical analysis of the resistance rates: for each unit, for each drug, over the two time scales.

For the key please refer to the text.

Table 51
p values for the difference in resistance across time

Area	Cefuroxime (1)	Gentamicin (2)
surgical (1)	0.014	0.085
medical (2)	0.612	0.044
ITU (3)	0.028	0.654

The trend for drug 1 (cefuroxime) is increasing over time and the trend for drug 2 (gentamicin) is decreasing. This is clearly significant ($p<0.05$) for the cefuroxime resistance in the surgical wards and the Intensive care areas and for gentamicin resistance in the medical units.

Resistance rates to each drug within ward areas

This was carried out using one-way analysis of variance for each drug and time combination. The p-values are shown in Table 52.

Table 52
p values for differences in resistance rates to each drug within the ward areas.

Time period	Cefuroxime (1)	Gentamicin (2)
1986-1989 (1)	0.003	0.077
1989-1991 (2)	0.007	0.002

There is therefore clear evidence of differences in each case although gentamicin resistance over 1986-89 it is a little marginal.

Differences in resistance rates to cefuroxime and gentamicin

The differences in resistance rates between cefuroxime and gentamicin is very clear indeed. When a two sample t test was carried out for each group and time combination the p values were all extremely small. From the figure it can be seen that the values for the two drugs hardly overlap at all.

COMMENTS ON THE BETA-LACTAM USAGE FIGURES COMPARED TO THE CEFUROXIME RESISTANCE

Surgical hospital

1. The highest usage was in the vascular unit (4) with 5.4g/patient and the resistance was high at 26%. Next was the general surgical unit 1 with 4g/patient and a resistance of 20.5%.
2. The highest costs of antimicrobials per patient were also in these two units (4 and 1).
3. Most of unit 1's Beta-lactam use was in the form of 3rd generation cephalosporins.
4. Most of the vascular unit's (4) use was of cefuroxime (2.2g of 5.9g).
5. The lowest resistance and lowest usage were in Gynaecology (7) with 1.6g/patient and 4.6%, respectively.
6. General surgery unit, 3 had a higher total usage (2.8g/patient) than unit 2 (1.8g/patient) yet the resistance to cefuroxime was 7.7% compared to 15.4%.

Medical hospital

1. The highest usage and rates of resistance were found in the Renal/CAPD ward (8) with 5.2g/patient and resistance of 24%, respectively.

2. In the general/haematology wards (10 and 13) both had high usage at 4.6 and 4.3g/patients respectively. They were also high users of 2nd/3rd generation cephalosporins.
3. The figures for the subgroups of haematology patients within units 10 and 13 were considerably higher because of the dilution by the numbers of ordinary general medical patients.
4. In terms of the total antimicrobial costs per patient the general medical/haematology units (10 and 13) and the Renal/CAPD (8) were the highest at £20.06, £24.17 and £23.29 respectively. This should be compared with the costs per patient in the general medical wards of less than £10.

ITU Areas

1. It is clear that large numbers of straight forward cases were diluting out the problem cases (true ITU cases) in the cardiothoracic ITU (14) with 3.6g/patient and in the Head injury unit (16) at 3.2g/patient. This is to be compared with the general ITU (15) patients at 19.9g/patient and the renal ITU patients at 13.9g/patient.
2. The costs of antimicrobials per patient were also similarly diluted in these areas with the cardiothoracic ITU at £13.80/patient and the Head injury unit at £8.98/patient compared with £88.04/patient for the general ITU and £69.92 for the renal ITU.
3. Despite this, the figures show the clear differences that exist between the usage of Beta-lactams and the total cost of antimicrobial between a general ITU patient and the general medical or surgical patient.
4. Please note the relatively large amount of Beta-lactam given to each baby (1.2g/baby) in the SCBU while realising that they are 50 times smaller than an adult. Yet the cefuroxime resistance is the lowest of all the ITU areas at 21%.

COMMENTS ON THE USAGE OF THE AMINOGLYCOSIDES

Surgical unit

1. The highest usage was within surgical unit 1 with 0.06g per patient, followed by the surgical unit 3 (0.05g/patient).
2. However the gentamicin resistance was 0.5% in unit 1 but 2% in unit 3.

Medical unit

1. The highest usage was in the Renal/CAPD unit (8) with 0.12g/patient. This amount, when compared to normal patients, should probably be even higher since the dosage in the renal patients is necessarily reduced because of their renal insufficiency.
2. The next highest users were the two general/haematology units (10 and 13) with usage of 0.04g/patient and 0.07g/patient, respectively.
3. As was the case with Beta-lactam use, the use within the subgroup of haematology patients will be considerably higher than this.

Intensive care areas

1. The highest usage was in the general ITU (15) with 0.2g/patient although the gentamicin resistance was only 2.2%.
2. Next highest usage was in the Renal ITU (17) with a use of 0.1g/patient. This figure is perhaps relatively lower than it might have been since most of the patients were in acute renal failure. The resistance to gentamicin was highest here at 5.9%.
3. The figures of usage within the cardiothoracic ITU (14) and Head injury unit (16) were falsely low because of a dilution out of the true ITU patients by those patients who were straightforward operative cases or minor head injuries.
4. The most remarkable figure was the usage within the SCBU (18) with 0.04g/patient. This in itself is four times more per patient than in e.g. the cardiothoracic ITU. But it is even more significant when it is realised that

the babies are on average at least 50 times lighter than an adult. Therefore an equivalent adult usage figure would be about 2g/patient. Yet the gentamicin resistance is low at only 2.5%.

STATISTICAL ANALYSIS OF THE ANTIMICROBIAL USAGE FIGURES

Scatter diagrams were constructed (data not shown) for the total Beta-lactam used per patient, total 2nd and 3rd generation cephalosporins per patient, total cefuroxime per patient and total antimicrobial costs per patient versus the cefuroxime resistance over the period of 1989-91.

In only the case of the surgical wards did an elliptical distribution occur.

Similarly scatter diagrams were constructed for the aminoglycoside use per patient versus the gentamicin resistance. No elliptical distributions were found.

This apparent lack of correlation between antimicrobial use and resistance is not surprising.

1. As has previously been pointed out, figures for antimicrobial use in the haematology patients, in the cardiothoracic ITU and Head injury unit are biased because of the dilution brought about by large numbers of problem-free patients. This results in a lack of correlation between use and resistance within the medical and ITU areas of the hospital.
2. Similar problems existed within the population of surgical patients. For instance the gentamicin use in surgical units 1 and 3 were very similar at 0.06g/patient and 0.05g/patient, respectively, yet their resistance rates to gentamicin are clearly different at 0.5% and 2%, respectively.

   The Beta-lactam usage in surgical unit 2 was 1.8g/per patient and in surgical unit 3 was 2.8g/per patient yet the resistance to cefuroxime was 15.4% in unit 2 and about half that at 7.7% in unit 3.

Perhaps the sort of surgery (and therefore the underlying conditions of the patients) in each of these surgical units was different in some way which then had an effect on their likelihood of acquiring a resistant coliform? The underlying condition of the patient has already been shown to be an important variable in the rate of resistance to both cefuroxime and gentamicin by the fact that the rates of resistance were higher in the ITU patients than in either those patients from a general surgical or general medical wards.

3. The relatively low rates of resistance to both gentamicin and cefuroxime in babies from the SCBU in the face of the relatively widespread use of both these drugs (in terms of the number of babies treated) suggests that it might be in the total amount of drugs that are used (per square metre, for instance) that was important.

4. In any case the relationship between antimicrobial use and resistance is not a simple one and requires a great deal more investigation of the other possible variables to elucidate it.

5. The usage figures are only over a 12 month period and it is therefore obviously important to obtain these over a much longer time period in order to view the trends in antimicrobial prescribing which might then allow us to explain why there has been a rise in resistance to cefuroxime but a decrease in the resistance to gentamicin.

CHARACTERISATION OF ACINETOBACTER BETA-LACTAMASES

CONVENTIONAL ISOELECTRIC FOCUSING

Bush (1988) described isoelectric focusing as "one of the most critical assays used to verify the identity of Beta-lactamases". Therefore IEF is a reasonable technique to employ in the first instance to look for obvious differences or similarities in the Beta-lactamases produced by these organisms.

Clearly it was important to determine which strains had Beta-lactamases which were obviously plasmid mediated e.g. TEM-1, TEM-2, SHV-1 etc. From the IEF screen it was found:

a) No strains carried a TEM-1 or a similar plasmid mediated Beta-lactamase.

b) All 43 strains studies possessed a (presumed) chromosomal Beta-lactamase which had a very basic pI.

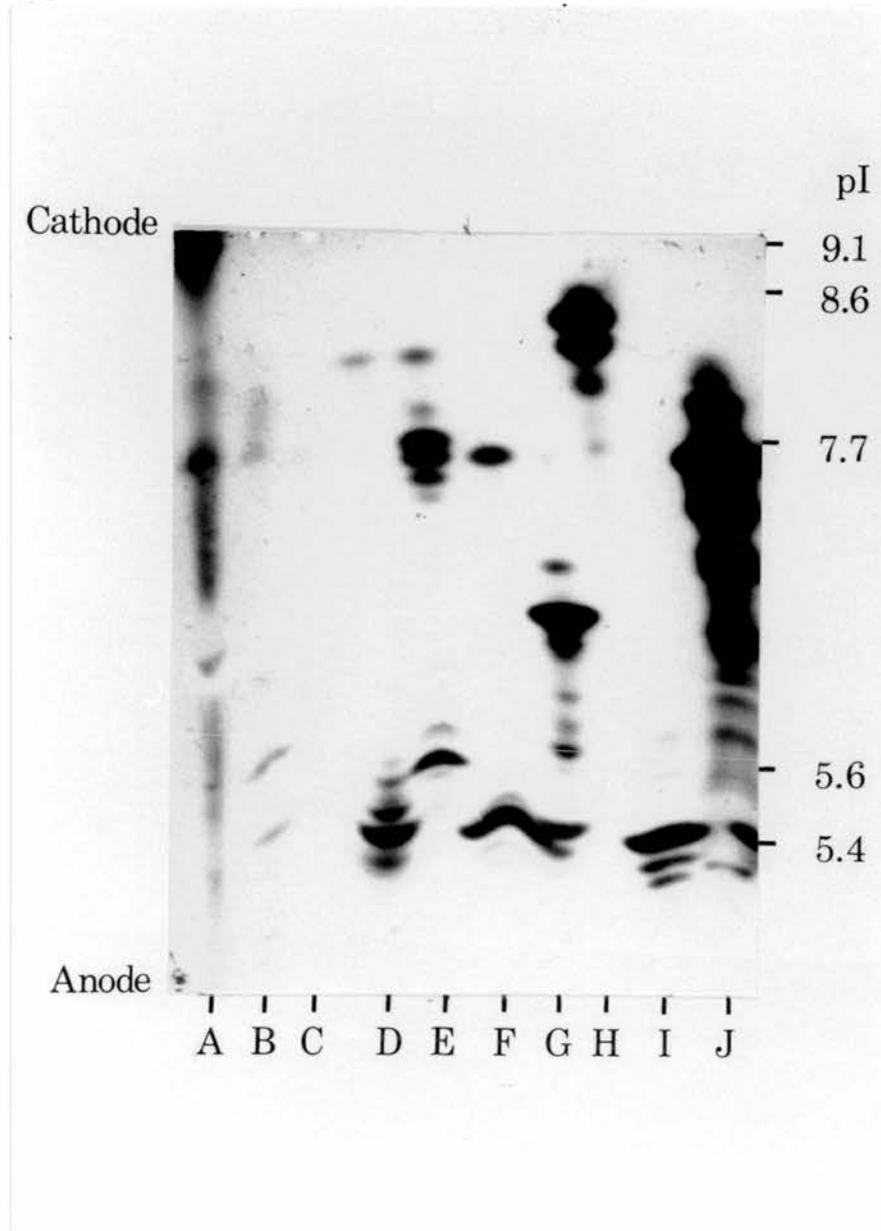
c) Only one strain (HI26) out of the 43 strains focused in a conventional sense with a pI of around 9.1.

d) The other 42 possessed ill-defined areas of activity often associated with streaks from the anode to the cathode. This made it extremely difficult to assess the pI of these enzymes.

e) It was also clear that the staining with nitrocephin was comparatively slow compared to the rapid staining of control enzymes such as TEM-1, P99 etc. This no doubt reflected the poorer activity of the *Acinetobacter* enzymes.

The problem is graphically portrayed in figure 11 where in Track A a typical *Acinetobacter* Beta- lactamase is seen in comparison with a series of other Beta-

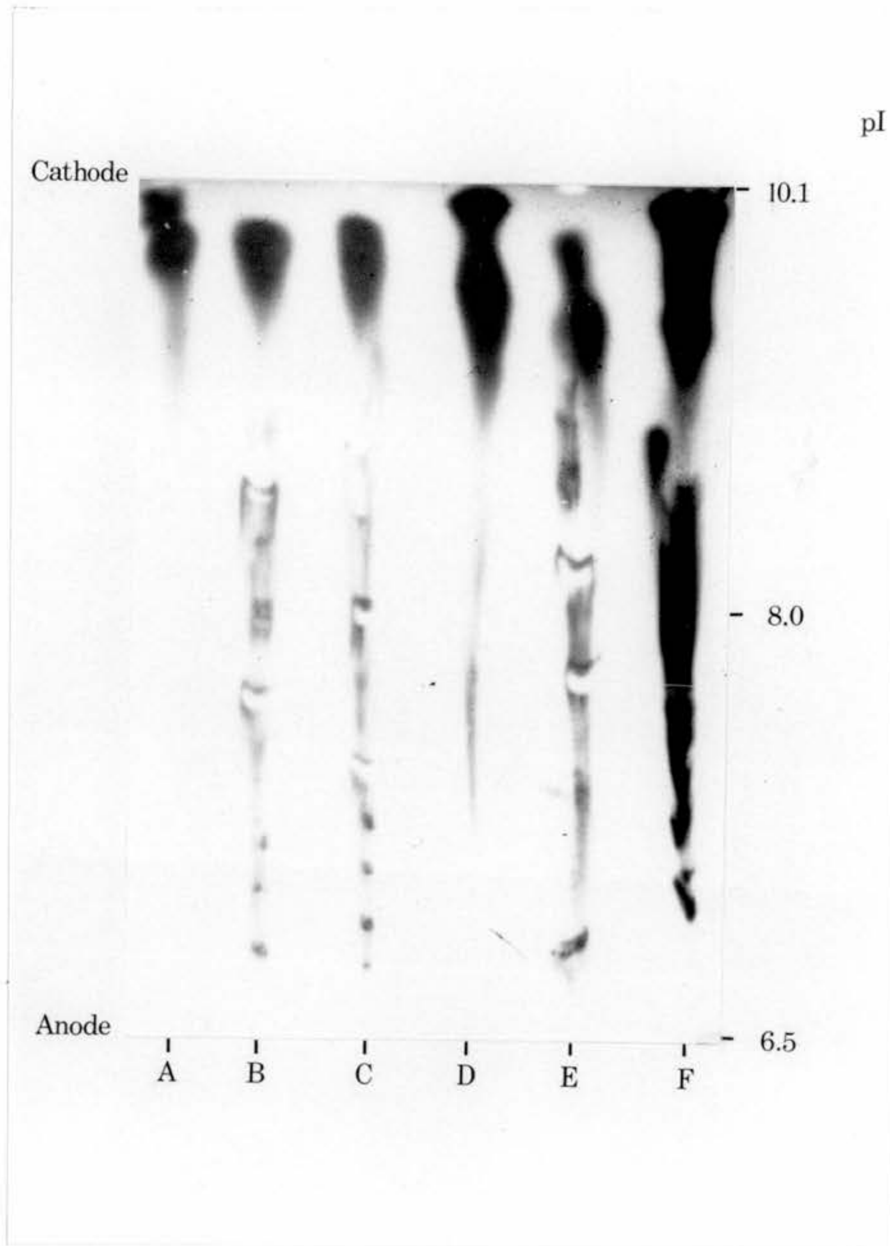
Figure 11



Conventional isoelectric focusing

Track A:	*Acinetobacter*, H10	Track F:	*Klebsiella oxytoca*, H23
Track B:	*Providencia*, H25	Track G:	*Klebsiella oxytoca*, H19
Track C:	*Acinetobacter*, 6B101	Track H:	*Citrobacter freundii*, H35
Track D:	TEM-1	Track I:	*Klebsiella oxytoca*,7B52
Track E:	TEM-2+OXA-2	Track J:	P99

Figure 12



Tracks A to F are *Acinetobacter* β-lactamases on conventional isoelectric focusing with a mixture of 3.5-10 and 9-11 ampholines

lactamases, both plasmid mediated and chromosomal in origin, with pI values that were easily assessed.

This obvious failure to focus properly in conventional polyacrylamide IEF systems is further shown by employing a 1:1 mixture of 3.5 - 10.0 and 9 - 11 ampholines. (figure 12)

SDS-FREE PAGE

Therefore an attempt to discriminate between different Beta-lactamases on the basis of their M_r (molecular size) was made.

Conventionally, proteins can be separated by their molecular size on an SDS - PAGE system. This involves denaturing the protein and linearising it with the SDS. This linearised and negatively charged protein will then migrate in an electrical field to a distance proportional to its length (if the right conditions are attained, e.g. gel porosity, a plot of $\log_{10} M_r$ versus relative mobility (R_f) reveals a straight line relationship). The protein is stained with either coosmassie blue or silver nitrate.

Such a system, therefore, employs reducing conditions, these are unsatisfactory when using crude preparations of Beta-lactamase since many other proteins are inevitably present. In order to use crude sample preparations and to retain the substrate nitrocephin as a rapid probe for the enzymes, it was therefore necessary to employ non-denaturing conditions. This required a system that was free from SDS. The protocol employed was as described in the Materials and Methods.

Initial experimentation with this system was with twelve mini-preparations of *Acinetobacter* Beta-lactamases run on 12% acrylamide gels with normal polarity for short running times of up to 4 hours e.g. 250 V, 40 mA for 1 h followed by 250 V, 70 mA for 3 h.

The result was a broad band of Beta-lactamase activity detected in front of the focused line of bromophenol blue. Similar results were obtained despite running the gels at lower voltages for longer periods e.g. 40 V for 2 h followed by 30 V for 14 h.

Finally six *Acinetobacter* enzymes were run in a SDS-free, 12% acrylamide gel with control Beta-lactamases i.e. TEM-1, TEM-2 and OXA-2 on normal polarity at 30 V for 17 h followed by 40 V for 9 h.

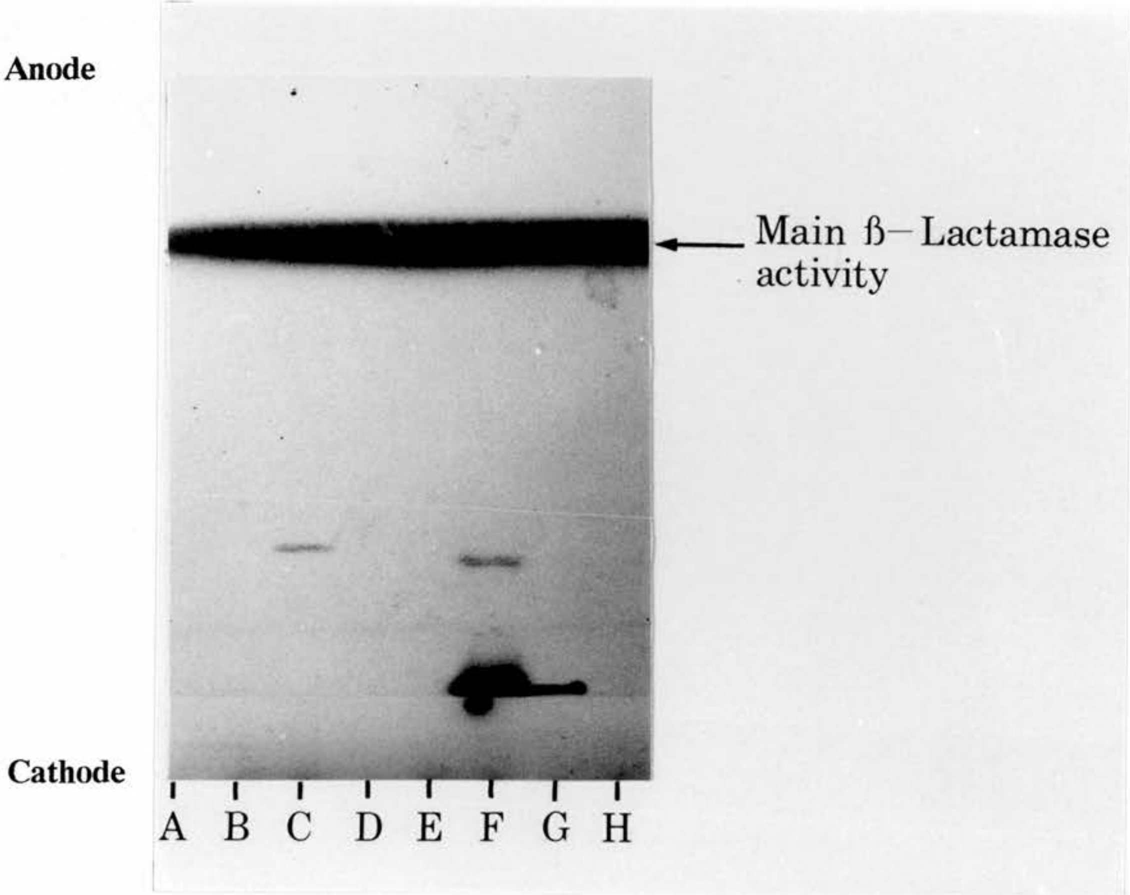
The result is shown in figure 13 . Almost all the Beta-lactamase activity detected was found on the anode side of the bromophenol blue line. The control Beta-lactamases TEM-1 (track C), TEM-2 (track F) and OXA-2 (track F) could all be visualised. The activity in track G, at the point of loading was thought to be an artefact.

From these experiments it was decided to abandon SDS - free PAGE as a means of discriminating between *Acinetobacter* Beta-lactamases. It was felt that these enzymes were neither solubilising within the conventional IEF polyacrylamide system nor in the SDS-free PAGE system. The reasons for this apparent lack of solubilisation were therefore pursued. This followed two separate lines of research, firstly into systems for solubilising the preparations and secondly into estimations of the M_r of these enzymes.

It should be noted however, that several of the assumptions made at this stage concerning the SDS-free PAGE electrophoresis were misplaced as will be discussed more fully later.

Figure 13

SDS-FREE PAGE GEL



Track A *Acinetobacter*, 6B253

Track B *Acinetobacter*,75

Track C TEM-1

Track D *Acinetobacter*,6B410

Track E *Acinetobacter*,6B324

Track F TEM-2 + OXA-2

Track G *Acinetobacter*,H33

Track H *Acinetobacter*,H10

ISOELECTRIC FOCUSING EMPLOYING A POLYACRYLAMIDE GEL INCORPORATING UREA AND TRITON X-100

To investigate the hypothesis of poor solubilisation in conventional IEF PAGE systems an IEF system incorporating urea and Triton X-100 was devised.

Clearly it was important that the denaturing conditions employed were sufficient to enable solubilisation but not enough to destroy the biological activity of the Beta-lactamase. This was crucial to enable the enzyme activity to be probed, as before, with the chromogenic substrate, nitrocephin.

An IEF gel containing approximately 12% polyacrylamide (final concentration) with 4M urea and 2.5% Triton X-100 was devised, its composition is shown in Table 53.

Table 53

Composition of the IEF gel containing urea, Triton X-100 & polyacrylamide

Material	Supplier	Volume or mass employed	Final concentration (approx)
Urea	BDH	10g	4 mol/L
Distilled water		10ml	
Acrylamide (27g) plus methylene bisacrylamide (3g) in 100ml distilled water	BDH	15ml	80g/L (A) 9g/L(bis)
Riboflavin (20mg/L)	Sigma	5ml	2mg/L
Triton X-100 (10%w/v)	BDH	10.2ml	2.5%
40% ampholines w/v pH 3.5-10 pH 9-11	BDH	 1.25ml 1.25ml	 2.0% w/v
TEMED	Sigma	0.05ml	0.9mg/L

The concentration (%C) of bisacrylamide (crosslinker) was increased to 10% to increase the pore size of the gel (i.e. where %C = g crosslinker per 100 g monomers).

Attempts to decrease the %T of the gel (i.e. %T = g monomers per 100 ml, where g monomers = g acrylamide + g crosslinker) resulted in gels that could not be poured in a vertical plane due to their lack of physical strength.

The problems of employing urea in these systems were noted. The major problem is the accummulation of cyanate ions in stock solutions as a result of chemical isomerisation. The cyanate ions react with the amino groups to form stable carbamylated derivatives resulting in an alteration of the charge of the proteins.

If this reaction is incomplete several artefactual species of proteins, with different charges, result. For this reason all urea solutions were made up fresh just prior to use.

Preparation of gels

The gels were poured in the same vertical glass plate cassette as for conventional IEF polyacrylamide gels.

The gel mixture was prepared by initially mixing 10g of urea in 10.2 ml of 10% w/v Triton X-100 in a 60°C waterbath followed by 10 ml of distilled water. To 15 ml of the stock acrylamide/bisacrylamide solution, 2.5 ml of a 50:50 mixture of pH 3.5 - 10.6 and pH 9 - 11 ampholines were added. The two mixtures were then added to each other with gentle agitation followed by 5 ml of riboflavin (20 mg/L) and 50 μl of TEMED. The mixture was thoroughly but gently mixed and applied to the vertical glass plates. The gel was allowed to stand overnight to achieve mechanical strength.

Sample preparation

Initially mini-preparations of the Beta-lactamases were employed (as described previously). Up to 100 μl were added for each sample depending on the nitrocephin spot test. Five μl of isoelectric point (pI) standard markers were also spotted onto the gel to quantify the pI gradient.

Running conditions

The gel was run at 1W (constant), 550 V (limiting) and 20 mA (limiting) for 18 h at room temperature.

Results

Initial gel runs revealed no detectable Beta-lactamase activity after staining with nitrocephin solution (500 mg/L)

It was therefore possible that the concentration of particularly, urea and Triton X-100 in the gel might have been denaturing the enzyme to such an extent that it was losing its biological activity. Controls were prepared of each enzyme containing equivalent concentrations of urea and Triton X- 100. These were probed for biological activity with the nitrocephin spot test over a time period of up to 18 h. In all cases the biological activity of the enzyme remained unaffected.

Acinetobacter strains

At this point chronologically we narrowed our interest down to ten strains of *Acinetobacter* from the survey. They had been selected on the basis of their resistance to aztreonam and had MIC values of $\geq$ 4 mg/L (see Table 54)

All subsequent work with IEF gels, M_r estimates and biochemical characterisation was carried out on 9 of these 10 strains. Conventional IEF on polyacrylamide showed that strain HI51 as well as possessing a chromosomal

Beta-lactamase also carried a TEM-2 plasmid-mediated Beta-lactamase. Therefore it was excluded from further study.

Table 54
Ten strains of Acinetobacter studied: sources & MIC to aztreonam

Strain	sub-species	New species	Source	AZ MIC
5B86	*var.lwoffii*	*A.lwoffii*	blood	16
6B230	*var.anitratus*	*A.junii*	blood	32
H17	*var.anitratus*	*A.baumannii*	urine	64
H26	*var.anitratus*	*A.baumannii*	sinus swab	32
H63	*var.anitratus*	*A.baumannii*	wound swab	128
H68	*var.anitratus*	*A.baumannii*	urine	64
H126	*var.lwoffii*	*A.junii*	wound swab	>256
H141	*var.anitratus*	*A.baumannii*	wound swab	64
H151	*var.lwoffii*	*A.junii / A.lwoffii*	blood	8.0
H162	*var.anitratus*	*A.baumannii*	wound swab	64

Sample solubilisation

A possible explanation for the failure of the initial experimentation with the urea/Triton polyacrylamide gels was that the sample itself required solubilisation before being placed on the gel. Therefore a sample concentrate was devised whose composition is shown in Table 55.

Table 55 Composition of the sample concentrate

Material	Supplier	Volume
40% ampholines w/v	LKB	
pH 3.5-10		100μL
pH 9-11		100μL
Triton X-100 (10% w/v)	BDH	200μL
β–mercaptoethanol	Sigma	100μL

Sample concentrate was added to each sample in the ratio of 1 μl of sample concentrate for each 5 μl of Beta-lactamase in an Eppendorf tube. The total sample volume was not more than 120 μl.

Results after sample solubilisation

After the above sample solubilisation, activity was again detected by nitrocephin staining and, rather than ill-defined areas of activity, lines of focusing were beginning to appear.

It was also noted that the time for the colour change to occur in the gel (from yellow to red) was taking a considerably greater length of time compared to conventional IEF gels. In fact staining took 30 - 60 minutes at 37°C. The gel during that period was wrapped in "clingwrap" to prevent it drying out.

Sample solubilisation and saturation with urea

The next step was to increase the solubilisation of the sample. To do this urea was added to the sample + sample concentrate in an Eppendorf until the sample was saturated.

The excess urea was pelleted by centrifugation (30s at 2874 g, MSE Microcentaur). After considerable experimentation, there appeared to be a critical

amount of urea required to give optimal "focusing". Too little resulted in poorer focusing and too much affected the activity of the enzyme. The technique was therefore very difficult to standardise.

This was especially true if ambient room temperatures fell below 10°C when the urea began to precipitate out of solution.

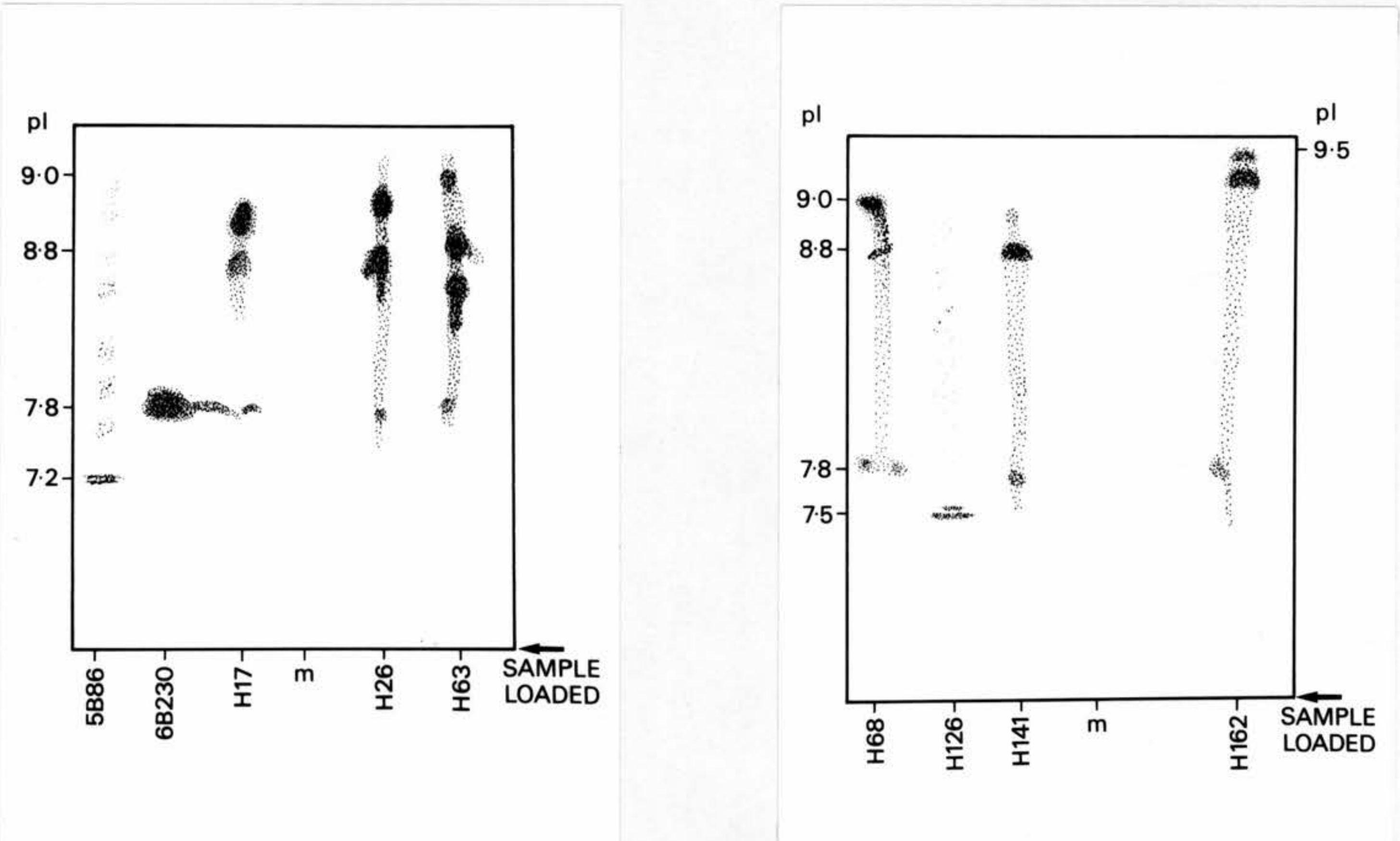
After running some 12 individual gels, there appeared some degree of similarity amongst 5 of the strains with clear differences within the other 4 strains. These are shown in Figure 14.

From these gels a tentative classification of the Beta-lactamases was made (Table 56). H17, H26, H63, H68 and H141 all had what appeared as 3 predominant areas of activity in each gel. Namely at pI 9.0, 8.8 and 7.8. These were all *A. baumannii* strains. 6B230 focused best with bands around pI 7.8. It was felt that all the other enzymes H17, 26, 63, 68 and 141 might well, if given the same conditions, focus in the same manner. 6B230 was a *A junii* strain. H162 another *A. baumannii* strain was very similar to the first group but also had pronounced bands of activity at pI 9.3 and 9.5. The other strains 5B86 (*A.lwoffii*) and H126 (*A.junii*) produced completely different bands of activity at pI 7.2 and pI 7.5 respectively.

Although a tentative classification was possible, the technique was clearly not optimal. There was still considerable distortion of the polyacrylamide during focusing. The slow staining was also a problem with 50 mg of nitrocephin being required to stain one gel properly.

It was clear that solubilility was still the major problem and the distortion of the gels perhaps was due to sieving in the polyacrylamide - suggesting that the M_r of these enzymes were high.

Figure 14



Isoelectric focusing of the β-lactamases produced by the 9 strains of *Acinetobacter*, employing a polyacrylamide gel incorporating urea and Triton X-100, with sample solubilisation. The enzyme activity was identified with nitrocephin. These gels should be compared with those seen in fig.16 which show the same enzyme preparations electrophoresed on a conventional IEF gel.

Table 56
Tentative classification of Acinetobacter chromosomal β-lactamases

ACE group	Strain(s)	pI(s)
Ia	H17, H26, H63, H68 & H141	7.8, 8.8, 9.0
Ib	6B230	7.8
2	H162	7.8, 9.3, 9.5
3	H126	7.5
4	5B86	7.2

BETA-LACTAMASE MOLECULAR MASS DETERMINATIONS

This was carried out in conventional low pressure gel permeation columns with large scale preparation of crude cell extracts.

Each enzyme was first applied to a calibrated G-75 Sephadex column. All but two enzymes appeared in the void volume and in those the procedure was repeated with a Sephacryl S- 300 column. The M_r of these enzymes is shown in Table 57.

Table 57
Molecular size of the Acinetobacter β-lactamases

Strain	Molecular size (Kd)
5B86	>1000
6B230	>1000
H17	640
H26	>1000
H63	580
H68	>1000
H126	32.5
H141	>1000
H162	60.5

It was therefore clear that only two enzymes H126 and H162 had an M_r that one would expect of chromosomal Beta-lactamases in Gram negative aerobic bacilli. Interestingly, the one with the lowest M_r (H126) at 32.5 Kd was the only enzyme which focused in a conventional IEF polyacrylamide gel. Seven of the others had an M_r ranging from 580 Kd to >1000 Kd. These enzymes were clearly different to any other Beta-lactamase so far described. This finding indeed suggested that the polyacrylamide matrix was too tight for optimal separation of these large enzymes. It also set the interesting question as to why these enzymes appeared so large in low pressure gel permeation systems. Was it because they

were attached to other cell membrane components? Or was it because they exist in multi-subunit form?

AGAROSE UREA AND SORBITOL (AUS) GELS

It was not very easily to increase the porosity of the polyacrylamide gel further. This would have required a lower concentration of acrylamide (we had already increased the concentration of the crosslinker). A lower concentration of acrylamide leads to a gel with poor mechanical stability - unsuitable for production in a vertical cassette. Increasing the crosslinker was also likely to increase the hydrophobicity of the gel. Therefore agarose, with a macroporous structure that minimises molecular sieving effects, appeared to be the matrix of choice, especially with proteins of the molecular size that we were wishing to study.

Vecoli *et al.* (1983) compared the use of an agarose gel IEF with that of a conventional polyacrylamide gel IEF in characterising low molecular weight Beta-lactamases. They showed good correlation with the isoelectric points in most samples tested. Differences were found in only two cases. HMS-1 produced two highly resolvable enzyme bands in agarose gels rather than the single faint band seen on a polyacrylamide gels. The PSE-1 enzyme showed a 0.2 pH unit shift between polyacrylamide and agarose (5.7 and 5.5 respectively).

Olsson & Laas (1981) described a method for isoelectric focusing in agarose under denaturing conditions. They pointed out the following:

1. Agarose of standard quality cannot be used for IEF since it contains a high content of charged groups, this gives rise to severe endosmosis and consequently disruption of the gel. Agarose-IEF has an extremely low concentration of negatively charged groups and the effect of these are counter-balanced by

positively charged groups that are covalently bound to the matrix. This results in a gel with very little endosmosis.

2. The presence of urea in an agarose gel disturbs the well organised structure of the agarose matrix. This matrix is extensively hydrogen bonded and urea is a typical H-bond breaking agent. This results in a much lower gelling temperature, an increased time for gelling and a gel of lower mechanical strength.

3. Urea and ammonium cyanate form an equilibrium pair. Therefore there is always a risk of carbamylation when using urea especially of the proteins under study. "The risk is fairly low with fresh urea solutions at room temperature, but increases as the temperature rises."

Olsson and Laas (1981) solved the problems of agarose/urea incompatibility by:

(a) increasing the agarose concentration in the gel to 2% (for 8M urea)

(b) incorporating 10% sorbitol into the gel.

(c) scavanging the cyanate ions by employing 1% β-mercaptoethanol in the agarose matrix.

(d) allowing the gel solution to set overnight at room temperature.

We therefore devised a gel containing agarose, urea, sorbitol and Triton X-100 based on the polyacrylamide, urea, Triton X-100 gel previously described ,taking into account the findings of Olsson and Laas (1981). The composition of this gel is shown in Table 58. It was found that a 1% agarose gel gave optimal results, the higher (2%) concentration resulted in fracturing of the gel followed by gel disintegration resulting from high field strength around the fractures.

Table 58
Composition of AUS gel

Material	Supplier	Volume or mass employed	Final concentration (approx)
Urea	BDH	10g	4.3 mol/L
Distilled water		24.6ml	
Agarose-IEF (VIII)	Sigma	377mg	1%
D-sorbitol	BDH	4g	10%
Triton X-100 (10%w/v)	BDH	10.2ml	2.5%
40% ampholines w/v pH 3.5-10	BDH	2.5ml	2.0% w/v
β-mercaptoethanol	Sigma	0.5ml	1%

AUS gel preparation

AUS gels were prepared by melting 377 mg of agarose (agarose for isoelectric focusing) in 15 ml of distilled water in a microwave oven (550 W) for 30 - 40 seconds. This was then allowed to cool to 60 ^{0}C in a waterbath.

Four g of D-sorbitol were added to 9.6 ml of distilled water and heated to 60^{0}C in the waterbath. Similarly 10.2 ml of 10% (w/v) Triton X-100 were added to 10 g of urea and placed in the 60^{0}C waterbath. The sorbitol solution and urea in Triton X-100 were then added and mixed. Just before incorporation of this mixture into the agarose, 2.5 ml of pH 3.5 - 10.6 ampholines (LKB) and 0.5 μl Beta-mercaptoethanol were added. The final mixture of 1% agarose, 4.3 mol/L urea and 10% sorbitol was then gently stirred at 60^{0}C and drawn up into a preheated glass pipette. The solution was then pipetted onto the hydrophobic surface of a GelBond sheet (LKB) which had been placed on the LKB 2217

ultrophor electrofocusing unit. A few drops of kerosene were spread over the surface of the cooling unit to ensure good contact between the GelBond and the cooling unit. The gel had approximately a 1 mm overall thickness. The gel was left overnight (with the lid firmly down to prevent the gel drying out) to achieve mechanical strength.

Sample preparation

The Beta-lactamase samples used for AUS gel electrophoresis were obtained from the pooled peak fractions obtained from the gel permeation experiments.

The sample size was equalised with time as described before. Five μl of Beta-lactamase were added to 1 μl of sample concentrate. The composition of the sample concentrate was as described in Table 55 apart from 200 μl of ampholines (40% w/v) pH 3.5 - 10.6 instead of a 50:50 mixture of pH 3.5 - 10.6 and pH 9 - 11.

As before, just prior to application to the gel the sample mixture was saturated with urea. The excess urea was pelleted by brief centrifigation.

The samples (and isoelectric point markers) were applied to the surface of the gel but near the middle - rather than 2 cm from the anode as with conventional polyacrylamide IEF gels.

Running Conditions

Electrode wicks (LKB) were applied to the gel, the anode soaked in O.1N phosphoric acid (BDH Analar) and the cathode soaked in O.1N NaOH (BDH Analar). The electrofocusing unit was maintained at 15 - 20°C since urea precipitates at temperatures below 15 ^{O}C.

A constant power was maintained at 30 W with the voltage and current set at the limiting values of 1500 V and 30 mA respectively.

Gels were electrophoresed until the pI markers had focused i.e. around 2.5 h.

Staining

AUS gels were stained with nitrocephin solution (500 mg/L) as described previously . As with the urea/polyacrylamide gels almost 100 ml of nitrocephin solution was required to visualise the bands of Beta-lactamase activity. The staining period being 30 minutes to 1 hour.

The problem was exacerbated by the need to use 1N NaOH as the cathode buffer. This strong alkali hydrolysed the nitrocephin causing the gel in the cathodic area to turn red. This made visualising the Beta-lactamase bands near the cathode difficult. The problem was partially overcome by overlaying this area with a weak acid prior to staining with nitrocephin.

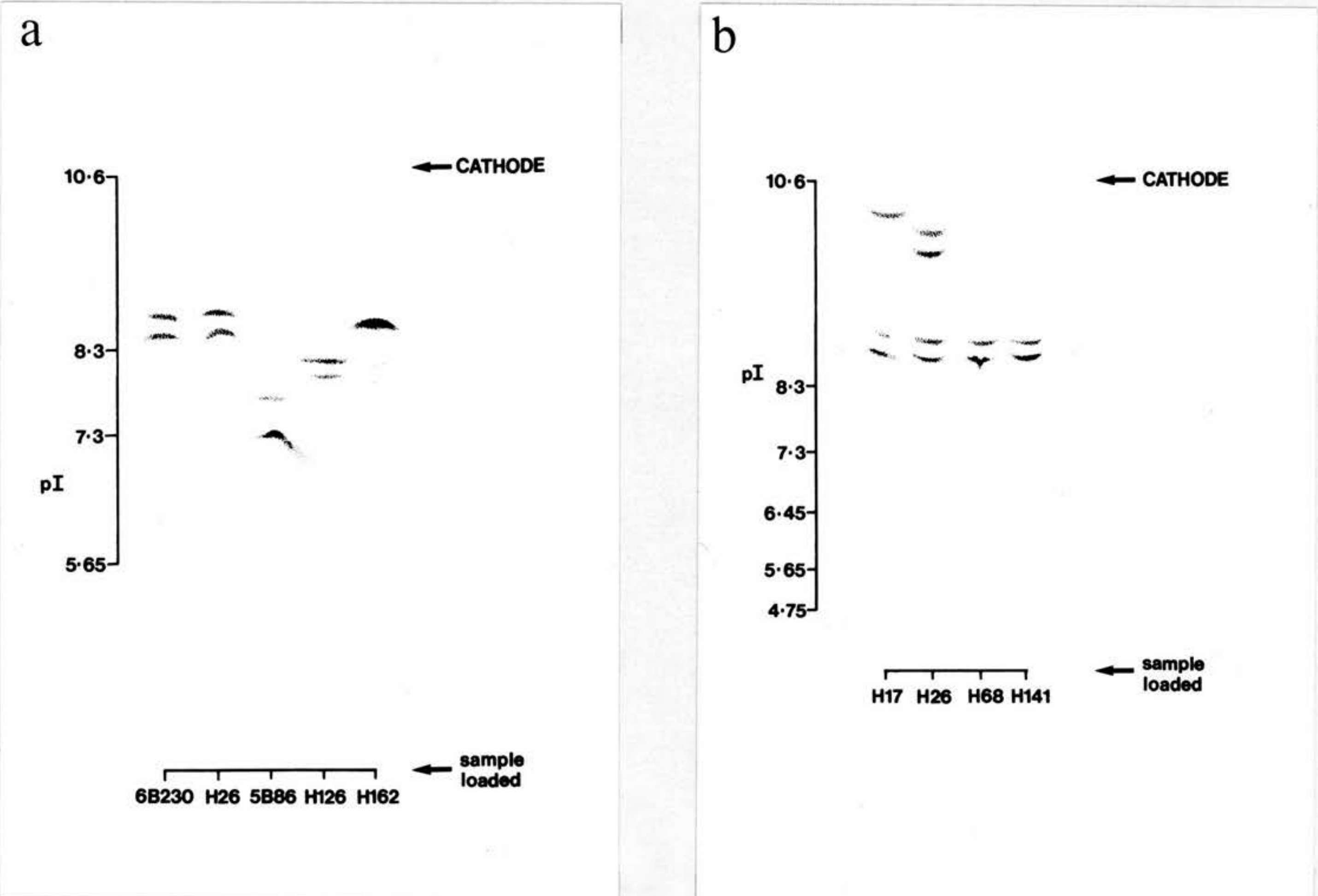
Results

The AUS gel system was initially tried without the sample solubilisation step but poor focusing resulted. After some experimentation eight of the nine Beta-lactamases focused on two separate gel runs. (See figure15). Figure 16 is also included to show the same enzymes electrophoresed on a conventional polyacrylamide IEF gel for comparison.

The Beta-lactamase from H63 (not shown) was found on another AUS gel run to be identical to 6B230.

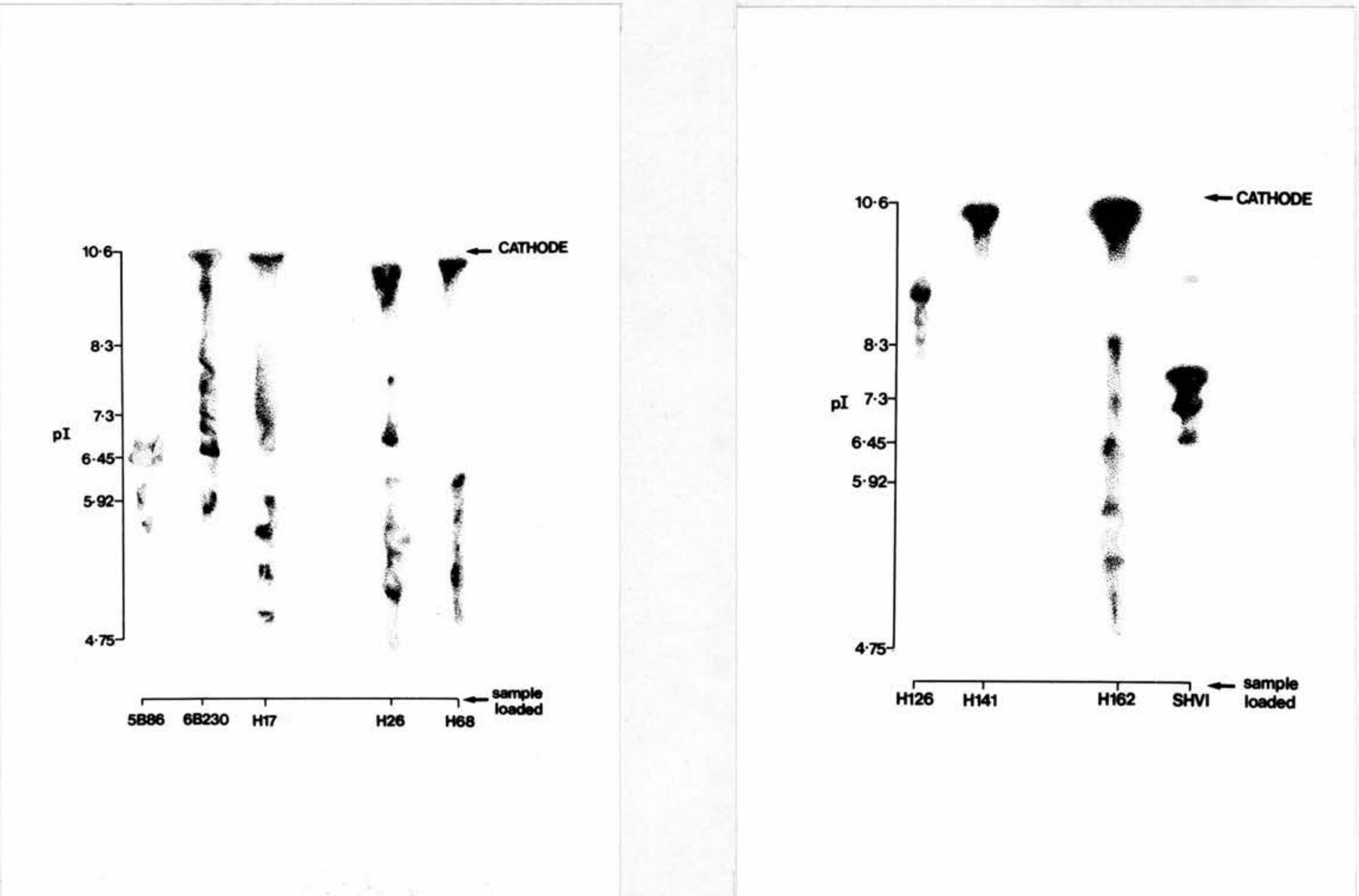
Therefore there appeared to be at least four different focusing patterns on AUS gels. Six of the nine enzymes have very similar focusing patterns; the Beta-lactamases from strains H17, H26, H63, H68, H141 and 6B230 all had major bands at pI 8.6 and 8.8. However the enzyme from H26, which was run on both Gel 15a and Gel 15b as an internal control, had additional minor bands at pI 9.8 and 10.1 on Gel 15b. H17 also had a minor band at pI 10.3. The other three enzymes were completely different, H162 had a single band at pI 8.7; H126 has a

Figure 15



Agarose, urea and sorbitol (AUS) IEF gels. Focused bands of *Acinetobacter* chromosomal β-lactamase, activity identified with nitrocephin

Figure 16



Conventional polyacrylamide IEF gels of *Acinetobacter* chromosomal β-lactamases and the plasmid β-lactamase SHV-1. Enzyme activity identified with nitrocephin.

major band at pI 8.2 and a minor band at pI 8.1 and 5B86 has a major band at pI 7.3 and a minor band at pI 7.7.

Table 59

ACE + types by AUS * gel isoelectric focusing

Strain	Species	β-lactamase ACE type
5B86	*A.lwoffii*	4
6B230	*A.junii*	1
H17	*A.baumannii*	1
H26	*A.baumannii*	1
H63	*A.baumannii*	1
H68	*A.baumannii*	1
H126	*A.junii*	3
H141	*A.baumannii*	1
H162	*A.baumannii*	2

ACE+ = Acinetobacter chromosomal enzyme
AUS* = Agarose / Urea / Sorbitol

Therefore at least four different *Acinetobacter* chromosomal enzymes (ACE) types were characterised by this technique, ACE 1 to ACE 4 (see Table 59). The same four groups (with the same order of pI's) were found with the polyacrylamide/urea IEF system but there was poorer focusing and more gel distortion.

BIOCHEMICAL CHARACTERISATION OF THE ACE ENZYMES

The biochemical characterisation of the nine *Acinetobacter* Beta-lactamases essentially follows the recommendations for the evaluation of novel Beta-lactamases as suggested by Bush & Sykes (1986) and Bush (1989 a).

Biochemical characterisation was first carried out by measuring the specific activity of the nine *Acinetobacter* strains against nine penicillin, cephalosporin and monobactam substrates.

Antimicrobial sensitivities

The MIC values of the strains to these substrates are shown in Table 60 . The results showed that all the *Acinetobacter* strains studied had a high degree of resistance to penicillins and first, second and third generation cephalosporins. There was some correlation between the resistance levels and the enzyme produced. All the strains encoding the ACE-1 enzyme had very similar MICs to all the drugs tested. H162, which encoded ACE-2, could not be distinguished from the ACE-1 strains on its resistance profile. However, strain H126, which encoded ACE-3 was generally more resistant to the drugs tested than the ACE-1 or ACE-2 producers. Specifically, H126 was more resistant to the third generation cephalosporins and the monobactam, aztreonam. On the other hand, strain 5B86, which encoded ACE-4, was generally less resistant than the other ACE producers. It was, particularly, less resistant to the penicillins and the first generation cephalosporins tested.

Substrate profiles

The enzyme preparations were partially purified by gel permeation. The spectrophotometric assay employed was that of O'Callaghan *et al.* (1972).

Table 60

MIC (mg/L) of ACE producing strains to a selection of cephalosporins, penicillins and a monobactam.

Strain	ACE type	AZ	CAZ	CTX	CXM	CER	CED	PENG	AMP	CARB
5B86	4	16	2	4	8	8	16	16	8	16
6B230	1	32	8	4	16	64	128	>32	128	64
H17	1	64	4	32	64	64	256	>32	32	32
H26	1	32	8	32	64	256	>256	>32	64	32
H63	1	128	16	64	128	128	>256	>32	128	128
H68	1	64	8	16	32	64	256	>32	32	32
H126	3	>256	64	64	64	128	256	>32	128	128
H141	1	64	8	16	64	64	256	>32	64	32
H162	2	64	8	32	64	64	256	>32	32	32

Table 61
Relative rates of hydrolysis* of the ACE enzymes.

Strain	ACE type	NIT	CER	CED	CXM	CTX	CAZ	AZ	PENG	AMP	CARB
5B86	4	100	1.8	-	-	-	-	-	-	-	-
6B230	1	100	7.9	1.3	-	-	-	-	0.1	-	-
H17	1	100	1.9	1.5	-	-	-	-	0.1	-	-
H26	1	100	4.7	-	-	-	-	-	0.3	-	-
H63	1	100	1.2	0.8	-	-	-	-	0.4	-	-
H68	1	100	1.4	0.9	-	-	-	-	0.3	-	-
H126	3	100	1.6	0.3	-	-	-	-	0.6	-	-
H141	1	100	0.9	0.3	-	-	-	-	0.1	-	-
H162	2	100	3.6	0.7	-	-	-	-	0.2	-	-

*Relative rates of hydrolysis values are expressed as a percentage of the value for nitrocephin.
- no detectable activity

Cephalosporins and the monobactam were used at concentrations of either 100 μM or 1000 μM and the penicillins at a concentrate of 1000 μM

The relative rates of hydrolysis of the nine enzymes are presented in Table 61. The values were expressed as a percentage of the value for nitrocephin which was the substrate most rapidly hydrolysed by all these enzymes (Bush & Sykes, 1986). Cephaloridine was also hydrolysed, albeit much more slowly, by all the enzymes. Cephradine was hydrolysed (again much slower than the nitrocephin) by all the enzymes apart from 5B86 and H26. All the enzymes apart from 5B86 hydrolysed penicillin G but at around 1000 times more slowly than nitrocephin. There was no detectable hydrolysis of cefuroxime, cefotaxime, ceftazidime, aztreonam, ampicillin or carbenicillin by any of the nine enzymes.

These results show no clear differences in the substrate profiles of the enzymes, however, they are clearly all cephalosporinases rather than penicillinases or broad spectrum enzymes.

Kinetic parameters of the ACE enzymes

The kinetic parameters (K_m and V_{max}) of all nine enzymes were calculated by measuring the rate of hydrolysis at limiting substrate (nitrocephin and cephaloridine) concentrations and constructing Lineweaver - Burk plots (data not shown). The K_m and V_{max} values are shown in Table 62.

The K_m values obtained with both nitrocephin and cephaloridine were broadly similar to each other. The K_m values to cephaloridine ranged from 150 μM to 710 μM and were similar to the values of *Acinetobacter* Beta-lactamases found by Morohoshi & Saito (1977) and Hikida *et al.* (1989) of 250 μM and 511 μM respectively. These results show that all four ACE enzymes have moderate affinity for cephaloridine and nitrocephin. All the K_m values were within the same order of magnitude and could not convincingly be distinguished from one

Table 62
Kinetic data for the ACE enzymes

		NITROCEPHIN			CEPHALORIDINE		
Strain	**ACE type**	**Vmax**	**Km**	**Vmax/Km**	**Vmax**	**Km**	**Vmax/Km**
5B86	4	0.027	220	100	0.005	710	5.8
6B230	1	0.83	280	100	0.044	150	9.7
H17	1	0.71	550	100	0.010	160	4.6
H26	1	0.36	250	100	0.014	150	6.3
H63	1	0.20	250	100	0.0048	170	3.8
H68	1	0.63	280	100	0.0083	180	2.2
H126	3	0.11	150	100	0.0083	380	2.7
H141	1	0.34	220	100	0.005	210	1.3
H162	2	0.20	250	100	0.010	260	5.0

Efficiency of hydrolysis values (Vmax/Km) are expressed as a percentage of the value for nitrocephin.
Km values are measured in μM and Vmax values are measured in μmol/minute/ml of enzyme.

another. Thus none of the ACE enzymes demonstrated greater substrate affinity than the others and K_m values were a poor discriminator.

All the enzymes hydrolysed nitrocephin at similar rates apart from ACE-4 (5B86) which is one order of magnitude lower than the others. All the enzymes hydrolysed cephaloridine at similar rates and between one to two orders of magnitude lower than the rates of hydrolysis for nitrocephin. The enzymes all have very similar relative efficiency values (V_{max}/K_m) for the hydrolysis of cephaloridine (Table 62).

Therefore hydrolysis rates and relative rates of efficiency were poor discriminators of the ACE enzymes.

Inhibition Studies

Table 63 shows the effect of the Beta-lactamase inhibitors aztreonam, cloxacillin and clavulanic acid, expressed as the concentration required for 50% inhibition (ID_{50}) of enzyme activity. The enzymes from all the strains were readily inhibited by cloxacillin. Most were inhibited within the range of 0.003 - 0.022 µM. However, two enzymes required at least 0.1 µM cloxacillin for 50% inhibition. One of these was an ACE-1 but this level of cloxacillin is still considered to be very low. Thus cloxacillin inhibition also seems to be a poor discriminator of the ACE enzymes, although a characteristic feature of them all is that they are cloxacillin-sensitive.

Table 63

***ID_{50}(µM) of ACE enzymes with Nitrocephin as substrate.**

Strain	ACE type	Aztreonam	Cloxacillin	Clavulanic acid
5B86	4	>100	0.18	>100
6B230	1	28	0.012	>100
H17	1	8	0.005	>100
H26	1	5	0.022	>100
H63	1	2	0.016	>100
H68	1	9	0.1	>100
H126	3	0.08	0.003	>100
H141	1	1.8	0.02	>100
H162	2	23	0.022	>100

*ID_{50}(µM) = amount of inhibitor required to inhibit 50% of hydrolysis of Nitrocephin

Similarly, the enzymes from all the strains were extremely resistant to clavulanic acid inhibition. The inability of any of these enzymes to be inhibited significantly by 0.1 mM clavulanic acid shows that clavulanic acid resistance is a characteristic feature of all these enzymes but is incapable of being used as a discriminator.

Differences in inhibition were, however, identified when aztreonam was used as an inhibitor. There was little difference in the ID_{50} for the ACE-1 and ACE-2 enzymes which were in the range of 1.8 to 28 μM. The ACE-3 enzyme from strain H126 was much more sensitive to aztreonam inhibition than the others (ID_{50} = 0.08 μM), while the ACE-4 enzyme from strain 5B86 was much more resistant (ID_{50} = >100 μM). Therefore the inhibitor profiles with aztreonam provide good discrimination of the ACE-3 and ACE-4 enzymes.

Table 64 shows the effect of 1 mM EDTA, 1 mM $HgCl_2$ and 0.1 mM p-chloromercurobenzoate (pCMB)

All the enzymes were inhibited to the same extent by a fixed concentration of $HgCl_2$ but were virtually unaffected by moderately high levels of pCMB. Thus no discrimination between these enzymes could be obtained from these profiles.

The effect of 1mM EDTA on the enzymes, with either nitrocephin or cephaloridine as the substrate, was variable although generally below 20%. EDTA had no effect on the *Acinetobacter* Beta-lactamase described by Hikida *et al.* (1989). However since many of the enzymes described here are of large M_r and, therefore may exist in subunit form, it is possible that metal ions play a role in their function. Therefore further experiments with a more purified enzyme will be required to elucidate these findings.

Table 64
Effect of inhibitors and metal ions on ACE enzymes with nitrocephin as substrate

Strain	ACE type	Percentage inhibition		
		EDTA*	$HgCl_2^+$	pCMB ++
5B86	4	<20	89	0
6B230	1	<20	98	0
H17	1	<20	96	0
H26	1	<20	97	0
H63	1	<20	98	0
H68	1	<20	95	0
H126	3	<20	95	0
H141	1	<20	98	0
H162	2	<20	98	0

EDTA* 1mM
$HgCl_2^+$ 1mM
pCMB++ 0.1mM

INDUCTION EXPERIMENTS

Induction experiments were carried out on all nine strains employing cefoxitin as the inducer at one quarter of the MIC value for the strain. The method employed was that described by Minami *et al.* (1980).

No discernable Beta-lactamase induction was found with any of the strains studied.

CONJUGATION EXPERIMENTS

Conjugation experiments were carried out on all nine strains as previously described in the Materials and Methods.

However, despite varying the times of incubation of the mixture of the donor and recipient, these conjugation experiments were unsuccessful and no transconjugants were detected.

Perhaps, plasmid curing experiments should have been carried out (for the sake of completeness) but it is most unlikely that these enzymes are plasmid mediated.

CONCLUSIONS

The above data therefore suggests that all the *Acinetobacter* (ACE) enzymes studied are non-inducible, chromosomal cephalosporinases of Bush Group One (CEP-N) (Bush, 1989b).

FAST PROTEIN LIQUID CHROMATOGRAPHY

The work on the development of the AUS isoelectric focusing technique showed that the chromosomal Beta-lactamases of *Acinetobacter* could be separated on the basis of their charge. However it also showed that this technique was not ideally suited to the screening of a large population of Acinetobacter. This was for two reasons. Firstly it was technically demanding and was not guaranteed to work every time and secondly it was enormously expensive in terms of the amount of nitrocephin used in each run. This was in the region of 50 - 100mg per gel and was therefore unsustainable. Therefore some other technique for easy separation was required.

The work on the M_r of these enzymes employing conventional low pressure gel permeation chromatography also encouraged us to pursue the reasons for the enzymes' apparent large size. To measure the M_r of these enzymes by conventional SDS-PAGE techniques requires the enzyme(s) to be purified to homogeneity since nitrocephin can no longer be used as a probe for the Beta-lactamase activity in these denaturing conditions. The most appropriate techniques to continue along these lines of investigations were :-

(i) Fast liquid Protein Chromatography (Pharmacia) employing initially high performance ion exchange chromatography.

(ii) Reversed polarity native PAGE for basic proteins on the PhastSystem (Pharmacia) employing nitrocephin as the probe for Beta-lactamase activity.

(iii) SDS-PAGE with silver staining on the PhastSystem.

Fast Protein Liquid Chromatography of the ACE Beta-lactamases

FPLC was an appropriate technique for the purification of these enzymes because of:

a) its high resolution

b) its high recoveries of sample mass with retention of structural integrity and biological activity

c) its high loading capacities - up to 50mg of protein could be loaded onto each 1ml column

d) its fast separation time - in exchange chromatography runs could be completed in 30 minutes with a 1 hour turnaround

These attributes coupled with the rapidity of the nitrocephin spot test makes FPLC the most powerful technique so far described for the separation and purification of Beta-lactamases.

Initially the remainder of the frozen pooled aliquots of the *Acinetobacter* Beta-lactamases derived from the low pressure gel permeation chromatography were applied to the FPLC system.

FPLC Method

Two ml of the partially purified enzyme (containing between 0.02 - 3.3 mg protein/ml) equilibrated in 50 mM sodium phosphate buffer pH 7.0 (Buffer A) were passed through a 0.2 micron filter (Gelman) and applied to a MONO S (HR 5/5, 1ml) cation exchange column. The system consisted of a LCC 500 Plus controller, UV-M monitor (protein measured at 280 nm) and FRAC 100 collector. The column was equilibrated with Buffer A.

The flow rate was 1 ml/min. Separation was achieved by elution with a linearly increasing concentration of sodium chloride (in Buffer A) to a concentration of 1M. Thirty-four fractions (1ml) were collected from each

separation. Each aliquot was probed for Beta-lactamase activity with the nitrocephin spot test as described previously. Beta-lactamase activity was therefore correlated to the retention volume and the protein peaks (if present).

The results from enzymes produced from 8 of the 9 strains can be seen in Table 65. Insufficient enzyme (ACE-4) from 5B86 remained from the original gel permeation and enzyme characterisation experiments.

Table 65

FPLC of aliquots of partially purified β-lactamase, from low pressure gel permeation, on MONO S ion exchange.

Strain no.	ACE type	Retention volume of activity (ml)
6B230	1	16
H17	1	16/17
H26	1	17/18
H162	2	15/16
H63	1	12/13
H68	1	12/13&15/16
H141	1	12/13&17
H126	3	4

It can be seen that there were at least 3 groupings of retention volumes with these 8 enzymes. The ACE-1 and ACE-2 enzymes appear indistinguishable by this technique with retention volumes of around 15 to 18 ml. However, there appeared to be a subgroup of ACE-1 enzymes e.g. with the enzymes from H63, H68 and H141 - major activity existed at 12 - 13 ml, but in the cases of H68 and H141 - activity also sometimes appeared at 15 - 17 ml. The ACE-3 enzyme produced from H126 was clearly different with a retention volume of 4 ml. The

latter result suggested that ACE-3 would be more appropriately separated on a MONO-Q high performance anion exchange column.

Very small or no protein peaks were visible at the fractions that corresponded with the areas of Beta-lactamase activity except in the case of ACE-3 which was essentially unseparated in the midst of other proteins in the void volume.

A more significant problem however became apparent, namely the loss of biological activity. All the 'purified' Beta-lactamases (except ACE-3) lost their activity within 24 - 48 hours whether they were stored at $4^{o}C$, $-20^{o}C$ or $-70^{o}C$; or if they were dialysed overnight against sodium phosphate buffer (50mM, pH 7).

Preservation of activity with β-lactoglobulin A

After 'purification' of the Beta-lactamase by ion exchange chromatography the loss of biological activity could be prevented by the addition of β-lactoglobulin A (to a concentration of 1%) to the fractions containing the activity.

It is therefore likely that the loss of biological activity was due to dilution and removal of the protein from its normal cellular environment. This is also inferred by the fact that H126 - ACE-3 did not lose its activity after passage through a MONO-S column, presumably because it remained in solution with many other proteins in the void volume.

The results of these findings therefore made further investigation into the characteristics of these proteins a little more difficult. To investigate their properties on reversed polarity SDS-free PAGE or by SDS-PAGE on the PhastSystem all of them had to be purified by ion exchange on the same day as their application to either technique.

Application of crude Beta-lactamase preparations onto the FPLC with a MONO-S column

All of the partially purified Beta-lactamase from pooled aliquots from the conventional gel permeation chromatography were employed during the preliminary experimentation and it was not possible to repeat the larger column work within the constraints of time.

It was noted that the columns could take up to 50 mg of protein per run and that the protein concentrations of crude Beta-lactamase preparations derived from the induction experiments contained in the range of 10 - 15 mg/ml. Therefore, 1 ml of crude enzyme diluted to 2 ml with 50 mM phosphate buffer (pH 7) was added to the MONO-S columns as before. Crude enzyme preparations of ACE-3 (H126) and ACE-4 (5B86) were made in 25 mM Tris/HCl pH 8 and applied to a MONO Q anion exchange column and run as described for MONO-S columns (the eluant being Tris/HCl pH 8).The results are seen in Table 66.

Application of crude preparations of the *Acinetobacter* Beta-lactamases gave very similar results to the application of the same partially purified enzymes except that in each case an ionic variant appeared in the void volume, albeit weak. The results also confirmed that ACE-2 is very similar to ACE-1 and that ACE-3 and ACE-4 are completely different. However, there did seem to be variation within the ACE-1 group with major activities either at a retention volume of around 16 to 18 ml or 12 to 13 ml. However there was usually weak activity, in each case, at the other retention volume e.g. H26 with major activity at 18 ml and weak activity at 2/3 ml and 11/12 ml; H63 with major activity at 12 ml and weak activity at 2/3 ml and 17/18 ml.

Table 66

FPLC of crude ACE enzymes on high performance ion exchange.

Strain no /column	ACE type	Retention volume of activities (ml)
MONO S		
Cation exchange		
6B230	1	2,3;15*/16
H17	1	2,3;12;17/18*
H26	1	2,3;11/12;18*
H162	2	2,3;15*/16
H63	1	2,3;12*;17/18
H68	1	2,3;12*/13
H141	1	2,3;13/14*
MONO Q		
anion exchange		
H126	3	2,3,4;13*
5B86	4	2,3,4;29*

*signifies the retention volume with maximum activity

REVERSED POLARITY SDS-FREE (NATIVE) PAGE ELECTROPHORESIS ON THE PHASTSYSTEM

The purified enzymes obtained from the FPLC ion exchange and crude enzyme preparations were applied to this gel system which had been devised to resolve basic proteins on a polyacrylamide mini-gel.

Method

The sample preparation and running conditions were those described by Olsson and Tooke (1988).

Sample preparation

Equal amounts of sample (in 50mM sodium phosphate buffer pH 7) were mixed with double strength sample buffer - 0.112 M acetate , 0.112 M Tris pH 6.4. 4 μL of sample were loaded onto the gel.

Buffer-strips

Table 67 shows the ingredients sufficient to make 25 buffer strips.

Table 67

Ingredient	Quantity
agarose-IEF	2g
β-alanine	4.4g
glacial acetic acid	4ml
distilled water	95ml

The components were mixed and boiled with stirring. The solution was cooled to 70°C and then poured into casting moulds and allowed to solidify by cooling to room temperature.

PhastGel mini-gel

The separations were run on PhastGel Homogeneous 20 (Pharmacia).

Running conditions

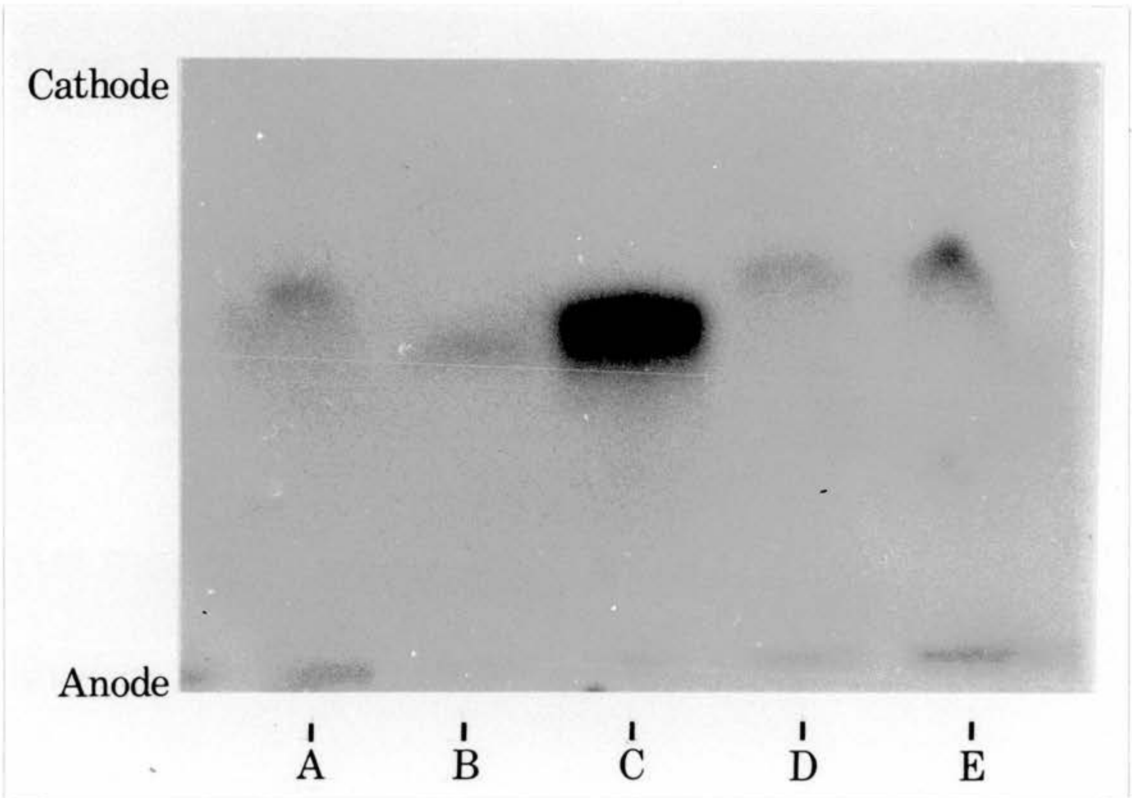
The gel was run with the polarity reversed. The separation conditions were: 200 V, 10 mA, 2.5 W at 15 ^{o}C for 100 Vh.

Staining

After electrophoresis, the enzymes were visualised by nitrocephin solution (500 mg/L).

Figure 17

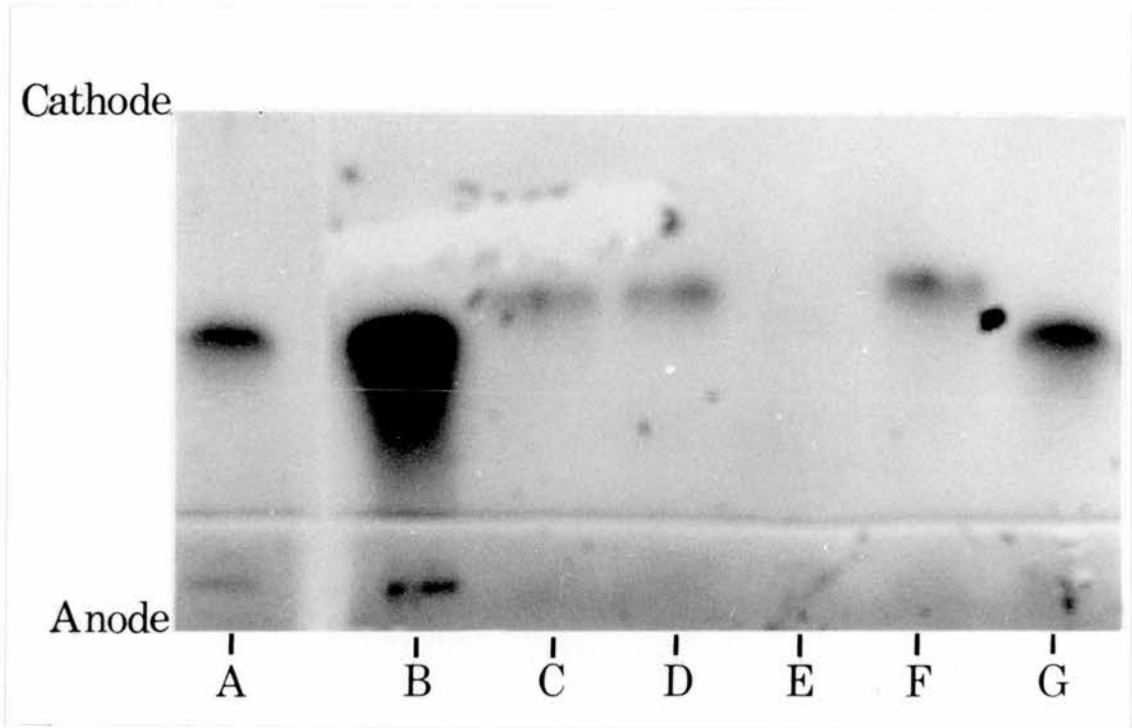
Reversed polarity SDS-free PAGE on the PhastSystem employing a Homogeneous 20 mini-gel loaded with partially purified β-lactamase preparations, stained with nitrocephin



Track A	H141
Track B	H126
Track C	SHV-1
Track D	H68
Track E	H63

Figure 18

Reversed polarity SDS-free PAGE on the PhastSystem employing a Homogeneous 20 mini-gel loaded with crude β-lactamase preparations, stained with nitrocephin



Track A	OXA-1 + TEM-2
Track B	SHV-1 + TEM-1
Track C	H68
Track D	H141
Track E	------
Track F	H162
Track G	OXA-2 + TEM-2

Results

Purified preparations of ACE-1 (H63, H68, H141 and 6B230) and ACE-2 (H162 not shown) migrated similar distances from the cathode whereas ACE-3 (H126) migrated slightly less far (see figure 17). The control SHV-1 migrated less far than ACE-1 but slightly further than ACE-3.

Interestingly 6B230 was very unstable and its activity as shown by nitrocephin lasted for a very short time only (<10 minutes).

These results were confirmed utilising crude enzyme preparations of ACE-1, ACE-2 and ACE-3 (not shown) which suggests that this system would be ideal for the screening of large numbers of *Acinetobacter* Beta-lactamases (See figure 18). The control Beta-lactamases of OXA-2, TEM-2, SHV-1 and TEM-1 migrated to appropriate positions in the gels i.e. TEM-1 and TEM-2 stayed at the cathode while SHV-1 and OXA-2 migrated to a point just behind the ACE-1 enzymes. There was insufficient ACE-4 to test on this system.

FURTHER PURIFICATION AND MOLECULAR MASS ESTIMATION OF THE ACE-1 BETA-LACTAMASE OF THE ACINETOBACTER BAUMANNII STRAIN H63 EMPLOYING FPLC AND THE PHASTSYSTEM

The above results with the FPLC suggested that the next stage was to attempt purification to homogeneity employing the FPLC with additional purification steps ending with M_r estimation on SDS-PAGE (which would also give an indication of purity).

The following purification scheme (see Harris,1989) was devised:

1. **1L of culture**
2. **Harvest** by centrifugation
3. **Disruption** by sonication
4. **Clarification** by centrifugation
5. **Ion exchange chromatography**

   FPLC with MONO-S
6. **Gel permeation** *

   FPLC with Superose 12
7. **Concentration**

   Ultrafiltration with an Amicon concentrator
8. **SDS-PAGE***

   Phast System minigel with silver stain

*Molecular size estimations were carried out at steps 6 and 8.

Steps 1 to 3 and Step 5 have been described previously, Steps 6 to 8 are described here.

Ion exchange on MONO S

For these experiments the fraction collector was set to collect individual peaks and not to collect a uniform volume as previously.

Gel permeation on FPLC

0.5 ml of the fractions containing the maximum Beta-lactamse activity from the ion exchange step were applied to a Superose 12, HR10/30 (Pharmacia) gel permeation column. The column was equilibrated with 50 mM sodium phosphate buffer pH 7 (Buffer A). The flow rate was 0.5 ml/min and each run lasted approximately 1 h. The Beta-lactamase activity was rapidly detected, as before, by the nitrocephin spot test.

Calibration of the Superose 12 column

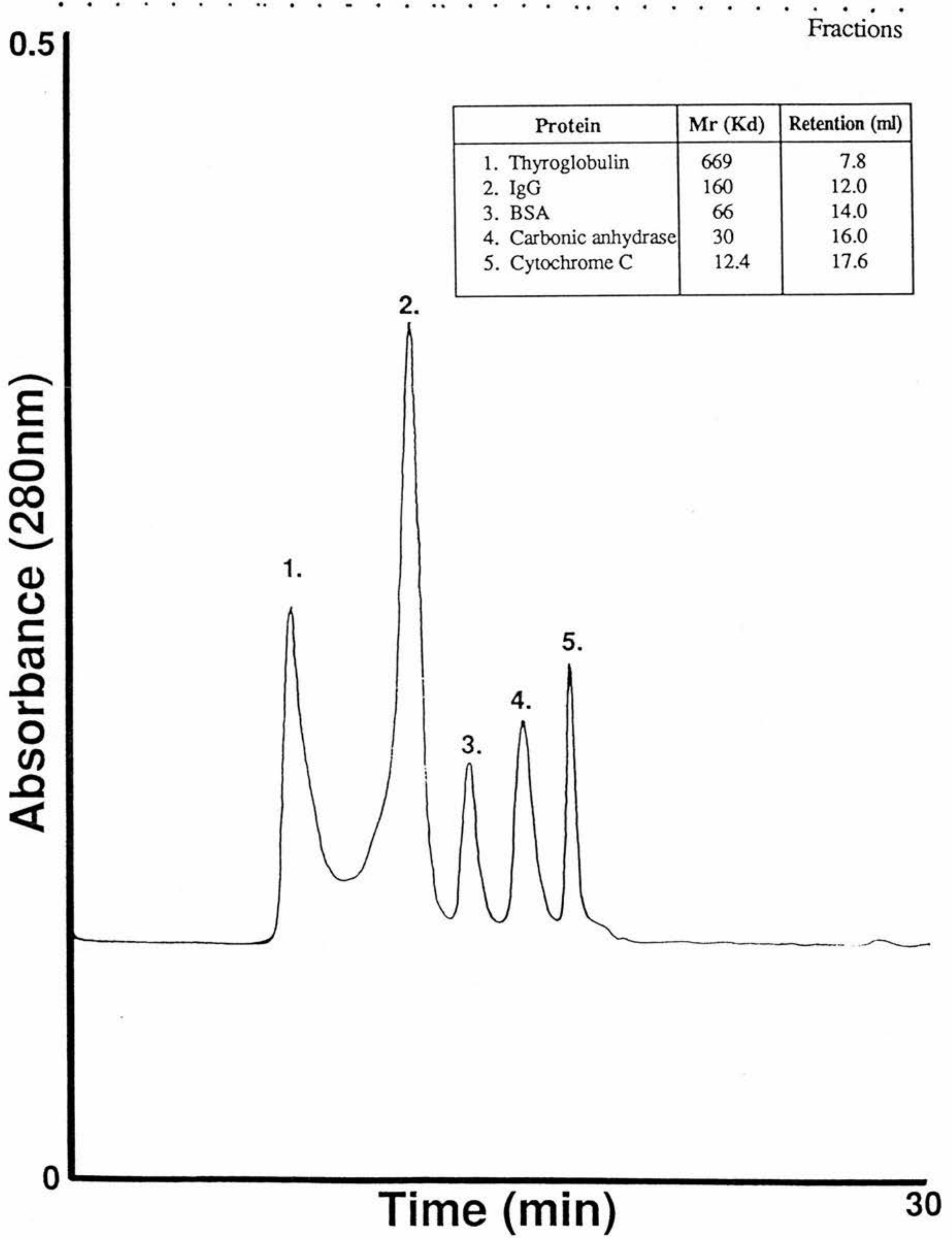
M_r estimations were carried out after the Superose 12 column had been calibrated. The column was equilibrated with Buffer A. 0.5 ml of a protein standard solution was applied to the column. The protein standard solution contained thyroglobulin, IgG, bovine serum albumin, carbonic anhydrase and Cytochrome C. The resultant protein trace is shown in Figure 19 . The retention volumes of the protein standards were plotted against log M_r and a standard curve obtained (Figure 20). The retention volumes of the Beta-lactamase peaks were then applied to the calibrated column and their M_r estimated from the standard curve.

Results

The ion exchange step on the Mono S resulted in 4 peaks of activity with retention volumes of : 2.0 ml (f2:"a"), 14.0 ml (f18:"b"), 15.1 ml (f22:"c") and 15.6 ml (f24:"d") . The activity at 2.0 ml was relatively weak .

0.5 ml of each of these peaks ("a","b","c"&"d") were then applied to the gel permeation column. The resultant protein traces and retention volumes of the Beta-lactamase activity are shown in Figures 21-25 .

Figure 19



Protein	Mr (Kd)	Retention (ml)
1. Thyroglobulin	669	7.8
2. IgG	160	12.0
3. BSA	66	14.0
4. Carbonic anhydrase	30	16.0
5. Cytochrome C	12.4	17.6

FPLC® trace of gel permeation with calibration of Superose 12, employing appropriate protein standards.

Figure 20

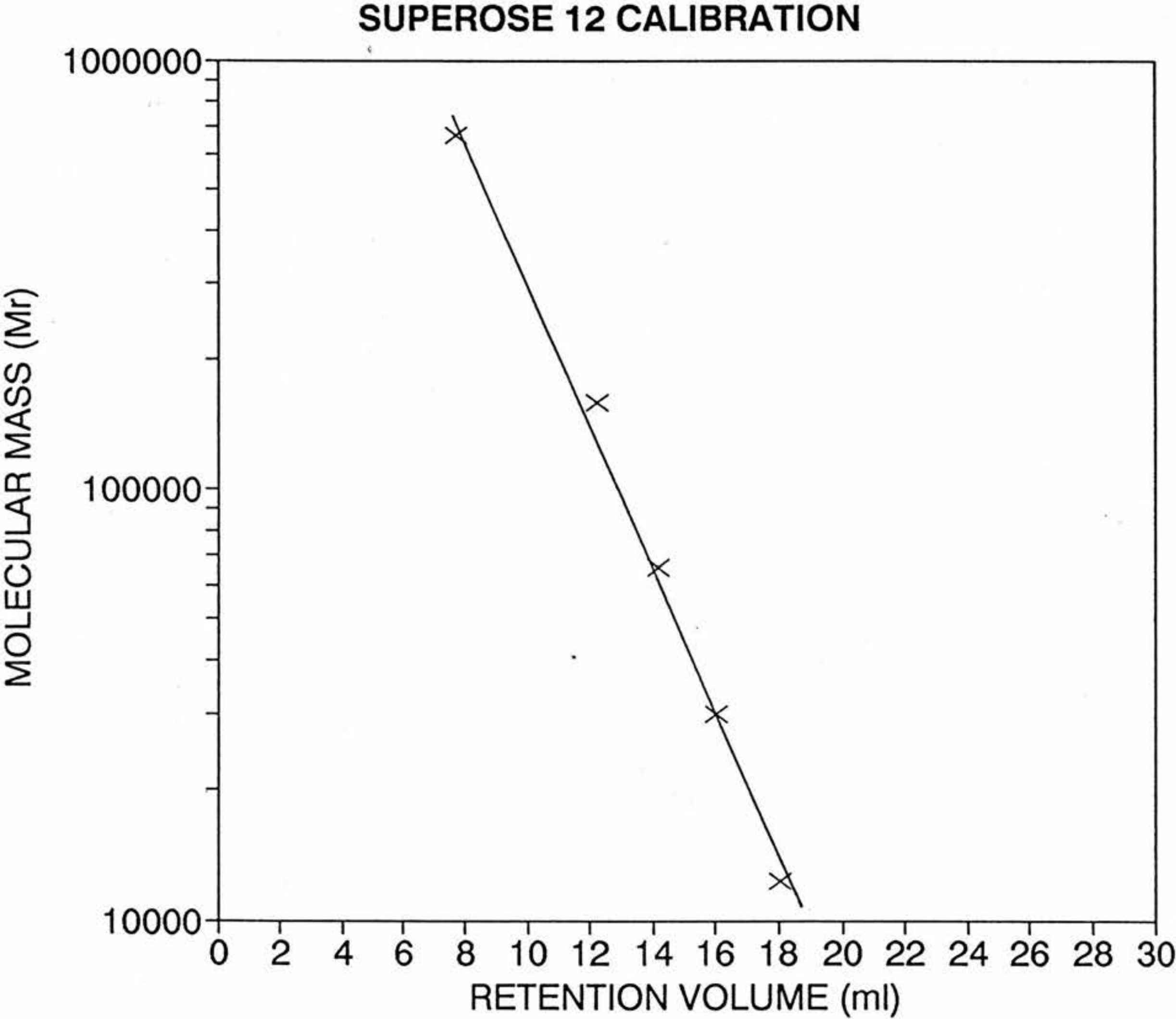
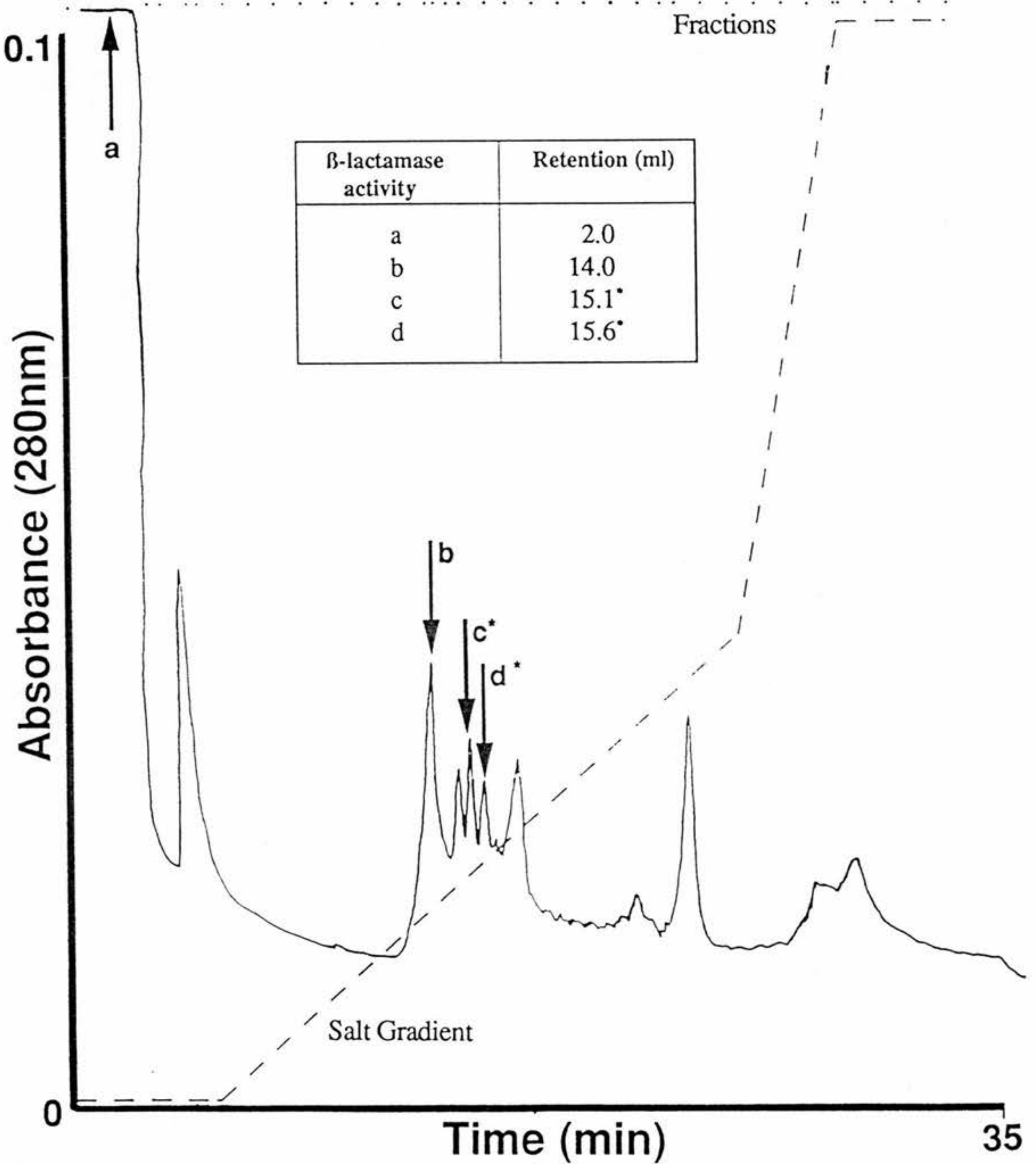


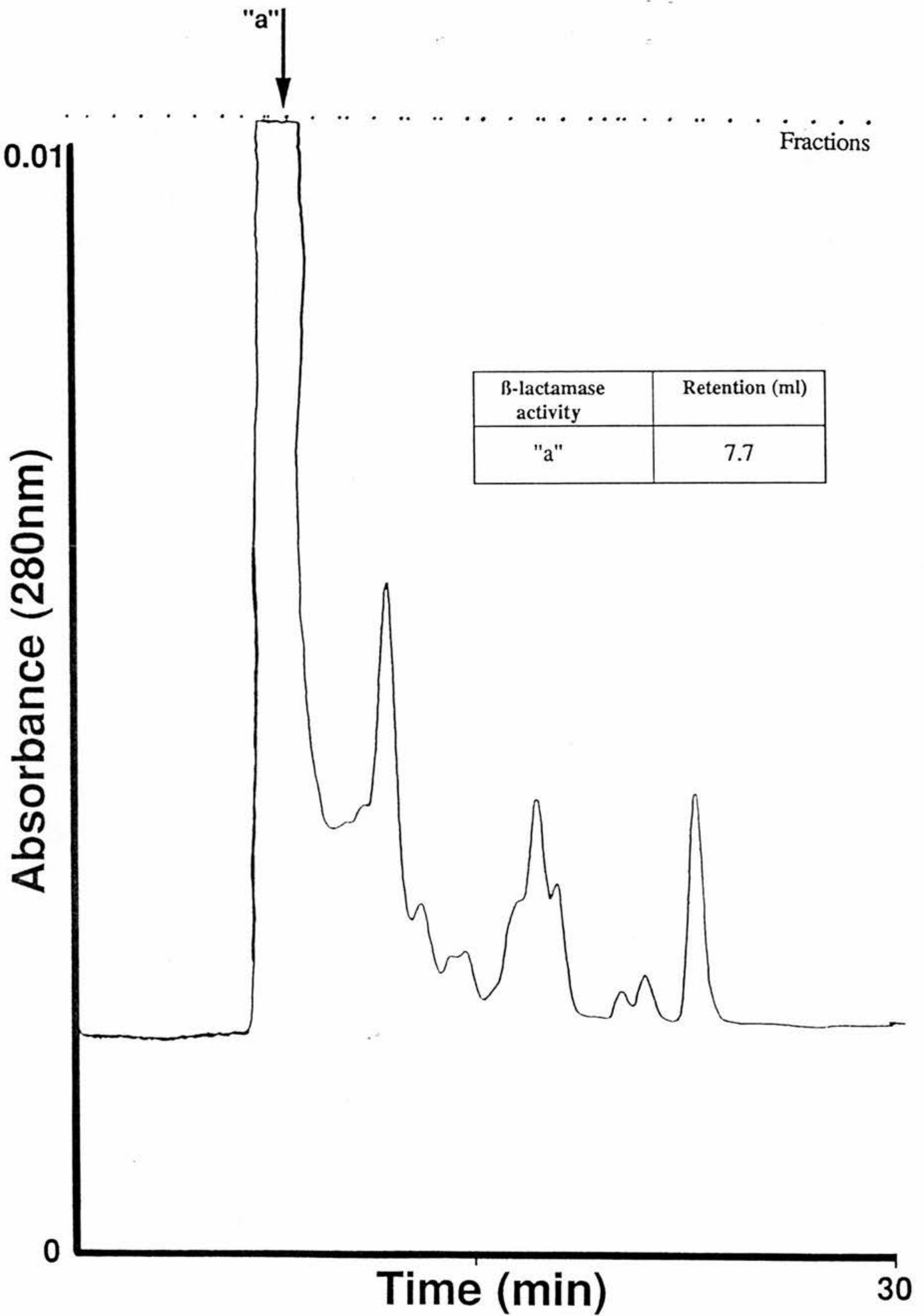
Figure 21



FPLC® trace of high performance ion exchange (Mono S) of the crude ß-lactamase preparation from strain H63. ß-lactamase activity was probed for by employing the nitrocephin spot test. The presence of ß-lactamase activity is indicated by the arrows (* represents the major activities)

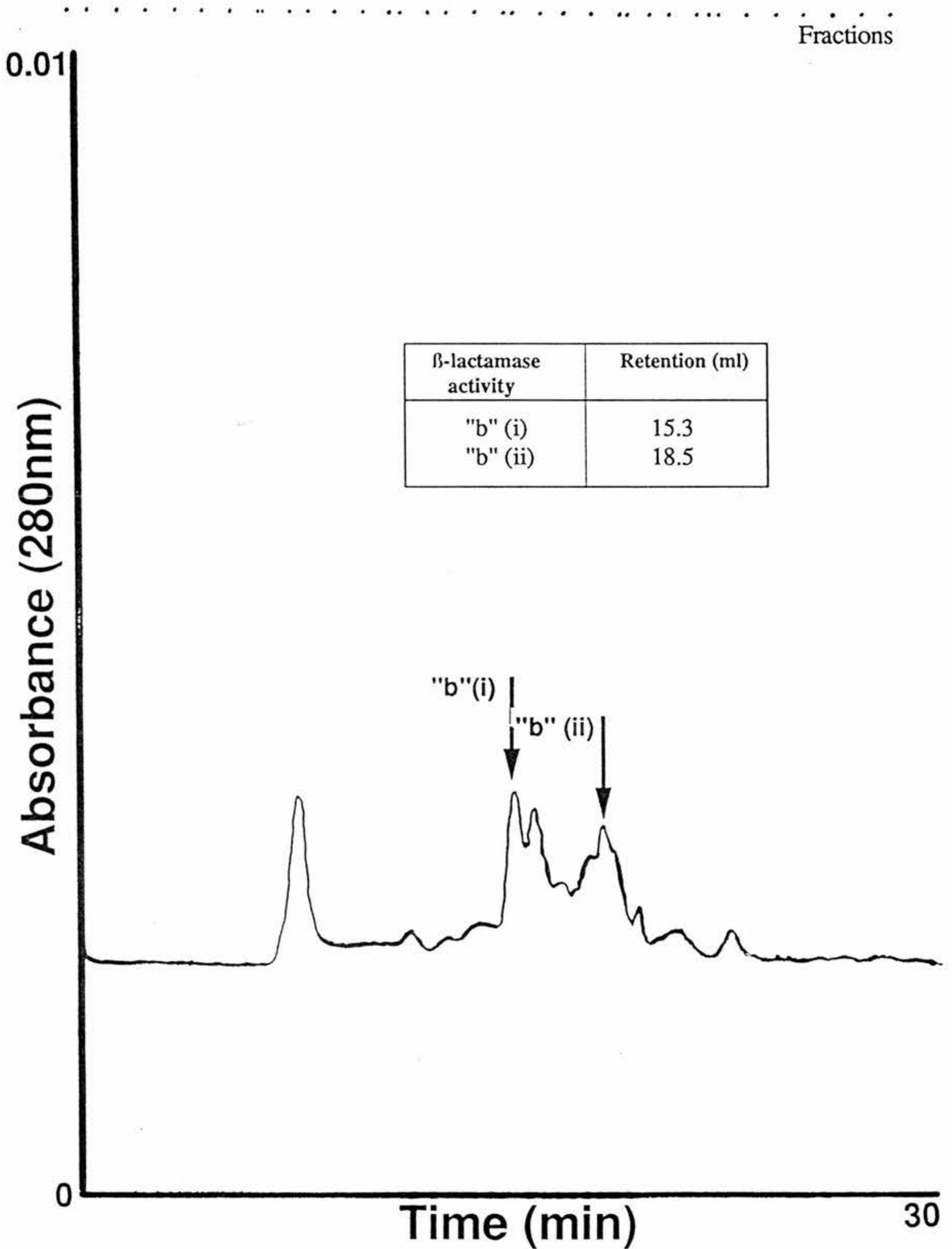
The broken line represents the increase in sodium chloride concentration.

Figure 22



FPLC® trace of gel permeation of activity "a" obtained from the ion exchange step. ß-lactamase activity was probed for by employing the nitrocephin spot test and is indicated by an arrow.

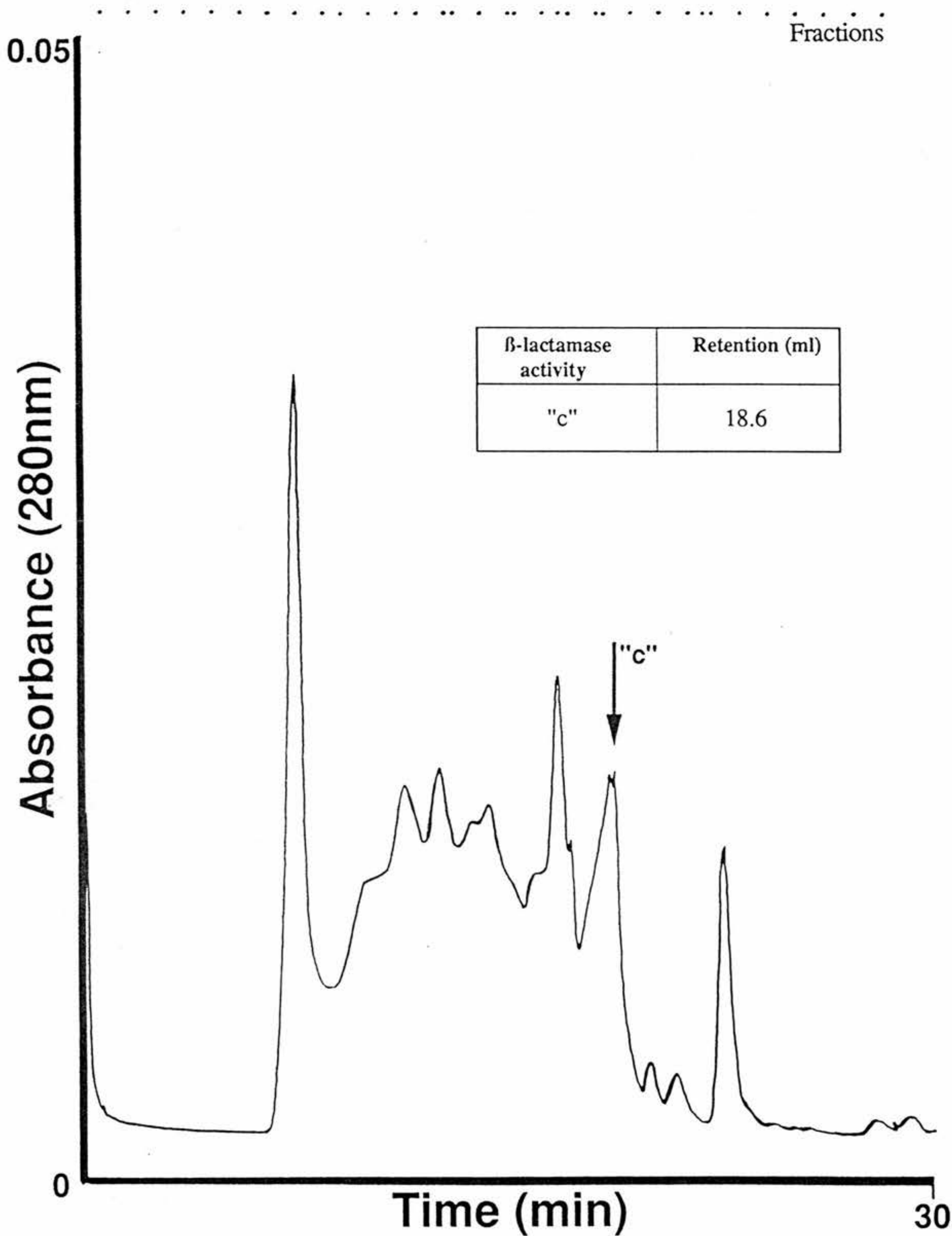
Figure 23



ß-lactamase activity	Retention (ml)
"b" (i)	15.3
"b" (ii)	18.5

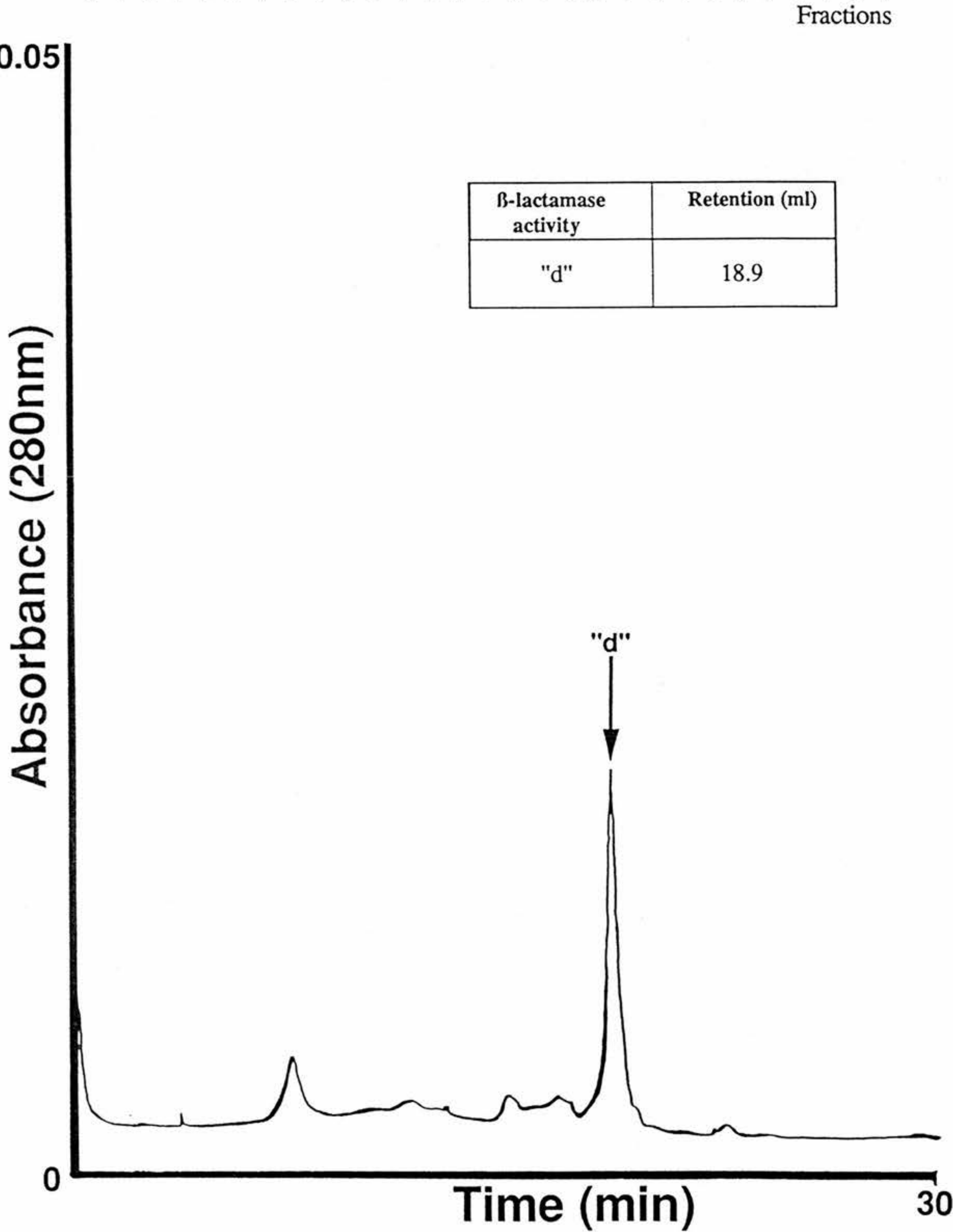
FPLC® trace of gel permeation of activity "b" obtained from the ion exchange step. ß-lactamase activity was probed for by employing the nitrocephin spot test and is indicated by the arrows.

Figure 24



FPLC® trace of gel permeation of activity "c" obtained from the ion exchange step. ß-lactamase activity was probed for by employing the nitrocephin spot test and is indicated by the arrow.

Figure 25



ß-lactamase activity	Retention (ml)
"d"	18.9

FPLC® trace of gel permeation of activity "d" obtained from the ion exchange step. ß-lactamase activity was probed for by employing the nitrocephin spot test and is indicated by the arrows.

The retention volume of "a" was 7.7 ml; "b" split into two major activities at retention volumes of 15.3 and 18.5 ml; "c" had a retention volume of 18.6 ml and "d", with a single protein peak, had a retention volume of 18.9 ml. The experiment was repeated several times with the same results, the retention volumes and M_r estimates are seen in Table 68 .

Table 68
Molecular size estimations on Superose 12.

Retention volume (ml)	M_r (Kd)
7.7	700
15.0	43
15.3	38
15.4	37
18.3	13.5
18.5	12.5
18.6	12.0
18.7	11.5
18.7	11.5
18.9	10.5

It can be seen that the M_r of the Beta-lactamase of H63 (ACE-1) seems to exist in at least 3 sizes. Firstly at 700 Kd, which correlates with its estimated M_r of 580 Kd on conventional gel permeation, but it also exists in two other sizes: one around 40 Kd and (in the case of H63) a predominant M_r of around 12 Kd.

These experiments were repeated with the ACE-1 enzyme from H68 and similar results were obtained but the predominant activity was an M_r of around 40 Kd with minor activity around 10 Kd.

M_r estimation of the enzyme from H63 by SDS-PAGE on the PhastSystem

250 µl of the single protein peak at retention volume 18.9 ml, from the gel permeation column, was concentrated to 30 µl with an Amicon centriprep concentrator.

A 8 - 25% gradient PAG mini-gel was employed, with a Sigma molecular size marker kit (14,000 - 78,000) as standards. The protein samples were boiled for 5 minutes in a mixture of SDS (2.5%), β-mercaptoethanol and bromophenol blue. Four µl of the standards and samples were applied to the mini-gel. The running conditions were as described in the PhastSystem Separation Technique File No.110 (Pharmacia). Protein bands were visualised with a silver stain (Pharmacia) according to the manufacturer's instructions. The electrophoresis run and staining of the gel took approximately 1 hour 20 minutes.

Results

The Phastgel is shown in Figure 26. Calibration of the gel is shown in Figure 27. The result shows a major band at M_r 14 - 15 Kd and minor bands at 22 Kd, 33 Kd and 35 Kd.

These results would suggest confirmation of the M_r of the enzyme in the region of 10 - 15 Kd and 30 - 40 Kd. However, the presence of 4 bands of protein in the SDS-PAGE suggested that the Beta-lactamase was not purified to homogeneity.

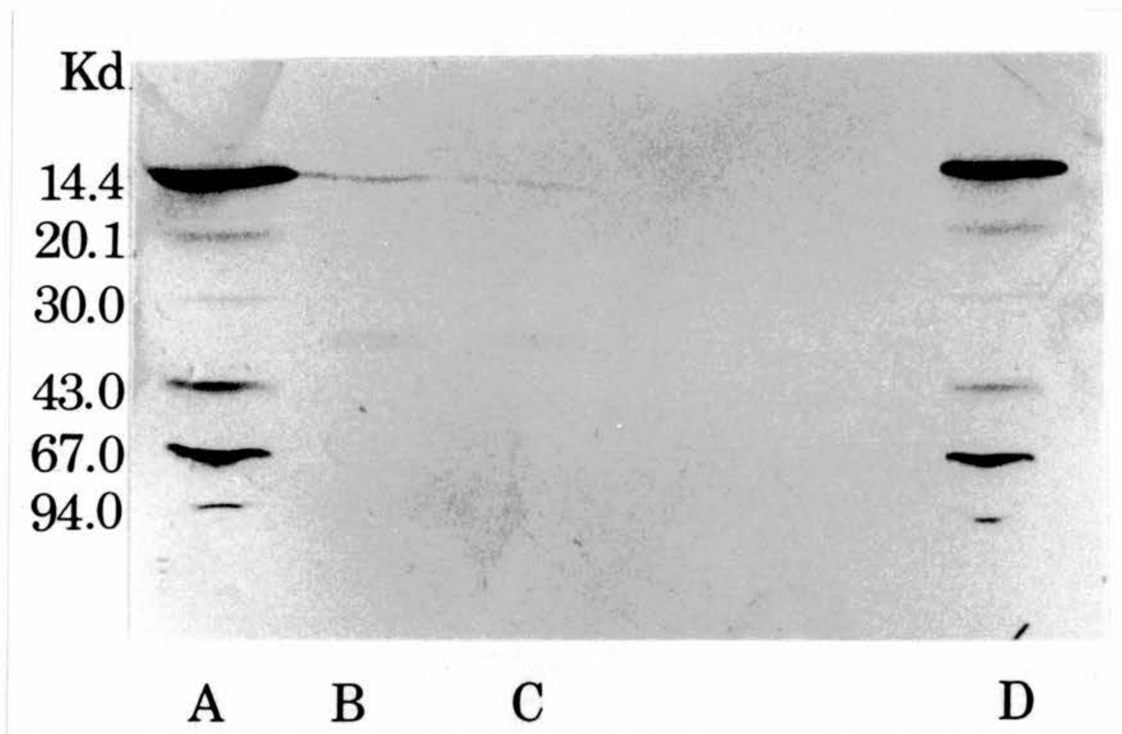
It should also be noted that this SDS-PAGE system was not particularly accurate at the low molecular size range.

These results while posing new questions about the nature of these proteins also helps answer and explain previous questions and observations.

Firstly, it explains why these enzymes retain their activity in the presence of 4 M urea. It would seem that they exist in high M_r aggregates which depending on the conditions break up into several different M_r subunits with

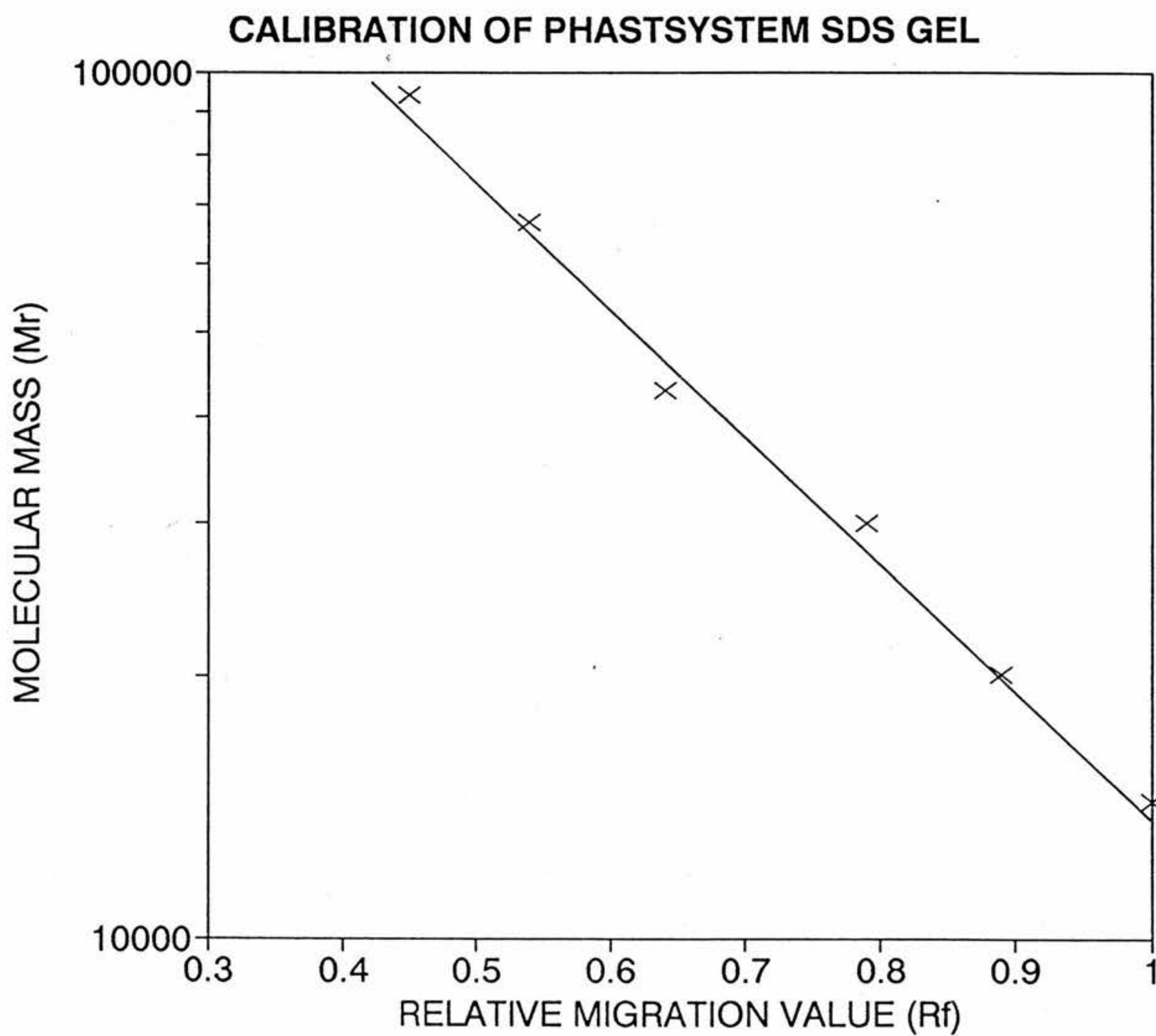
Figure 26

M_r estimation of the β–lactamase from H63 by SDS-PAGE on the PhastSystem



Tracks A & D	molecular size markers
Tracks B & C	β-lactamase samples

Figure 27



differing ionic charges. This could explain their behaviour in ordinary IEF systems and the variable bands obtained in AUS gel systems.

Secondly, it explains why other workers have described *Acinetobacter* chromosomal Beta-lactamases as being of M_r around 35 - 40 Kd (Morohoshi & Saito, 1977 and Hikida *et al.* 1989).

While all the above evidence suggests that these enzymes exist as aggregates and therefore seem (under certain conditions) to have a high M_r it was still important to determine whether these proteins were membrane bound or present free in the periplasm.

PREPARATION OF CELL MEMBRANE FRACTIONS OF ACINETOBACTER BAUMANNII, H68.

In order to determine the major position of these enzymes within the *Acinetobacter* cellular structure, washed cell membrane (WCM) preparations were prepared.

The growth, harvesting, disruption and WCM preparation of these cells are described in the Materials and Methods. Disruption by both the French pressure cell and ultrasonication were employed to ascertain the best method for the preparation of these enzymes (with a view to future purification). Some doubt has been cast recently on the use of ultrasonication in the preparation of Beta-lactamases (Nichols & Hewinson, 1989).

Total enzyme activity and specific activities (μmol/mg/protein) were measured at various steps of the procedure. The percentage of the total enzyme activity present at these various steps was calculated with 100% enzyme activity taken to be present in the low speed supernatants, after the broken cell suspensions had been treated by centrifugation.

The results of these experiments are shown in Table 69.

Table 69

Purification of *Acinetobacter* (H68) β–lactamase showing relative amounts within the periplasmic space or membrane-associated, employing either ultrasound or French pressure cell disruption.

Step / Method	Total volume (ml)	Concn. protein (mg/ml)	Activity/ml (μmol)	Total activity (μmol)	Sp.activity (μmol/mg)	% of total enzyme activity
Ultrasonication						
low speed supernatant	20	15.4	0.3	6.0	0.019	100
high speed 2 supernatant	17	10.4	0.246	4.18	0.024	69.7
high speed 2 pellet	15	4.0	0.033	0.495	0.008	8.3
high speed 3 pellet (WCM)	10.5	5.2	0.028	0.294	0.005	4.9
French pressure cell						
low speed supernatant	30	37	0.9	27.0	0.024	100
high speed 2 supernatant	26	27.6	0.91	23.66	0.033	87.6
high speed 2 pellet	30	9.5	0.036	1.08	0.004	4.0
high speed 3 pellet (WCM)	14	19.3	0.21	2.94	0.011	10.8

Firstly, it was clear that the best method for the initial purification of these enzymes is disruption by French pressure cell, with higher specific activities being obtained by this method.

Secondly it was quite clear that (at least in the case of H68) the Beta-lactamase is mostly present in the periplasmic space with, 87.6% of the enzyme activity there and only 10.8% residing in the washed cell membrane component (in the case of French pressure cell disruption).

Therefore it is likely that these enzymes exist as aggregated proteins and are not attached to the other cell membrane components.

DISCUSSION

CEFUROXIME RESISTANCE SCREEN

Four hundred strains of Gram negative oxidase-negative aerobic bacilli were collected over a two year period (January 1986 to December 1987). From them, 200 strains with clear resistance to cefuroxime (MIC≥32mg/L), were studied further. Their MIC's to 12 different antimicrobials were obtained.

The results were very much as expected. Within this group of cefuroxime-resistant organisms there was total resistance to ampicillin and cephradine. There was 84% resistance to the other 2nd generation cephalosporin cefoxitin. Aztreonam and the 3rd generation cephalosporins cefotaxime and ceftriaxone had significantly better levels of resistance at 25.5%, 24% and 29% respectively. Ceftazidime had a resistance slightly higher at 35%. Not surprisingly the resistance to imipenem and ciprofloxacin, drugs which at that time were not on the market, had resistance levels of 1% and 2% respectively.

The resistance levels to the aminoglycosides tested (netilmicin and gentamicin) were similar at 19.5% and 26.5%.

CEFUROXIME RESISTANT SPECIES

The proportion of different species forming this group of 200 cefuroxime resistant organisms was also not surprising with 50% *Enterobacter* spp., 12% *Serratia* spp., 4.5% *Citrobacter* spp. with 21.5% *Acinetobacter* spp. In other words the majority of these strains were organisms known to produce inducible R&S Class I chromosomal Beta-lactamases (Sanders & Sanders, 1988).

The relationship of resistance to particular bacterial species was also very much as expected.

BETA-LACTAMASES PRODUCED BY CEFUROXIME – RESISTANT ORGANISMS

Of the 200 strains studied 192 produced a typical species-specific chromosomal Beta-lactamase. Of these 192, 139 produced a typical chromosomal enzyme alone while 52 produced a chromosomal enzyme and the plasmid-associated enzyme TEM-1 while one strain produced a chromosomal enzyme and SHV-1. Eight strains produced TEM-1 alone.

Interestingly within the 43 *Acinetobacter* strains, no plasmid-mediated enzymes were detected. This is in contrast to the study of Joly-Guillou *et al.* (1988) where out of 100 strains, 34 produced a TEM-1 Beta-lactamase and 7 produced a CARB-5 Beta-lactamase. However this was carried out in an general group of bacteria and not those specifically with cefuroxime resistance.

Therefore it is likely that the cefuroxime resistance in the vast majority of the strains studied by us was due to the presence of a chromosomal Beta-lactamase.

TRANSFERABLE 3RD GENERATION CEPHALOSPORIN RESISTANCE

A study in collaboration with Dr D J.Payne amongst the majority of strains which exhibited resistance to the third generation cephalosporins showed *no* evidence of transferable third generation resistance.

ANTIMICROBIAL RESISTANCE RATES WITHIN SPECIFIC WARD AREAS

Phillips (1988) had the following points to make concerning studies on the prevalence of antimicrobial resistance within various populations:

"...having detected resistance, the diagnostic microbiologist has the problem of determining its prevalence. The microbiologist will need to :

1. exclude multiple isolates from the same patient assuming that he can identify both of these
2. attempt to separate organisms by their sources (community vs nosocomial; intensive care units, paediatrics)
3. separate organisms by type of infection (e.g. urinary tract infection, septicaemia)

Then, if rates of resistance are to be compared with these or other institutions, or even longitudinally within the same institute, prevalence must be related to the numbers and types of patients seen. Thus it is not surprising that results are seldom available for outside scrutiny".

Our study consisted of some 14,000 isolates of Gram negative, oxidase negative aerobic bacilli that had been isolated from 18 different units within a large Scottish teaching hospital (which contained many of the regional services for the East of Scotland) over a 5 year period.

This is certainly the largest study of its kind undertaken in the UK.

With reference to the points made by Phillips (1988):

1. Multiple isolates from the same patient were excluded as far as was possible
2. The organisms were separated into the areas where they had originated from within the hospital. It was not possible to split the isolates into those patients who had recently come from the community or who had acquired their organisms while in the hospital.

3. Separation of the organisms into infection types could have been carried out but was not. It was decided not to do this because there were enough data to discuss without introducing yet another variable.
4. The resistances were related to the bed occupancy of each area and the antimicrobial usage in that area over a period of time.
5. It was not possible to relate the resistance to specific organism type since their identification was not accurate, to even the genus level.

VALIDITY OF THE STOKES' METHOD FOR DETERMINING THE ANTIMICROBIAL SENSITIVITY

Some may feel that the use of Stokes' method for the determination of the antimicrobial sensitivity in this study makes it less credible. I would make the following points.

1. This was a retrospective study using the existing information that was available in the archives of our computer system.
2. This method is good enough to be used in the day to day running of the Clinical Microbiology Laboratories of the great majority of hospitals throughout the UK. The results generated by these tests are used to initiate or change treatment in large numbers of infected patients.
3. There is considerable debate as to what method microbiologists should use to determine antimicrobial resistance (See Edwards, 1991 and Hamilton-Miller, 1991) and what relevance the results have to clinical practice.
4. The resistance figures for the three major areas studied (surgical, medical and ITU) were found to be similar in the general population (determined by Stokes' method) and in the blood culture isolates (determined by MIC estimation).

5. It would have been technically difficult (in terms of manpower and time) and financially prohibitive to have collected these 14,000 isolates and carried out MIC determinations in all of them.
6. Others have used their disc sensitivity test results in resistance studies (Phillips *et al.*, 1990).
7. The same people were employed using the same method on isolates from a single institution over the 5 year period.

   Therefore, although there will be discrepancies in a study this size, the methods employed are entirely valid.

RESULTS

Cefuroxime resistance over a five year period

The results showed clear differences between various units within the hospital.

The overall resistance in the Surgical hospital was 12.7% with a range between 4.7% and 23.3%. In the Medical hospital these figures were similar to those in the Surgical hospital at 11.8% with a range between 6.7% and 20.4%.

The overall resistance in the Intensive care areas was significantly greater at 33.1% with a range between 18.9% and 42.2%.

Gentamicin resistance over a five year period

The results for gentamicin resistance did show variation between units but it was not so marked as with cefuroxime, nor was the level of resistance particularly high.

The overall resistance in the Surgical hospital was 1.7% with a range from 0.7% to 3.8%. In the Medical hospital these figures were 2.4% with a range

from 1.0% to 4.7% and in the ITU areas they were 3.9% with a range from 2.6% to 6.5%.

Cefuroxime and gentamicin resistance trends over five years

In two of the three areas (Surgical hospital and Intensive care areas) there was a significant increase in the resistance to cefuroxime.

In one of the three areas (Medical hospital) there was a significant decrease in the resistance to gentamicin.

The relationship between antimicrobial usage and the prevalence of resistance

In some cases there *did* seem to be a simple relationship between antimicrobial use and the resistance to that antimicrobial, for instance in the vascular surgery unit the cefuroxime resistance was 26% (the highest within the Surgical hospital) and the Beta-lactam use was 5.4g per patient (which was the highest in the Surgical hospital).

Similarly the cefuroxime resistance was 4.6% in the gynaecology unit and the usage was 1.6g/patient (both figures were the lowest for the Surgical hospital).

However in other instances this relationship was clearly not so simple. For example general surgical units 1 and 3 had similar usage of aminoglycosides at 0.06g/patient and 0.05g/patient respectively. However the gentamicin resistances were 0.5% in unit 1 and 2% in unit 3.

The Beta-lactam usage in surgical unit 2 was 1.8g/patient and in surgical unit 3 was 2.8g/patient yet the resistance to cefuroxime was 15.4% in unit 2 and 7.7% in unit 3.

Scatter diagrams showed that there was no clear correlation between antimicrobial usage and resistance in most of the areas studied.

These results are not entirely surprising. Phillips *et al.* (1990) made the following comment: "Most discussions of antibiotic resistance concentrate on its increasing incidence correlated with antibiotic usage. We have found that the trends are more complex and more interesting. For some species resistance has increased while for others, extensively subjected to the same selective pressures it has declined".

McGowan (1983) also pointed out that the relationship between antimicrobial use and resistance was far from simple.

He made several important observations:

1. The pathogens causing nosocomial infection over the last 50 years have changed, with surveys showing a decrease in the frequency of relatively susceptible pathogens such as the pneumococcus but with an increase in relatively resistant organisms such as *Pseudomonas* and *Serratia*.
2. These changes have largely been attributed to the use of the antimicrobial agents.
3. However there are many other factors that have contributed to these changes:
   (i) The development of new therapies and technology have decreased mortality therefore patients survive instead of dying. These patients often (as either the result of their disease or therapy) have impaired host defences. This relative susceptibility allows organisms of limited virulence to cause problems that normally would not occur in a normal host. Many of these opportunistic pathogens are inherently resistant to the commonly prescribed antimicrobials.

   However, they probably colonise these patients **primarily** because of the patient's poor host defences rather than because the organism is resistant to antibiotics.

"For example, the respiratory flora of severely ill patients often changes after hospitalisation to include a higher prevalence of Gram negative bacilli. This change depends primarily on the severity of the patient's illness rather than on antimicrobial usage", ending he quoted Sen *et al*. (1982).

(ii) A second factor involved in the change in pattern of organisms is the increasing use of invasive monitoring and instrumentation in patient care. Some organisms have attributes that allow them to survive in or around these devices or equipment. He noted that these characteristics may be entirely independent of the organism's susceptibility or resistance to antimicrobial agents.

Subsequently the above observations have been supported by the work of Craven *et al.* (1988) and Maki (1989) who have studied the risk factors for infection and mortality within the ITU. These and other relevant points have been discussed previously in the Introduction.

Taking into consideration the points made by McGowan (1983) one can easily explain why the resistance to both cefuroxime and gentamicin is greater in the ITU areas than in either the Surgical or Medical hospital. It also uncovers possible reasons for the presence of differences (or lack of differences) in resistance rates which cannot simply be explained in terms of the antimicrobial usage.

In other words the resistance in each area must be related not only to the antimicrobial use but to the underlying condition of the patient; the procedures that the patient underwent; the length of hospital stay; age; sex and the location within the hospital etc. For instance the types of operations and underlying conditions of the patients in the different surgical units may well have had an important effect on their subsequent acquisition of a resistant coliform.

However it was not possible, nor will it be possible, to obtain these data and relate them to the antimicrobial resistance figures in our study.

Further evidence that the relationship between the use of antimicrobials and the resistance was seen in data not presented. Each of the Medical and Surgical "units" described consists of an identical male and female ward. Therefore the resistance figures are available for males and females in both hospital areas. The differences between males and females is quite striking. A male is almost twice as likely to have a resistant coliform than a female, in both the medical or surgical areas. These findings can be partially (but not completely) explained by the fact that a female is more likely to have a urine specimen with a sensitive coliform in it than a male. But a great many other factors may be involved, and require further investigation.

The other fascinating piece of data produced by this survey comes from the special care baby unit. This unit admits all the seriously ill and premature babies from the East of Scotland. It admits in the region of 500 babies per annum, many of whom are ventilated. In terms of the percentage of the population of a ward receiving aminoglycosides, this unit has the highest at about 50%. The absolute level of aminoglycoside use per patient is almost as high or higher than for adult areas of the hospital. Yet the resistance to gentamicin is reasonably low at 2.5%.

Similarly in terms of their Beta-lactam use many of the babies are treated, but their resistance rate is lower than any of the other ITU wards.

This leads to the hypothesis that it is the amount of antimicrobial used per metre^2 that is an important determinant of resistance rather than the amount of antimicrobial used per patient.

In summary therefore we have shown that :

1. There are clear differences in the rates of resistance to both cefuroxime and gentamicin in Gram negative aerobic, oxidase-negative bacilli within different areas of a large teaching hospital over a 5 year period.
2. The differences are not only due to the antimicrobial use within these areas but also due to the underlying conditions of the patients and to other variables which are as yet not identified.
3. We have shown that over the five year period there was an increase in the resistance to cefuroxime which was clearly significant in the Surgical hospital and Intensive care areas. On the other hand the resistance to gentamicin showed a decreasing trend but this was only clearly significant in the case of the Medical hospital.
   (Unfortunately these trends could not be related to the trends in antimicrobial prescribing since this information was not available).
4. The resistance to cefuroxime in these organisms was clearly much greater than the resistance to gentamicin.

Therefore the long term outlook for the continued use of Beta-lactam drugs such as cefuroxime seems bleak with an inexorable rise in their resistance. Phillipon (1988) has also described an increase in resistance to a Beta-lactam (cefotaxime) in the Pitie-Salpetriere Hospital in Paris from 1980-1985. He noted that within strains of *Enterobacter cloacae* resistance to cefotaxime had risen from 6.4% to 20.7% and within *Serratia* spp. it had risen from 0.8% to 22%.

The outlook for the aminoglycosides is however less bleak. These drugs have been in clinical practice for about twenty years and in our study the resistance rates are still rather low. One might argue that the lower resistance rate is simply due to the fact that less aminoglycosides are being used - but this is not the whole story. In the special care baby unit where most aminoglycoside (in patient terms) is used, the resistance to

gentamicin resistance actually fell from 4.5% to 3.3% over the five year period.

The lower resistance to the aminoglycosides may be related to the fact that these agents are toxic and therefore require careful serum monitoring i.e. they are used much more judiciously than their Beta-lactam counterparts.

5. Finally this study has shown that quoting global figures of resistance to various antimicrobial agents for a single hospital, pooled figures for groups of hospitals and pooled figures for countries or groups of countries, are likely to be totally meaningless and serve only to hide the considerable variation that exists in resistance rates to antimicrobial agents within different subgroups of patients.

ACINETOBACTER CHROMOSOMAL BETA-LACTAMASES

At first sight it would appear that the properties of the presumed chromosomal Beta-lactamases of the genus Acinetobacter have been fully characterised without the need for any reference to the work presented in this thesis.

Isoelectric points (pI) in polyacrylamide gel systems were first described by Matthew and Harris (1976) and reiterated by Sykes and Matthew (1976). Other workers since then have described the pI's of *Acinetobacter* Beta-lactamases as ranging from 7.5 to >10 (Medeiros *et al.,* 1985); >8 (Joly-Guillou *et al.,* 1987) and 9.9 (Hikida *et al.*, 1989). Similarly the M_r of Acinetobacter Beta-lactamase has been described as 30,000 by Morohoshi and Saito (1977) and as 38,000 by Hikida *et al*, (1989).

The kinetics of hydrolysis to various Beta-lactam antibiotics have been most fully described (initially) by Morohoshi and Saito (1977) and subsequently to the newer agents by Hikida *et al.* (1989). These authors also examined the effect of a variety of Beta-lactamase inhibitors.

There is little doubt however from the data previously published that these Acinetobacter enzymes are cephalosporinases (Sykes & Matthew, 1976; Morohoshi & Saito, 1977; Medeiros, 1984; Bauernfeind, 1986; Neu, 1986b; Joly-Guillou *et al*, 1987; Hikida *et al*, 1989).

The inducibility of these cephalosporinases by specific Beta-lactam inducers however, has been far from clear.

On the basis of the work by Morohoshi and Saito (1972), Bush (1989b) classified the *Acinetobacter* chromosomal Beta-lactamase in Group One, i.e. a cephalosporinase not inhibited by clavulanic acid (CEP-N). The subsequent substrate profile and inhibition studies of Hikida *et al.* (1989) suggested that this classification is correct.

ISOELECTRIC POINTS ON CONVENTIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Matthew and Harris (1976) listed pI's of around 8.6 for two Beta-lactamases produced by strains of *Acinetobacter*: *A.inotti* 1786E and *Acinetobacter* sp. 1787E. These same two strains appear in Sykes and Matthew (1976) as *A.inotti* (1786E) pI 8.6 and *A.mallei* (1787E) pI 8.7. The latter strain (*A.mallei*) 1787E was therefore not an *Acinetobacter* but a *Pseudomonas mallei*. This organism had somewhat controversially been placed in the genus *Acinetobacter* by Cowan and Steel (1965) - it became *P.mallei* in subsequent editions (Cowan & Steel, 1974).

Therefore, this enzyme can no longer be considered as one produced by the genus *Acinetobacter*. The former strain (*A.inotti*) 1786E is equally interesting. An extensive taxonomic literature search has failed to find any previous or subsequent mention of the specific epithet "inotti". This strain is part of the Glaxo Research Laboratories' collection and one of the original authors (Miss A. M. Harris) kindly sent us strain 1786E for further study. The freeze dried vial was still marked as *A.inotti* but was identified by the API 20NE system as *A.lwoffii* (new specific epithet: *A.junii*). On reflection, we suggest that "lwoffi", when written could easily have been wrongly transcribed to "inotti" and perhaps this error took place somewhere between its original entry in the strain book and the subsequent publications.

Medeiros *et al.* (1985) were the first to mention that not all of the chromosomal Beta-lactamases of *Acinetobacter* focused on conventional polyacrylamide isoelectric focusing systems. Of 14 strains of *A.calcoaceticus*, the pI was stated to be 8.8 for one enzyme, 9.0 for one, 9.7 for one, 10.0 for four, >10 for three and a "blur" for four. Joly-Guillou *et al.* (1987) described 30 strains of *Acinetobacter* with a chromosomal cephalosporinase of pI >8. This again suggests poor focusing of these enzymes on conventional polyacrylamide IEF

systems. More recently, Hikida *et al.* (1989) described a pI of 9.9 for a purified cephalosporinase from a *A.calcoaceticus* (ML 4961). However, these authors employed broad range ampholines of pH 3.5 - 10.0 in their IEF gel. This suggests that the enzyme had migrated to, or almost to, the cathode. Unfortunately, there was no photograph of the IEF gel in their publication..

In our initial screening study we found only one Beta-lactamase from 43 strains of *Acinetobacter* focused in a conventional polyacrylamide IEF system. So, clearly this system did not work.

SDS-FREE PAGE

Our first thought was to attempt to separate these enzymes in another polyacrylamide system but to separate them on (what we initially believed to be) their M_r. Therefore the enzymes were applied to SDS-free PAGE. This allowed the Beta-lactamase activity to be probed by nitrocephin. Experiments employing this system revealed that most of the Beta-lactamase activity electrophoresed to the anode! This led us to believe (correctly but perhaps for the wrong reasons) that the problem was one of solubility. Subsequently, after experiments with the same enzymes utilising a SDS-Free PAGE run on the PhastSystem the following observations could be made:

(i) we were not separating these enzymes in the above system by their M_r but by their charge.

(ii) therefore it was unlikely to have worked since the electrodes needed to be reversed! Otherwise the enzymes would not have migrated from the cathode. On reviewing the photographs it could be seen that some of the *Acinetobacter* enzyme preparations did appear at the cathode where they had been loaded.

(iii) the enzymes apparent lack of solubility in the system (i.e. a broad band at the anode) was probably because they needed some SDS in

the sample buffer i.e. enough to allow them to solubilise but without loss of their biological activity.

IEF IN POLYACRYLAMIDE INCORPORATING UREA AND TRITON X-100

The next logical step was therefore to attempt to solubilise these enzymes in a conventional polyacrylamide gel system. This was initially tried by incorporating approximately 4M urea and 2.5% Triton X - 100 into the gel matrix. However it was not until the sample itself was solubilised - with urea, Triton X-100, β-mercaptoethanol and ampholines - that some focusing occurred.

This allowed an initial tentative classification of these enzymes.

It was at this point of the work that nine particular enzymes produced by nine *Acinetobacter* strains had been collected. These nine strains: 5B86, 6B230, H17, H26, H63, H68, H126, H141 and H162 were therefore employed in all the further experimentation discussed in this thesis.

Although some initial classification was possible it was clear that the technique was not optimal with both considerable distortion of the gels and extremely slow staining of the enzyme activity with the nitrocephin.

Concomitantly the initial M_r estimations had been carried out on conventional low pressure gel permeation. This showed that only two enzymes had M_r's of the expected values for chromosomally-mediated Beta-lactamases i.e. H126 at 32.5Kd and H162 at 60.5Kd. All the others (7) had values which ranged from 580Kd to >1000Kd.

Interestingly the lowest M_r of 32.5Kd produced by H126 was the only enzyme which focused normally on a conventional polyacrylamide IEF system. Therefore it seemed likely that the large M_r of these enzymes was at least partially responsible for their poor solubility in polyacrylamide gel systems.

AGAROSE, UREA AND SORBITOL (AUS) GELS

It therefore seemed pertinent to change the separation matrix from polyacrylamide to agarose since the molecular masses of these enzymes were so large that sieving (and therefore distortion) was likely to occur in the polyacrylamide gels. Vecoli *et al.* (1983) had previously employed agarose IEF for the investigation of Beta-lactamases with some success and Saravis and Zamcheck (1979) described thin layer IEF gels containing agarose which had resolved proteins with a molecular mass of greater than $2x10^6$. The problems of incorporating urea with agarose in these gels had been outlined previously by Olsson and Laas (1981). This included lower mechanical strength of the gels and the risk of carbamylation of the proteins under study. We therefore incorporated sorbitol and β-mercaptoethanol (as they had) into the gels - which also contained 1% agarose, 2.5% Triton X-100 and 4M urea. As in the polyacrylamide/urea gel system focusing only occurred after the initial solubilisation of the samples.

This system resulted in all nine of the Beta-lactamases studied focusing. It therefore allowed a classification of these enzymes into 4 groups - ACE-1 to ACE-4. These are shown in Table 70 .

Table 70.

Classification of ACE enzymes by pI on AUS gel electrophoresis

Strain no	Species	pI (s) of β-lactamase	ACE type
5B86	*A.lwoffii*	7.3 , 7.7	4
6B230	*A.junii*	8.6 , 8.8	1
H17	*A.baumannii*	8.6 , 8.8 (10.3)	1
H26	*A.baumannii*	8.6 , 8.8 (9.8 , 10.1)	1
H63	*A.baumannii*	8.6 , 8.8	1
H68	*A.baumannii*	8.6 , 8.8	1
H126	*A.junii*	8.2 , 8.1	3
H141	*A.baumannii*	8.6 , 8.8	1
H162	*A.baumannii*	8.7	2

This was similar to the groupings obtained from the previous studies employing urea and polyacrylamide gels. However more questions were posed than answered.

In some gels the Beta-lactamases produced by H17 and H26 had additional minor bands between pI 9 and 10.5. It was possible that this was the result of carbamylation, however the major problem was in actually visualising the bands of activity in this area. This was because the cathodic buffer (1N NaOH) hydrolysed (quite rapidly) the nitrocephin around this area.

There are two major problems associated with this novel IEF technique which make it unlikely that it will be used in the future for the screening of large numbers of *Acinetobacter* Beta-lactamases. Firstly it is technically demanding and secondly it is extremely expensive in terms of the quantities of precious nitrocephin that it consumes during each experiment.

Clearly, therefore alternative techniques needed to be sought both to corroborate the above results and to provide a quicker and easier means of discriminating between these enzymes.

In summary however, the AUS technique had shown that there were differences between these enzymes (at least 4 groups) with the ACE-1 type predominant within the strains of *A.baumannii* tested.

The fact that we have shown at least four different enzyme types on the basis of pI in *Acinetobacter* is not surprising. We found at least five different chromosomal Beta-lactamases in *Enterobacter* spp. in this thesis and Medeiros (unpublished data) described at least 8 different types of chromosomal Beta-lactamases in *Enterobacter* spp. on the basis of their differences in pI. Bakken *et al.* (1988) described at least four different (presumed chromosomal) enzymes in *Aeromonas* spp. and Matthew and Harris (1976) noted that there were several species of bacteria with more than one Beta-lactamase type.

HIGH PERFORMANCE ION EXCHANGE CHROMATOGRAPHY ON FPLC

Ion exchange chromatography on a FPLC system had several important attractions:

(i) It separates the enzymes on the basis of their charge

(ii) High resolution

(iii) High recoveries of sample with retention of structural and biological activity

(iv) High loading capacities

(v) A fast separation time with completion within 30 minutes

The above factors coupled with the rapid assay capability offered by the use of nitrocephin made this the most powerful tool for the purification and study of Beta-lactamases.

The use of FPLC in the purification of Beta-lactamases has been described by Kesado *et al.* (1989) in the purification of the Beta-lactamase from *Clostridium butyricum* and in the purification of the Beta-lactamase from *Pseudomonas paucimobilis* by Corkhill *et al.* (1991). We have shown ion exchange chromatography on FPLC to be the most powerful method to date in rapidly separating multiple Beta-lactamases from the same strain (Payne *et al.* 1990).

Initial experiments were carried out employing cation exchange with a MONO-S column utilising the aliquots that had been obtained from partially purifying these Beta-lactamases on the large gel permeation columns (and in estimating their M_r). Eight of the nine Beta-lactamases were investigated, as insufficient ACE-4 enzyme from 5B86 remained.

The results revealed that ACE-3 enzyme produced by H126 was completely different from the other ACE-1 and ACE-2 enzymes, infact its retention volume (in the void volume) suggested the employment of anion exchange chromatography for its further purification. The ACE-1 and ACE-2 enzymes appeared indistinguishable by this method, however within the ACE-1 group of enzymes there seemed to be two areas of activity at retention volumes of 12 - 13ml and 15 - 17ml. Clearly the reasons for this needed further investigation.

It was at this point that a classical problem of protein purification occurred - namely loss of the purified protein's activity.

There are many possible reasons for protein inactivation: removal of proteins from their normal cellular environment subjects them to a wide variety of conditions and processes which can lead to loss of activity or alteration of their structure. These include: dilution; change in solution conditions; exposure to degradative enzymes; oxygen; heavy metals; surfaces; and change in physical conditions (e.g. freezing and thawing) (Deutscher, 1990).

Stability trials and storage conditions were therefore initiated. These revealed that

(i) One freeze/thaw cycle resulted in complete inactivation of the enzymes

(ii) Activity persisted for 24-48h if the enzymes were kept at 4 ^{0}C though their activity waned over time

(iii) The loss of activity could be prevented completely by the addition of pure β-lactoglobulin A to a concentration of 1% in the purified enzyme preparations.

These preparations could then be stored for up to 6 months at -20^{0}C without appreciable loss of activity.

However the rapid inactivation of these enzymes in their (perhaps) native state meant that any structural studies e.g. application to SDS-PAGE or native PAGE gels had to be carried out on the same day as the ion exchange steps.

MONO-S AND MONO-Q CHROMATOGRAPHY WITH CRUDE BETA LACTAMASE PREPARATIONS

The frozen aliquots of the partially purified Beta-lactamases that had been obtained from the large scale gel permeation experiments were entirely used up in the previous experiments. Due to time constraints it was not possible to repeat these large scale and time consuming procedures. Therefore the application of crude enzyme preparations onto these ion exchange columns was investigated. ACE-1 and ACE-2 enzymes on a MONO-S (cation exchange) and ACE-3 and ACE-4 on MONO-Q (anion exchange).

These experiments revealed that ACE-1 and ACE-2 were very similar but ACE-3 and ACE-4 were completely different. It also revealed that within the ACE-1/2 group there seemed to be ionic variants with only one variant predominating. Weak activity was found in the void volume in each and then

there was activity at both around 12-13ml and 18ml (retention volumes) but strong activity was at either 12-13ml (e.g. in H68) or 17/18ml (e.g. H17).

These results (up to a point) certainly confirm the ACE enzyme groupings although ACE-1 and ACE-2 appeared identical.

On AUS IEF however ACE-2 was clearly different from the ACE-1 enzymes by producing a single focused band; and its M_r (60.5Kd) is much smaller than the ACE-1 enzymes (580->1000Kd). Therefore further experiments are required before amalgamating the ACE-1 and ACE-2 groupings.

Clearly, if it had not been due to the rapid inactivation of these enzymes, it would have been interesting to have applied the various aliquots, obtained from the above ion exchange chromatography, onto both ordinary polyacrylamide and AUS IEF gels. This might have shed light on the multiple banding (and variable banding) obtained on these systems.

REVERSED POLARITY ELECTROPHORESIS ON SDS-FREE (NATIVE) PAGE ON THE PHASTSYSTEM

Both crude Beta-lactamase preparations and the purified preparations obtained from the high performance ion exchange experiments were applied to native PAGE minigels in the PhastSystem.

This particular gel system had been devised for the rapid discrimination of basic proteins (Olsson & Tooke, 1988). It is a rapid system with each run of electrophoresis and staining taking less than 2 hours to complete. It has not previously been employed (in this form) to discriminate Beta-lactamases, however Huovinen (1988) utilized isoelectric focusing on the PhastSystem for the rapid identification of plasmid mediated Beta-lactamases.

The initial experiments with both crude and pure Beta-lactamase preparations showed similarity between all the ACE-1 enzymes tested and ACE-2

but a clear difference between them and ACE-3 (insufficient ACE-4 was available for testing).

Therefore although further experimentation is required it would seem that this rapid method will prove an extremely useful screening technique for the study of Beta-lactamases obtained from clinical isolates of *Acinetobacter.*

MOLECULAR MASS ESTIMATIONS

As previously mentioned Morohoshi and Saito (1977) gave the M_r of their cephalosporinase from *A.anitratum* NCTC 7844 as 30Kd when measured by gel permeation on a sephadex G-75 column. Hikida *et al.* (1989) described the M_r of their cephalosporinase from *A.calcoaceticus* ML4961 as 38Kd. This had been estimated by SDS gel electrophoresis after purification of the enzyme with strong cation exchange and conventional gel permeation.

Conventional low pressure gel permeation

We estimated the M_r of the Beta-lactamases of nine strains of *Acinetobacter*, initially on Sephadex G-75 and then if required on Sephacryl S-300. Only one enzyme (ACE-3 from strain H126) had a similar M_r (32.5Kd) to that described by Morohoshi and Saito (1977) and Hikida *et al.* (1989).

The ACE-2 enzyme from strain H162 had an M_r of 60.5Kd, but the other seven enzymes ranged from 580Kd to > 1,000Kd. This made these Beta-lactamases the largest yet described - after the L1 chromosomal Beta-lactamase of *Xanthomonas maltophilia* with an M_r of 123Kd (Bicknell *et al.,* 1985).

Their size raised the interesting question as to why these enzymes seemed so large on conventional gel permeation systems - do they exist in multi-subunit form or are they attached to other cell membrane components?

If they are attached to other cell membrane components then they are unlike almost all previously described chromosomal Beta-lactamases of Gram negative bacteria which are normally highly soluble (Medeiros, 1984).

In Gram positive bacteria membrane-associated Beta-lactamases are well known (Coles & Gross, 1967; Collins, 1979). Only one such membrane-associated Beta-lactamase has previously been described in Gram negative bacteria. This was the Beta-lactamase of *Pseudomonas pseudomallei* (Livermore *et al*,, 1987). These workers based the membrane association not on the enzyme's M_r (which was 29.5 Kd estimated on SDS-PAGE) but on the finding that 83% of the Beta-lactamase activity was associated with the pellet of membrane material.

Other workers have made similar claims for the Beta-lactamase produced by *Capnocytophaga* sp. (Foweraker *et al.* ,1990). However they based this assumption simply because isoelectric focusing could only be achieved if a non ionic detergent was added to the gel "*suggesting* the presence of a hydrophobic enzyme akin to a membrane-bound Beta-lactamase of Gram positive bacteria". They did *not* prepare membrane fractions and measure Beta-lactamase activity. Therefore their claims are largely speculation.

We carried out whole-cell membrane preparations of the Beta-lactamase from strain H68 of *A.baumannii* which had a M_r (estimated on conventional gel permeation) of >1000Kd. This experiment revealed almost 90% of the Beta-lactamase activity was present in the periplasm with about 10% associated with the membrane component. This suggests that these Beta-lactamases exist in multi-subunit or aggregated form.

PURIFICATION SCHEME FOR AN ACE-1 ENZYME (PRODUCED BY H63) WITH M_r ESTIMATIONS EMPLOYING GEL PERMEATION ON THE FPLC AND SDS-PAGE ON THE PHASTSYSTEM

So far we had elucidated the following about ACE-1 Beta-lactamases

(i) apparent large size on conventional gel permeation

(ii) at least three charge variants produced after high performance cation exchange

(iii) soluble in the periplasm not attached to cell membrane

Experiments were therefore carried out to estimate the M_r of this enzyme by gel permeation on FPLC and SDS-PAGE. In order to carry out the latter the Beta-lactamase had to be purified to homogeneity since denaturing conditions are required. Therefore a purification scheme for this enzyme was devised.

After application of the ACE-1 Beta-lactamase (from the strain H63) onto a gel permeation column on FPLC it was consistently shown that this enzyme existed in at least 3 molecular sizes. One major and two minor. Firstly a small quantity of the enzyme exists at a size of 700Kd (which correlated with its estimated size of 580Kd on conventional low pressure gel permeation). It also exists at a size of around 40Kd, but its predominant size in this particular system is around 12Kd.

The same procedures were repeated with the ACE-1 Beta-lactamase produced by H68 and very similar results were obtained except that the predominant activity existed at around 40Kd with minor activity around 10Kd.

From a gel permeation run with the ACE-1 enzyme of H63, a single peak of protein, which correlated with most of the Beta-lactamase present and therefore presumed to be homogenous protein, was applied to an SDS-PAGE mini-gel on the PhastSystem. This denatured protein was stained using silver nitrate. The

results revealed a major band of M_r 14-15Kd but with minor bands not only at 35Kd but also at 22Kd and 33Kd. This to some extent, confirms the sizes found in the gel permeation studies. However it is not clear whether these other bands indicate that the protein is not totally pure or whether they represent irregular amounts of disaggregated Beta-lactamase. It would perhaps have been useful to have electroblotted the proteins separated on the SDS-PAGE gel and then probed with nitrocephin to see if all the bands were in fact active Beta-lactamase. Alternatively a method described by Tai *et al.* (1985) might have been tried. They found that Beta-lactamase activity could be restored in SDS-PAGE gels after electrophoresis by the addition of a carrier protein.

It should be noted that this particular SDS-PAGE system is not as accurate at the low molecular size range as it is at the higher ranges.

These results suggest the following thesis:

(i) these Beta-lactamases exist under certain conditions as high molecular size aggregates

(ii) under different conditions they break up into different "subunits" with differing ionic charges which may exist in different M_r

(iii) under certain conditions these proteins exist predominantly in a low molecular size subunit of around 10-15Kd or alternatively in a 35-40Kd size

These results also offer possible explanations for various observations that have been made about these enzymes by others and in this thesis.

(a) their existance in large M_r aggregates (in certain conditions) would explain why they are poorly soluble in conventional polyacrylamide IEF systems

(b) it would also explain why their activity remains in the face of denaturing conditions such as 4M urea in AUS gels and the denaturing effects of the sample preparation for these gels

(c) it would explain why their eventual focusing in AUS gels resulted in some variation in the banding observed

(d) it explains why other workers have described *Acinetobacter* chromosomal enzymes have M_r estimated at 35-40Kd (Morohoshi & Saito, 1977; Hikida *et al.,* 1989).

It is perhaps a little ironic that initially we believed that these Beta-lactamases had the largest M_r described and now they seem to have one of the smallest.

BIOCHEMICAL CHARACTERISATION OF THE ACE ENZYMES

This was carried out by following the recommendations of Bush and Sykes (1986) and Bush (1989a).

All biochemistry was carried out employing partially purified enzyme obtained from large scale gel permeation.

Substrate profiles

Relative rates of hydrolysis of all nine enzymes showed generally poor hydrolytic activity. Nitrocephin was hydrolysed by all the enzymes as was cephaloridine to a similar extent by all the enzymes. All the enzymes apart from ACE-4 (from 5B86) hydrolyse penicillin G albeit some 1000 times slower than nitrocephin.

These results showed no clear differences between the Beta-lactamases but that they were clearly all cephalosporinases.

Kinetic parameters

The K_m, V_{max} and efficiency of hydrolysis values were calculated for each enzyme. The major substrates were nitrocephin and cephaloridine. The

relative efficiency of hydrolysis values were expressed as a percentage of the value for nitrocephin.

The K_m values for both nitrocephin and cephaloridine showed that all the enzymes had moderate affinity for these substrates.

The V_{max} values were also similar for all these enzymes, against both the substrates, but with the rate for cephaloridine one to two orders of magnitude lower than those for nitrocephin.

All the enzymes had similar relative efficiency values (K_m/V_{max}) for the hydrolysis of cephaloridine.

Therefore kinetic parameters did not show any convincing differences between each of the ACE Beta-lactamases.

However the K_m values for cephaloridine which varied from 150 to 710μM were very similar to the values of 250μM and 511μM described for their enzymes by Morohoshi and Saito (1977) and Hikida *et al.* (1989) respectively.

Inhibition studies

These revealed that all the enzymes were inhibited by cloxacillin but none was inhibited by clavulanic acid. All were inhibited by a fixed concentration of $HgCl_2$ but none by moderately high levels of pCMB. ID_{50} results did however discriminate between the ACE-3, ACE-4 and ACE-1 / ACE-2 groups. The ID_{50} to aztreonam for ACE-4 was significantly higher than that for the ACE-1 / ACE-2 enzymes tested and the ID_{50} for the ACE-3 enzyme was significantly lower than those for the ACE-1 / ACE-2 enzymes.

The effect of 1mM EDTA on the activity of all these enzymes was variable but generally less than 20% inhibition was observed when investigating partially pure enzyme preparations. The inhibition study was repeated using a purer preparation obtained from high performance ion exchange chromatography of the ACE-1 enzyme produced from H63. This consistently showed very little

inhibition by 1mM EDTA (<10%) and therefore metal ions do not seem to play a part in the function of this Beta-lactamase.

In summary these inhibition profiles place these enzymes firmly in the Bush Group One (CEP-N) i.e. chromosomal Beta-lactamases typical of Gram negative bacteria.

INDUCTION STUDIES

The inducibility of these cephalosporinases by specific Beta-lactam inducers has been far from clear. Morohoshi and Saito (1977) described how the enzyme activities of five strains of *A.anitratum* "increased by about five to ten-fold" following treatment with 100μg of benzylpenicillin, cephaloridine or 6-aminopenicillanic acid for 1 hour at 37°C. Hikida *et al.* (1989) quoted Morohoshi and Saito (1977) as having shown that *Acinetobacter* cephalosporinases are inducible, but they did not carry out induction experiments themselves.

They are quoted as being inducible by Sykes and Matthew (1976), Medeiros (1984) and Neu (1986b). Baurenfeind (1986) classified them as inducible or constitutive whereas Joly-Guillou *et al.*(1988) stated that inducibility in *Acinetobacter* spp. had not yet been demonstrated. Sykes and Smith (1979) stated that they were non-inducible.

I carried out induction experiments with all nine strains employing cefoxitin as the inducer at one quarter the MIC value for that strain. The method described by Minami *et al.* (1980) was employed.

No discernable Beta-lactamase induction was found with any of the strains studied. In discussions with D. Livermore and C. C. Sanders (personal communications) it was stated that evidence of induction had not yet been found in any strain of *Acinetobacter* so far tested. This includes the use of cefoxitin, imipenem and penicillin as inducers. Much more convincing evidence would

therefore be required before it can be claimed that these enzymes are inducible, since current evidence suggests that they are not.

However, recent work carried out by Kleber's group in Leipzig (Blechschmidt *et al.*, 1989; Borneleit *et al.*, 1991) may result in a reassessment of the role of Beta-lactamase induction in Acinetobacter.

They studied two strains of Acinetobacter (69V and CCM 5593). These strains were obtained from the Czechoslovak Collection of Microorganisms, Brno. It is not clear where they originated - from environmental samples or clinical specimens.

They suggested that the Beta-lactamases produced by these strains were

(i) inducible

(ii) predominantly extracellular in location rather than in the periplasm

They believe that the extracellular location of these enzymes does not result from periplasmic leakage since the other normally periplasmic enzymes e.g. alkaline phosphatase and glucose dehydrogenase are not found extracellularly in these strains. Nor do they feel that it is the result of cell lysis since the level of the cytoplasmic enzyme, malate dehydrogenase is not raised in the cell free medium.

If the induction experiments were carried out only measuring the Beta-lactamase activity in the periplasm - no evidence for induction was found, however measuring the activity in the supernatant revealed that induction was taking place.

They also described concomitantly with the increase in extracellular Beta-lactamase a steady increase in the release of lipopolysaccharide.

They suggest that the Beta-lactamase export was dependant on "enzyme over production in a cooperative manner" with a "self promoted perturbation of the outer membrane by overproduction of the enzyme leading to a semi-selective increase in membrane permeability".

It is clearly important to ascertain whether these findings can be repeated in clinical isolates of Acinetobacter since if correct, these data will throw further light on these fascinating but complicated enzymes.

PROTEIN AGGREGATION

If the thesis that many of these *Acinetobacter* Beta-lactamases exist in aggregated form is correct, then one must pose the question: why?

Under particular circumstances newly synthesised proteins do not fold properly *in vivo* and the result is amorphous protein aggregates (Bowden & Georgiou, 1990).

Protein aggregation is seen:

(i) in cells that are grown at elevated temperatures (Goldenberg &King, 1981)

(ii) during the synthesis of abnormal polypeptides (Prouty & Goldberg, 1972)

(iii) in cells expressing cloned gene products (Marston, 1986; Kane & Hartley, 1988; Mitraki & King, 1989; Schein, 1989)

Bowden and Georgiou (1990) stated that solubilisation of the aggregated proteins resulting from the expression of recombinant proteins usually required strong denaturing conditions indicating that the polypeptide chains were incorrectly folded.

Detailed studies *in vitro* have shown that protein aggregation arises from the association of partially folded intermediates and is dependant on the protein concentration (Brems, 1988; Jaenicke & Rudolph, 1989).

Haase-Pettingel and King (1988) have shown that the aggregation of P22 tail spike protein in *Salmonella typhimurium* is caused by the association of polypeptide molecules that fail to enter the folding pathway. The extent of *in vivo* aggregation depends on the accumulation of a soluble, partially folded,

intermediate.

This may either fold to the native conformation or participate in intermolecular association processes that lead to protein aggregates. Folding and aggregation are parallel pathways in kinetic competition with each other. So the ratio between native and aggregated products is determined by the rate of accummulation of the folding intermediate which is dependant on the rate of translation and the rates of polypeptide folding and association.

The relation between intracellular conditions such as ionic strength or interactions with cellular components, and protein aggregation is not clear (Bowden & Georgiou, 1990).

However growth temperature has been shown to effect drastically aggregation of the P22 tail spike protein, this may be related to the rate of translation and the interactions of the partially folded polypeptides with chaperonins (which exhibit strong temperature dependence) (Rothman, 1989).

Little is yet known about the folding and assembling of secreted proteins in procaryotes (Richey *et al.*, 1987; Hardy *et al.*, 1988).

It is believed however, that in the cytoplasm the precursor form of secreted polypeptides must be maintained in a conformation suitable for export (Randall & Hardy, 1989).

This secretory process appears to be modulated in two ways.

(i) The presence of the signal sequence which among other roles retards folding (Liu *et al.*, 1988)

(ii) By interactions with chaperonins e.g. GroEL, GoES, trigger factor and SecB (Lecker *et al.*, 1989)

The folding events that follow membrane translocation are not well defined (Georgiou & Bowden, 1990).

Some work has been carried out in the secretion of Beta-lactamases.

Minsky *et al.* (1986) established in *E.coli* with TEM-1 Beta-lactamase that an intermediate bound on the periplasmic side of the inner membrane is formed prior to the release of the protein in soluble form into the periplasmic space.

Bowden and Georgiou (1990) again studying TEM-1 in *E.coli* found that aggregation occurred when the rate of expression exceeded 2.5% of the total protein synthesis rate.

Therefore further work is required to establish whether the apparent aggregation of *Acinetobacter* β-lactamases is due to abnormal folding.

Interestingly, the β-lactamases of *Staphylococcus aureus* behave in a very similar fashion, in conventional polyacrylamide IEF gel systems, as the *Acinetobacter* enzymes (personal communication, Beecham Research Laboratories). Taking this into account with the work of Borneleit *et al.*(1991) leads one to speculate on the evolutionary place of the *Acinetobacter* β-lactamases. Perhaps they are intermediate between the Gram positive and Gram negative groups?

ROLE OF PRESUMED CHROMOSOMAL BETA-LACTAMASES OF ACINETOBACTER IN BETA-LACTAM RESISTANCE

Although it seems likely that these enzymes are largely responsible for the observed resistance to Beta-lactam drugs, it is also probable that other factors e.g. altered permeability or alterations in the penicillin-binding proteins may be equally important in some strains.

The ultimate test must involve the cloning of the Beta-lactamase gene into a suitable shuttle vector which would then allow the true role of this gene in the resistance to be ascertained.

THE DIRECTIONS FOR FUTURE RESEARCH

As has previously been alluded to, the future work on these fascinating enzymes should take at least three paths.

1. Microbiology

It is crucial that the *Acinetobacter* strains studied are correctly identified to the species level perhaps by the physiological and biochemical characterisation scheme delineated by Grimont and Bouvet (1991).

2. Molecular biology

This must involve the cloning of the Beta-lactamase gene (from perhaps more than one strain of the *Acinetobacters* studied here) into a suitable cloning vector.

This would allow:

(i) The role of the gene in the resistance mechanism to be determined and

(ii) After placement into a suitable sequencing vector, the nucleotide sequence could be established.

Once the sequence(s) is (are) known, then the position of these *Acinetobacter* Beta-lactamases in the evolutionary tree of Beta-lactamases can be ascertained.

The sequence data would also be important in allowing correlation with X-ray crystallographic data (if available). These data would allow study of the structure- activity relationships of these enzymes. Such information is crucial to the basic understanding of antimicrobial drug resistance and therefore in elucidating new ways (i.e. new antimicrobials) of bypassing this particular microbial defence mechanism.

3. Protein chemistry

The four ACE types enzymes should be purified to homogeneity and their N-terminal sequences determined. If possible X-ray crystallography should be carried out.

The purification to homogeneity will be necessary to allow more precise biochemical and kinetic characterisation of these enzymes e.g. the estimation of K_{cat} (turnover numbers) of these enzymes.

The purification will also hopefully clarify the molecular size of these proteins

The results presented in this thesis suggest that these enzymes exist in aggregated form. To investigate further these enzymes, and in particular to ascertain whether they actually do exist as aggregates or whether the aggregation was merely an artefact of the systems that I have used to study them, two techniques will be employed.

(i) *Analytical ultracentrifugation*

Determination of the sedimentation equilibrium provides the single most accurate and powerful method for the determination of the native molecular size of a protein. It is simple, non-destructive and relatively rapid to perform (Laue & Rhodes, 1990).

(ii) *Gel electrophoresis*

Native gel electrophoresis on gels containing differing concentrations of polyacrylamide will be carried out.

ENDPIECE

And now I shall complete the circle.

We started this thesis in the John Radcliffe Hospital in Oxford, with the first serious therapeutic use of penicillin and went on to discuss the fascinating evolution that has taken place both in the host and the parasite over the last 50 years. This took us through the evolution of Intensive therapy and the problems that infection poses in such units. We saw the shift from aggressive pathogens to less aggressive pathogens, from Gram positive to Gram negative, and back to Gram positive; infecting fit healthy patients to infecting very compromised patients.

This set the scene for the up and coming nosocomial pathogen - *Acinetobacter*.

In the initial cefuroxime resistance screen *Acinetobacter* was the commonest species present after *Enterobacter* .

We then looked at the resistance to cefuroxime and gentamicin in Gram negative, oxidase negative, aerobic bacilli within the whole hospital over a period of 5 years. This showed enormous variation (in the case of cefuroxime resistance) between different units but also underscored the particular problems in the Intensive therapy areas where the resistance was highest.

We then studied in some detail the fascinating β-lactamases of the genus *Acinetobacter*, discovering that they are completely different from any other β-lactamases previously described.

We pondered their possible role as an intermediary between Gram positive and Gram negative β-lactamases - but this is largely speculative.

Finally we have discussed the very basic, complicated yet fascinating, work on the chemistry of protein folding and aggregation.

Perhaps now I should close that circle by returning to where we started. It would then complete our journey from the bedside to the test tube back to its ultimate relevance - the patient.

During an eight week period in the summer of 1986 the Clinical Bacteriology department of the RIE noticed a sudden increase in the isolation of *Acinetobacter* spp. from the tips of arterial and central venous pressure lines from patients within the Cardiothoracic ITU.

This resulted in 14 cases, 3 with septicaemia and one related death. The strains were of the same biotype and resistance phenotype. There had been no isolation of similar strains from specimens of urine or tracheal secretions.

Interestingly, 11 of the 14 cases had been first on the list with a high proportion the first on the list on a Monday.

These lines are part of a complicated series of tubes which are attached to a plastic dome which in turn is in contact with a pressure transducer. The transducer measures either the arterial or venous pressure of the patient depending where the tip of the line is placed.

These lines and transducers are very complicated to set up and take some skill to do so quickly.

A new procedure had been instituted, just prior to this outbreak, and a new technician had just taken up post.

Previously the lines and transducer sets had been primed with saline as and when the need arose. However the unit had lost a great many of their senior staff nurses to Australia and the Middle East. Therefore due to the inexperience of the technician and the loss of these skilled nurses, a transducer set was primed each evening, to be used for any emergency that night or used for the first person on the operating list next day (or the following Monday if a weekend).

On investigation the same strain of *Acinetobacter* was isolated from the stored transducer sets and from the soap and sink in the preparation area.

It is therefore likely that the technician who set up most of these transducer sets, heavily contaminated his hands from the transducer heads and then contaminated the fluid that was being used to prime the sets.

The *Acinetobacter* then happily grew at room temperature within the giving set until it was introduced into the patient.

Cleaning the transducer heads with alcohol prior to storage and setting up the sets immediately prior to their use, resulted in no more cases.

Therefore we can see how subtle changes in the behaviour of the host allowed the parasite to gain a foothold. Such are the nuances of the host-parasite relationship.

I shall end with two quotes:

" *The DNA itself is lifeless, its language cold and austere. What gives the cell its life and personality are enzymes ... Nothing in nature is so tangible and vital to our lives as enzymes, and yet so poorly understood and appreciated by all but a few scientists* ". Arthur Kornberg.

And a quote from Peter Medawar - a constant source of encouragement. On the qualities needed by a scientist:

" *A sanguine temperament that expects to be able to solve a problem; power of application and that kind of fortitude that keeps them erect in the face of much that might otherwise cast them down; and above all, persistence, a refusal, bordering upon obstinancy, to give up and admit defeat* ".

Amen

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Appendix I

MICs to 400 strains of Gram negative aerobic bacilli to 12 antimicrobial agents.

NB:

ROC = CEFTRIAXONE

AZT = AZTREONAM

CRD = CEPHRADINE

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H001	1	XANM	4202000	>256.00	32.00	32.00	>256.00	16.00	>256.00	>256.00	0.50	>256.00	8.00	<0.06	>256.00
H002	2	ENB	3306573	>256.00	8.00	32.00	0.25	256.00	>256.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H003	3	SERL	4307763	64.00	0.25	0.25	0.50	8.00	64.00	<0.12	<0.12	256.00	1.00	<0.06	0.50
H004	4	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H005	5	ENBC	3305573	256.00	2.00	4.00	0.50	>256.00	64.00	4.00	1.00	>256.00	0.50	<0.06	0.50
H006	6	ENBC	3305573	>256.00	0.50	0.50	0.50	128.00	16.00	<0.12	<0.12	>256.00	1.00	<0.06	0.50
H007	7	PROSTU	0254000	>256.00	0.50	4.00	0.50	128.00	256.00	2.00	4.00	>256.00	4.00	0.12	0.50
H008	8	ENBC	3305573	>256.00	4.00	8.00	8.00	128.00	256.00	4.00	8.00	>256.00	1.00	0.12	32.00
H009	9	ENBC	3305573	>256.00	0.25	0.25	8.00	8.00	4.00	<0.12	0.25	128.00	1.00	0.12	32.00
H010	10	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	2.00	256.00	0.50	0.25	1.00
H011	11	SERM	5347761	128.00	<0.12	<0.12	2.00	8.00	64.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25
H012	12	KLEP	5215773	256.00	<0.12	<0.12	0.25	8.00	4.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H013	13	SER	5207763	256.00	<0.12	0.50	1.00	16.00	>256.00	<0.12	<0.12	>256.00	4.00	<0.06	0.50
H014	14	ENBC	3305573	>256.00	2.00	4.00	8.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	32.00
H015	15	ENBC	3305573	>256.00	8.00	16.00	8.00	64.00	256.00	4.00	8.00	>256.00	1.00	<0.06	32.00
H016	16	ENBC	3305573	>256.00	8.00	16.00	0.25	64.00	256.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H017	17	ACIA	0004042	32.00	32.00	32.00	0.50	256.00	64.00	64.00	4.00	256.00	1.00	1.00	0.25
H018	18	ACIA	0004042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H019	19	KLEOX	5254733	>256.00	4.00	1.00	8.00	8.00	128.00	32.00	0.12	128.00	1.00	<0.06	32.00
H020	20	KLEP	5215773	>256.00	32.00	64.00	8.00	64.00	>256.00	8.00	32.00	>256.00	1.00	<0.06	32.00
H021	21	ENBC	3305573	>256.00	0.50	1.00	0.25	128.00	32.00	0.25	0.25	>256.00	4.00	0.12	0.25
H022	22	ENBC	3305573	>256.00	1.00	1.00	<0.12	64.00	64.00	0.25	0.25	>256.00	2.00	0.12	<0.12
H023	23	KLEOX	5255773	>256.00	0.50	<0.12	8.00	1.00	64.00	2.00	<0.12	64.00	1.00	0.12	64.00
H024	24	PROVU	0070020	64.00	<0.12	<0.12	<0.12	1.00	64.00	<0.12	<0.12	128.00	4.00	<0.06	<0.12
H025	25	XANM	0452341NE	>256.00	64.00	64.00	4.00	256.00	256.00	8.00	32.00	256.00	>256.00	1.00	4.00
H026	26	ACIA	0004042	64.00	16.00	16.00	8.00	128.00	64.00	32.00	8.00	>256.00	1.00	1.00	32.00
H027	27	CITF	1004572	>256.00	1.00	2.00	0.25	64.00	16.00	1.00	1.00	>256.00	0.50	<0.06	0.25
H028	28	ENBS	3305373	128.00	<0.12	<0.12	0.25	256.00	16.00	<0.12	<0.12	256.00	0.50	<0.06	0.25
H029	29	SERL	1106723	32.00	<0.12	<0.12	0.25	8.00	32.00	<0.12	<0.12	128.00	1.00	<0.06	<0.12
H030	30	ENBC	3305573	>256.00	4.00	8.00	0.25	256.00	256.00	4.00	4.00	>256.00	0.50	0.12	0.25
H031	31	ENBC	3305573	>256.00	4.00	8.00	0.25	256.00	>256.00	4.00	4.00	>256.00	0.50	0.12	0.25
H032	32	ACIA	0004042	64.00	16.00	16.00	8.00	64.00	32.00	32.00	4.00	256.00	1.00	1.00	32.00
H033	33	ACIA	0004042	64.00	8.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	1.00	0.50	16.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H034	34	SERM	4106721	64.00	0.12	<0.12	2.00	16.00	128.00	<0.12	<0.12	256.00	0.50	0.12	0.50
H035	35	CITF	1604773	>256.00	0.50	2.00	0.25	128.00	8.00	1.00	8.00	>256.00	1.00	0.12	0.25
H036	36	ENBC	3305573	>256.00	<0.12	<0.12	16.00	2.00	2.00	<0.12	<0.12	8.00	0.50	0.12	64.00
H037	37	ECO	5144572	>256.00	0.50	2.00	0.25	64.00	64.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H038	38	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	4.00	>256.00	2.00	<0.06	8.00
H039	39	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	4.00	>256.00	2.00	<0.06	8.00
H040	40	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.50	1.00
H041	41	ENB	3107573	>256.00	0.50	4.00	4.00	128.00	256.00	1.00	0.50	>256.00	0.50	<0.06	32.00
H042	42	ENBC	3305573	>256.00	16.00	32.00	8.00	128.00	>256.00	8.00	8.00	>256.00	1.00	<0.06	64.00
H043	43	ENBC	3105573	>256.00	0.50	1.00	0.25	>256.00	32.00	0.25	0.50	>256.00	0.50	0.12	0.25
H044	44	CITF	1604573	>256.00	8.00	4.00	0.50	256.00	64.00	4.00	32.00	>256.00	1.00	0.12	0.50
H045	45	ECO	5144552	>256.00	<0.12	<0.12	0.50	0.50	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H046	46	ECO	5144552	8.00	0.12	<0.12	0.50	4.00	8.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H047	47	ENBC	3305773	>256.00	1.00	2.00	0.50	256.00	64.00	1.00	1.00	>256.00	0.50	<0.06	0.50
H048A	48	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	32.00	4.00	256.00	0.50	0.50	1.00
H048B	49	ENBC	3305573	>256.00	1.00	2.00	0.50	256.00	64.00	1.00	1.00	>256.00	1.00	<0.06	0.50
H049	50	SERM	4106763	128.00	<0.12	0.25	4.00	16.00	32.00	<0.12	<0.12	>256.00	4.00	0.12	0.25
H050	51	SERM	4107721	128.00	<0.12	0.25	4.00	8.00	16.00	<0.12	<0.12	>256.00	4.00	0.12	0.25
H051	52	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	8.00	>256.00	1.00	<0.06	8.00
H052	53	KLEB	5245773	>256.00	1.00	0.25	16.00	1.00	64.00	4.00	<0.12	64.00	0.50	<0.06	64.00
H053	54	ENBC	3305573	>256.00	1.00	1.00	0.25	>256.00	64.00	1.00	0.50	>256.00	0.50	<0.06	0.25
H054	55	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	8.00	8.00	>256.00	1.00	0.12	8.00
H055	56	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	256.00	8.00	8.00	>256.00	1.00	<0.06	8.00
H056	57	MORM	0174000	128.00	<0.12	<0.12	0.50	8.00	16.00	<0.12	<0.12	>256.00	2.00	<0.06	0.25
H057	58	ENBC	3305573	256.00	1.00	1.00	0.50	>256.00	64.00	1.00	0.50	>256.00	0.50	<0.06	0.25
H058	59	ENB	3304573	>256.00	8.00	8.00	0.25	>256.00	128.00	4.00	4.00	>256.00	0.50	0.12	8.00
H059	60	ENBC	3305573	>256.00	<0.12	0.25	0.50	4.00	32.00	<0.12	<0.12	32.00	0.50	0.12	8.00
H060	61	ENBC	3305573	>256.00	4.00	16.00	0.50	>256.00	128.00	4.00	4.00	>256.00	0.50	0.12	0.25
H061A	62	ENBC	3105573	>256.00	4.00	8.00	8.00	64.00	128.00	4.00	4.00	>256.00	0.50	<0.06	32.00
H061B	63	ENBC	3105573	>256.00	8.00	32.00	8.00	128.00	>256.00	4.00	8.00	>256.00	0.50	<0.06	32.00
H062	64	KLEB	5245773	>256.00	1.00	0.25	16.00	1.00	64.00	4.00	<0.12	256.00	1.00	<0.06	64.00
H063	65	ACIA	0004042	128.00	64.00	32.00	2.00	128.00	128.00	128.00	4.00	>256.00	0.50	1.00	1.00
H064	66	ENBC	3305573	>256.00	2.00	4.00	8.00	256.00	>256.00	0.50	0.50	>256.00	2.00	<0.06	32.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H065	67	MORM	0154000	>256.00	0.12	<0.12	16.00	4.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	32.00
H066	68	ENBC	3305533	>256.00	2.00	2.00	8.00	128.00	256.00	0.50	0.50	>256.00	1.00	<0.06	32.00
H067	69	ENBC	3305573	>256.00	8.00	16.00	0.50	64.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H068	70	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	8.00	256.00	0.50	0.25	1.00
H069	71	XANM	0472341	64.00	16.00	8.00	128.00	256.00	128.00	4.00	0.50	>256.00	>256.00	16.00	32.00
H071	72	PSEORZ	0205002	64.00	16.00	16.00	128.00	64.00	32.00	32.00	4.00	>256.00	1.00	2.00	32.00
H072	73	KLEP	1014773	16.00	<0.12	<0.12	0.25	2.00	1.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H073	74	ENB	3304573	>256.00	2.00	4.00	8.00	64.00	64.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H074	75	MORM	0174000	>256.00	<0.12	1.00	1.00	4.00	16.00	0.25	1.00	>256.00	4.00	<0.06	1.00
H075	76	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	1.00
H076	77	ACIA	0004042	256.00	1.00	1.00	4.00	16.00	>256.00	0.25	<0.12	>256.00	0.50	0.12	1.00
H077	78	ENBC	3305573	>256.00	1.00	2.00	0.50	>256.00	32.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H078	79	PSELU	5467741	64.00	0.12	0.25	2.00	16.00	64.00	<0.12	<0.12	>256.00	4.00	<0.06	0.50
H079	80	ACIA	1041473NE	64.00	8.00	16.00	8.00	32.00	32.00	32.00	4.00	256.00	1.00	0.50	2.00
H080	81	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.25	1.00
H081	82	ACIA	0205042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	256.00	0.50	0.25	1.00
H082	83	MORM	0174000	256.00	0.50	2.00	0.50	8.00	32.00	0.50	2.00	>256.00	2.00	<0.06	1.00
H083	84	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	8.00	8.00	>256.00	2.00	<0.06	8.00
H084	85	ECO	5044572	32.00	<0.12	0.50	0.50	64.00	32.00	0.25	0.25	128.00	0.50	<0.06	0.50
H087	86	SERL	5107768	16.00	<0.12	0.25	2.00	16.00	32.00	<0.12	<0.12	256.00	1.00	0.12	1.00
H088	87	MORM	0174000	>256.00	0.25	0.50	0.50	256.00	>256.00	0.50	1.00	>256.00	4.00	0.12	0.50
H089	88	XANM	5202000	>256.00	256.00	64.00	256.00	>256.00	>256.00	>256.00	2.00	>256.00	>256.00	4.00	128.00
H090	89	KLU	5344573	32.00	<0.12	<0.12	0.50	4.00	2.00	<0.12	<0.12	64.00	0.50	<0.06	0.50
H091	90	ECO	1144172	1.00	<0.12	<0.12	0.50	1.00	1.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H092	91	ENBC	3305573	>256.00	64.00	64.00	0.50	128.00	>256.00	16.00	16.00	>256.00	2.00	<0.06	0.25
H093	92	ENB	3104573	>256.00	16.00	64.00	0.25	64.00	>256.00	8.00	8.00	>256.00	0.50	<0.06	0.25
H094	93	MORM	1174000	64.00	<0.12	<0.12	1.00	8.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H095	94	MORM	0174000	>256.00	<0.12	1.00	1.00	8.00	64.00	0.25	1.00	>256.00	8.00	<0.06	1.00
H097	95	PROVU	0474021	>256.00	<0.12	<0.12	0.25	2.00	128.00	<0.12	<0.12	256.00	8.00	<0.06	0.25
H098	96	ECO	5144552	2.00	<0.12	<0.12	5.00	0.50	4.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H099	97	ENBC	3105573	>256.00	0.50	1.00	0.25	>256.00	64.00	0.50	0.50	>256.00	0.50	0.12	0.25
H100	98	ENB	3304573	64.00	8.00	8.00	32.00	64.00	16.00	8.00	4.00	256.00	1.00	2.00	8.00
H101	99	ACIA	0005042	>256.00	16.00	32.00	8.00	128.00	64.00	64.00	4.00	>256.00	1.00	1.00	128.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H102	100	KLEP	5215763	64.00	<0.12	<0.12	0.25	2.00	1.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H103	101	ENBC	3305573	>256.00	4.00	16.00	8.00	128.00	>256.00	4.00	4.00	>256.00	4.00	<0.06	32.00
H104	102	ENBC	3305573	>256.00	4.00	8.00	8.00	128.00	256.00	4.00	2.00	>256.00	2.00	<0.06	32.00
H105	103	ENBC	3305573	>256.00	8.00	16.00	0.25	>256.00	>256.00	4.00	4.00	>256.00	2.00	<0.06	<0.12
H106	104	ENBC	3305573	>256.00	16.00	16.00	0.25	128.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H107	105	ECO	5144573	2.00	<0.12	<0.12	0.50	1.00	16.00	<0.12	<0.12	32.00	0.50	<0.06	0.25
H108	106	ENBC	3305573	>256.00	4.00	8.00	0.25	128.00	256.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H109	107	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	256.00	4.00	2.00	>256.00	2.00	<0.06	64.00
H110	108	ENBC	3305573	>256.00	0.25	0.50	0.50	32.00	16.00	0.25	0.25	64.00	1.00	<0.06	8.00
H111	109	ECO	5144572	2.00	<0.12	<0.12	0.50	2.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H112	110	ECO	5144552	>256.00	<0.12	<0.12	0.50	1.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H113	111	ECO	5144512	128.00	0.25	0.25	0.50	8.00	16.00	1.00	0.50	128.00	0.50	<0.06	0.25
H115	113	ECO	5104572	>256.00	0.50	1.00	0.50	32.00	16.00	1.00	1.00	256.00	1.00	0.12	0.25
H116	114	ECO	5144512	2.00	0.12	<0.12	2.00	1.00	1.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H117A	115	ACIA	0005042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.25	1.00
H117B	116	PSECEP	1473773NE	64.00	16.00	16.00	1.00	32.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H118	117	ENBC	3305573	>256.00	16.00	8.00	0.25	128.00	256.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H119	118	ENBA	5304773	>256.00	4.00	2.00	1.00	32.00	>256.00	0.50	8.00	>256.00	4.00	0.12	0.50
H120	119	HAFA	5104112	128.00	8.00	4.00	0.25	8.00	64.00	0.50	8.00	>256.00	1.00	<0.06	<0.12
H121	120	ENBC	3305573	4.00	0.12	<0.12	0.50	16.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H122	121	ENBC	3305573	>256.00	4.00	4.00	8.00	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H123	122	HAFA	5305112	128.00	4.00	4.00	<0.12	8.00	64.00	1.00	16.00	256.00	1.00	<0.06	0.25
H124	123	ACIA	0005042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	4.00	256.00	0.50	0.50	1.00
H125	124	XANM	4202000	128.00	64.00	64.00	64.00	>256.00	>256.00	32.00	1.00	>256.00	>256.00	1.00	128.00
H126	125	ACIL	0000071NE	128.00	32.00	64.00	0.25	>256.00	64.00	>256.00	64.00	>256.00	0.50	0.50	0.25
H127	126	ENBC	3305573	>256.00	1.00	4.00	0.25	128.00	256.00	0.25	0.25	>256.00	0.50	<0.06	0.25
H128	127	ACIA	0200042	32.00	8.00	8.00	1.00	32.00	32.00	16.00	2.00	128.00	0.50	0.25	1.00
H129	128	CITD	3344552	>256.00	8.00	4.00	0.50	4.00	256.00	16.00	0.50	128.00	0.50	<0.06	0.50
H130	129	PSE	1555773NE	32.00	8.00	8.00	1.00	64.00	32.00	16.00	2.00	128.00	0.50	0.50	1.00
H131	130	ACIL	0200000	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	256.00	1.00	0.50	1.00
H132	131	ENBC	3305573	>256.00	4.00	4.00	8.00	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H133	132	CITF	1404532	>256.00	2.00	2.00	0.50	128.00	4.00	1.00	1.00	>256.00	1.00	<0.06	1.00
H134	133	ECO	5144552	256.60	<0.12	<0.12	0.50	2.00	4.00	<0.12	0.25	8.00	0.50	<0.06	0.50

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H135	134	CITF	1404572	>256.00	2.00	2.00	0.25	64.00	16.00	1.00	8.00	>256.00	1.00	<0.06	0.25
H136	135	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	4.00	256.00	0.50	0.50	1.00
H137	136	ENBC	3305573	>256.00	16.00	32.00	0.25	>256.00	>256.00	8.00	8.00	>256.00	8.00	<0.06	0.25
H138	137	CITD	3344573	>256.00	4.00	2.00	0.50	>256.00	256.00	8.00	0.50	64.00	1.00	<0.06	0.50
H139	138	KLEP	1215773	>256.00	0.25	0.25	4.00	32.00	32.00	0.25	0.50	64.00	0.50	0.25	16.00
H140	139	ACIA	0004042	64.00	16.00	16.00	0.25	64.00	32.00	32.00	2.00	256.00	0.50	0.12	0.25
H141	140	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	8.00	256.00	0.50	0.50	1.00
H142	141	MORM	0174000	64.00	<0.12	1.00	0.50	4.00	16.00	<0.12	0.50	>256.00	2.00	<0.06	0.50
H143	142	ECO	5104572	>256.00	0.25	1.00	1.00	16.00	16.00	0.50	1.00	128.00	0.50	<0.06	1.00
H144	143	ECO	5044552	>256.00	<0.12	<0.12	0.50	2.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H145	144	MORM	0174000	256.00	<0.12	0.25	0.50	4.00	16.00	<0.12	0.25	>256.00	4.00	<0.06	0.50
H146	145	ACIA	0205042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	4.00	256.00	0.50	0.50	1.00
H147	146	ACIA	0004042	8.00	4.00	4.00	8.00	8.00	8.00	4.00	1.00	64.00	1.00	0.12	0.25
H148	147	SERO	5346773	8.00	0.25	0.25	1.00	8.00	32.00	<0.12	<0.12	64.00	1.00	0.12	1.00
H149	148	MORM	0174000	128.00	<0.12	0.25	0.50	4.00	16.00	<0.12	0.50	>256.00	4.00	<0.06	0.50
H150	149	ENBC	3305773	>256.00	2.00	2.00	0.50	256.00	64.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H151	150	ACIL	0000050NE	8.00	2.00	2.00	0.50	8.00	4.00	8.00	0.50	16.00	1.00	0.12	0.25
H152	151	XANM	4202000	128.00	64.00	16.00	32.00	128.00	>256.00	8.00	0.50	256.00	256.00	2.00	1.00
H153A	152	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	128.00	2.00	2.00	>256.00	1.00	<0.06	64.00
H153B	153	ENBC	3305573	>256.00	4.00	8.00	0.25	64.00	128.00	2.00	2.00	>256.00	2.00	<0.06	0.25
H154	154	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.50	1.00
H155	155	ENBA	5305773	>256.00	2.00	4.00	0.50	256.00	64.00	1.00	1.00	>256.00	0.50	<0.06	0.25
H156	156	ENBC	3305573	>256.00	2.00	1.00	8.00	256.00	128.00	0.50	0.25	>256.00	1.00	<0.06	64.00
H157	157	ACIA	0006042	16.00	2.00	8.00	16.00	16.00	16.00	8.00	2.00	128.00	1.00	0.50	0.50
H158	158	ACIA	0004042	8.00	2.00	4.00	64.00	16.00	8.00	4.00	1.00	64.00	1.00	0.12	1.00
H159	159	ACIA	0205042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H160	160	ENBC	3305573	>256.00	2.00	1.00	8.00	256.00	128.00	0.50	0.25	>256.00	1.00	<0.06	64.00
H161	161	CITF	1604773	>256.00	<0.12	<0.12	8.00	64.00	64.00	<0.12	<0.12	64.00	1.00	<0.06	32.00
H162	162	ACIA	0004042	32.00	16.00	32.00	0.50	256.00	64.00	64.00	8.00	256.00	0.50	0.50	0.25
H164	163	ENBC	3305573	>256.00	8.00	16.00	8.00	64.00	128.00	4.00	4.00	>256.00	2.00	<0.06	32.00
H165	164	KLEP	5215773	8.00	<0.12	<0.12	0.25	2.00	16.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H166	165	ENBC	3305573	>256.00	1.00	1.00	8.00	256.00	32.00	0.50	<0.12	>256.00	2.00	<0.06	32.00
H167	166	ACIA	0004042	64.00	16.00	16.00	1.00	64.00	16.00	64.00	2.00	256.00	0.50	0.50	0.50

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H168	167	ACIA	0004042	128.00	32.00	16.00	1.00	64.00	16.00	64.00	8.00	>256.00	0.50	0.50	0.50
H169	168	ENBC	3305573	>256.00	1.00	1.00	8.00	256.00	16.00	0.25	0.25	>256.00	2.00	<0.06	32.00
H170	169	ENBC	3305572	>256.00	1.00	1.00	8.00	256.00	8.00	0.50	<0.12	>256.00	2.00	<0.06	32.00
H171	170	SERM	4117521	64.00	<0.12	<0.12	2.00	32.00	256.00	0.50	<0.12	>256.00	1.00	0.25	0.25
H172	171	ENBC	3305573	>256.00	2.00	2.00	0.25	64.00	16.00	0.50	<0.12	>256.00	1.00	<0.06	0.25
H173	172	CITF	1404570	>256.00	1.00	1.00	0.25	64.00	4.00	0.50	<0.12	>256.00	1.00	<0.06	0.25
H174	173	ENBC	3305573	>256.00	16.00	32.00	4.00	256.00	16.00	8.00	4.00	>256.00	1.00	<0.06	32.00
H175	174	ECO	5044572	32.00	<0.12	<0.12	0.25	2.00	256.00	<0.12	<0.12	32.00	2.00	<0.06	0.25
H176	175	ENBC	3305532	128.00	0.50	0.50	0.25	128.00	128.00	0.25	<0.12	>256.00	2.00	<0.06	0.25
H177	176	KLEP	5215773	32.00	0.25	<0.12	0.25	2.00	64.00	<0.12	<0.12	8.00	2.00	<0.06	0.25
H178A	177	ENBC	3304573	>256.00	4.00	8.00	0.25	128.00	16.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H178B	178	SERM	5107761	>256.00	<0.12	<0.12	2.00	16.00	256.00	<0.12	<0.12	256.00	2.00	0.25	16.00
H179	179	MORM	0374000	>256.00	0.50	2.00	4.00	4.00	16.00	0.50	0.50	>256.00	4.00	<0.06	16.00
H180	180	ENBC	3305573	>256.00	1.00	1.00	8.00	128.00	16.00	0.25	<0.12	>256.00	2.00	<0.06	32.00
H181	181	MORM	0174000	>256.00	0.50	2.00	4.00	4.00	64.00	0.50	4.00	>256.00	4.00	<0.06	64.00
H182	182	ENBC	3305573	>256.00	8.00	32.00	0.25	32.00	128.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H183A	183	KLEP	5215773	32.00	<0.12	<0.12	0.25	1.00	1.00	<0.12	<0.12	8.00	2.00	<0.06	0.25
H183B	184	CITF	1004572	32.00	<0.12	<0.12	0.50	32.00	8.00	<0.12	<0.12	64.00	2.00	<0.06	0.25
H184	185	ECO	5144572	>256.00	<0.12	<0.12	4.00	2.00	2.00	<0.12	<0.12	8.00	2.00	<0.06	32.00
H185	186	ENBC	3305573	>256.00	<0.12	0.25	8.00	64.00	8.00	<0.12	<0.12	32.00	2.00	<0.06	32.00
H186	187	ENBC	3305573	>256.00	<0.12	<0.12	8.00	64.00	8.00	<0.12	<0.12	32.00	2.00	<0.06	32.00
H187	188	CITF	3144533	32.00	<0.12	<0.12	0.50	4.00	2.00	<0.12	<0.12	4.00	2.00	<0.06	0.50
H188A	189	ENBC	1305573	64.00	<0.12	<0.12	0.50	128.00	4.00	<0.12	<0.12	128.00	1.00	<0.06	0.50
H188B	190	ENBC	1305573	128.00	<0.12	<0.12	0.50	64.00	16.00	<0.12	<0.12	>256.00	2.00	<0.06	0.25
H189	191	MORM	0154000	256.00	0.25	2.00	0.50	4.00	16.00	0.50	1.00	>256.00	2.00	<0.06	0.50
H190	192	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H191	193	ENBC	3305533	>256.00	128.00	128.00	8.00	128.00	256.00	16.00	32.00	>256.00	1.00	<0.06	32.00
H192	194	ENBC	3305573	>256.00	2.00	8.00	0.50	64.00	64.00	0.50	1.00	>256.00	1.00	<0.06	0.25
H193	195	ENBC	3305573	256.00	4.00	8.00	4.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H195	196	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H196	197	ECO	1144502	>256.00	<0.12	<0.12	0.50	8.00	8.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H197	198	ENBC	3305573	>256.00	32.00	32.00	0.25	128.00	>256.00	16.00	8.00	>256.00	4.00	<0.06	0.25
H198	199	SERM	5307720	>256.00	1.00	1.00	2.00	32.00	>256.00	0.25	<0.12	>256.00	4.00	0.12	0.50

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H199	200	KLEOX	5045773	>256.00	1.00	0.25	1.00	0.50	64.00	4.00	<0.12	64.00	0.25	<0.06	0.50
H200	201	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	2.00	64.00	4.00	256.00	0.50	0.50	1.00
H201	202	ECO	5144542	64.00	0.12	0.25	0.50	4.00	64.00	0.50	0.25	64.00	0.50	<0.06	0.50
H202	203	ENB	1105573	>256.00	4.00	4.00	0.25	128.00	256.00	2.00	1.00	>256.00	0.50	<0.06	0.25
H203	204	ECO	5140002	64.00	<0.12	0.25	0.50	4.00	2.00	0.50	<0.12	64.00	0.50	<0.06	0.50
H204	205	ECO	5144552	>256.00	16.00	16.00	2.00	64.00	64.00	32.00	2.00	256.00	0.50	0.12	2.00
H205	206	ECO	5144522	128.00	0.25	1.00	0.50	16.00	32.00	1.00	1.00	256.00	0.50	<0.06	0.25
H206	207	MORM	0154000	128.00	<0.12	<0.12	1.00	4.00	64.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H207	208	ENBA	5304773	>256.00	<0.12	0.25	0.50	128.00	64.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25
H208	209	KLEOX	5245773	32.00	<0.12	<0.12	0.25	16.00	64.00	<0.12	<0.12	8.00	0.50	0.12	0.25
H209	210	SERM	5107521	128.00	<0.12	0.50	1.00	16.00	64.00	<0.12	<0.12	>256.00	2.00	0.12	1.00
H211	211	MORM	0174000	64.00	0.25	<0.12	0.50	4.00	>256.00	<0.12	<0.12	>256.00	4.00	<0.06	0.25
H212	212	ECO	5144552	>256.00	<0.12	<0.12	0.50	2.00	4.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H213	213	ENBC	3305573	256.00	<0.12	0.25	0.50	256.00	>256.00	<0.12	<0.12	256.00	2.00	<0.06	0.25
H214	214	ENB	1105573	>256.00	4.00	4.00	0.25	128.00	2.00	8.00	2.00	>256.00	0.50	<0.06	0.25
H215	215	ENB	1105573	256.00	4.00	4.00	0.50	256.00	32.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H217	217	MORM	0174000	64.00	<0.12	<0.12	1.00	4.00	256.00	<0.12	<0.12	256.00	4.00	<0.06	1.00
H218	218	N/P	7345773	256.00	16.00	16.00	0.50	128.00	16.00	16.00	2.00	>256.00	1.00	1.00	0.50
H219	219	ECO	4144402	16.00	<0.12	<0.12	0.25	2.00	64.00	<0.12	<0.12	32.00	0.25	<0.06	0.25
H220	220	MORM	0174000	64.00	<0.12	<0.12	0.50	4.00	64.00	<0.12	<0.12	256.00	4.00	<0.06	0.25
H221	221	MORM	0174000	128.00	<0.12	<0.12	0.50	4.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	0.50
H222	222	MORM	3374000	128.00	<0.12	<0.12	1.00	4.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H223	223	PSEO	0201002	16.00	8.00	8.00	0.50	32.00	16.00	8.00	0.50	128.00	0.50	0.50	0.25
H224	224	MORM	0174000	128.00	<0.12	<0.12	0.50	4.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	0.50
H225	225	ENBC	3305573	16.00	<0.12	<0.12	0.50	256.00	2.00	<0.12	<0.12	128.00	4.00	<0.06	0.50
H226	226	ENB	1105573	256.00	4.00	4.00	0.50	128.00	256.00	4.00	2.00	>256.00	0.50	<0.06	0.50
H227	227	MORM	0174000	128.00	<0.12	<0.12	1.00	8.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H228	228	ENB	3305173	>256.00	0.50	0.50	0.50	64.00	16.00	0.25	0.25	256.00	2.00	<0.06	0.25
H229	229	PSEO	0201002	32.00	16.00	16.00	0.50	128.00	64.00	64.00	2.00	256.00	1.00	1.00	<0.12
H230	230	MORM	0174000	128.00	<0.12	<0.12	0.50	8.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H231	231	ENB	3105573	256.00	4.00	8.00	0.25	256.00	>256.00	8.00	2.00	>256.00	0.50	<0.06	0.50
H232	232	ENB	3305173	64.00	<0.12	0.25	0.25	64.00	8.00	<0.12	<0.12	64.00	1.00	<0.06	0.25
H233	233	ENBC	3305573	>256.00	32.00	64.00	0.50	64.00	>256.00	8.00	16.00	>256.00	1.00	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H234	234	ENBA	5105773	>256.00	2.00	4.00	1.00	256.00	64.00	1.00	2.00	>256.00	2.00	<0.06	1.00
H235	235	ENBA	5305773	>256.00	2.00	4.00	1.00	256.00	64.00	1.00	2.00	>256.00	2.00	<0.06	1.00
H236	236	ECO	5144562	256.00	0.25	1.00	0.50	16.00	16.00	2.00	1.00	256.00	0.50	<0.06	0.25
H237	237	ECO	5144552	64.00	<0.12	0.25	0.50	4.00	16.00	1.00	0.25	64.00	0.50	<0.06	0.25
H238	238	ENBA	5305773	>256.00	2.00	4.00	1.00	256.00	64.00	1.00	1.00	>256.00	2.00	<0.06	1.00
H239	239	ECO	5144562	256.00	0.25	1.00	0.50	16.00	16.00	1.00	1.00	256.00	0.50	<0.06	0.25
H240	240	ECO	5144552	>256.00	<0.12	0.25	0.50	16.00	8.00	0.50	0.25	64.00	0.50	<0.06	0.25
H241	241	ECO	5164552	>256.00	<0.12	<0.12	2.00	0.50	16.00	<0.12	<0.12	32.00	0.50	<0.06	2.00
H242	242	ENBC	3305573	64.00	<0.12	<0.12	0.50	64.00	8.00	<0.12	<0.12	128.00	2.00	<0.06	0.25
H243	243	MORM	0374000	>256.00	<0.12	1.00	1.00	8.00	64.00	0.25	1.00	>256.00	4.00	<0.06	1.00
H244	244	ECO	5144572	>256.00	1.00	1.00	0.50	32.00	16.00	1.00	0.50	256.00	0.50	<0.06	0.25
H245	245	CITF	1404572	16.00	1.00	<0.12	0.50	64.00	2.00	<0.12	<0.12	32.00	1.00	<0.06	0.25
H246	246	ENBC	3305573	>256.00	16.00	32.00	0.25	64.00	256.00	4.00	8.00	>256.00	1.00	<0.06	4.00
H247	247	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	256.00	4.00	8.00	>256.00	1.00	<0.06	4.00
H248	248	ACIA	0006000	<0.12	<0.12	<0.12	<0.12	<0.12	2.00	<0.12	<0.12	2.00	0.12	<0.06	<0.12
H249	249	KLEOX	0004353	64.00	16.00	16.00	2.00	64.00	64.00	64.00	4.00	256.00	0.50	0.50	1.00
H250	250	MORM	0374000	256.00	0.25	2.00	0.50	4.00	32.00	0.50	2.00	>256.00	4.00	<0.06	0.50
H251	251	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	256.00	2.00	2.00	>256.00	1.00	<0.06	64.00
H252	252	ENBC	3305533	>256.00	4.00	4.00	0.50	256.00	256.00	1.00	1.00	>256.00	2.00	<0.06	0.25
H253	253	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.25	1.00
H254	254	KLU	5344572	256.00	<0.12	1.00	0.50	32.00	16.00	1.00	0.50	256.00	0.50	0.12	0.25
H255	255	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	4.00	>256.00	0.50	0.50	1.00
H256	256	ACIA	0004042	64.00	16.00	16.00	4.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	1.00
H257	257	CITF	3544772	>256.00	4.00	4.00	1.00	128.00	64.00	4.00	4.00	>256.00	0.50	<0.06	1.00
H258	258	ACIA	0004042	16.00	8.00	4.00	0.25	64.00	16.00	8.00	0.50	128.00	0.50	0.25	0.25
H259	259	ACIA	0004042	64.00	16.00	32.00	0.50	128.00	64.00	32.00	2.00	>256.00	0.50	0.25	0.25
H261	260	ENBA	5305773	>256.00	<0.12	0.25	0.50	>256.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	0.25
H262	261	ENBA	5305773	>256.00	4.00	4.00	0.25	>256.00	256.00	4.00	4.00	>256.00	4.00	<0.06	0.25
H263	262	ECO	5144572	64.00	0.25	0.25	0.50	16.00	16.00	1.00	0.50	128.00	1.00	<0.06	0.25
H264	263	ENBC	3305573	>256.00	0.50	0.50	0.50	64.00	16.00	<0.12	<0.12	>256.00	2.00	<0.06	0.25
H265	264	ECO	5144552	>256.00	<0.12	<0.12	0.50	1.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H266	265	SERM	5307721	256.00	0.50	1.00	1.00	32.00	>256.00	<0.12	<0.12	>256.00	4.00	0.12	0.50
H267	266	ENB	2305573	256.00	8.00	8.00	0.25	64.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H268	267	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	1.00
H269	268	PROP	0036020	>256.00	0.25	<0.12	<0.12	2.00	256.00	<0.12	<0.12	256.00	8.00	<0.06	<0.12
H270	269	ACIA	0004040	8.00	2.00	8.00	16.00	16.00	16.00	4.00	1.00	64.00	1.00	0.25	0.25
H271	270	ECO	5144552	>256.00	<0.12	<0.12	0.50	1.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H272	271	ENB	3304573	256.00	1.00	1.00	0.25	128.00	64.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H273	272	ACIA	0004040	16.00	2.00	8.00	16.00	16.00	16.00	4.00	1.00	64.00	1.00	0.25	0.25
H274	273	PSEC	1453773	32.00	8.00	8.00	0.50	64.00	32.00	16.00	1.00	256.00	0.50	0.25	<0.12
H275	274	ECO	4144572	4.00	<0.12	<0.12	0.50	4.00	2.00	<0.12	<0.12	8.00	1.00	<0.06	0.25
H276	275	KLEOX	0255773	>256.00	2.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	0.50	<0.06	0.25
H277	276	SERL	5304723	32.00	<0.12	<0.12	0.25	8.00	64.00	<0.12	<0.12	128.00	1.00	<0.06	<0.12
H278	277	ACIA	0004042	64.00	16.00	16.00	1.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	0.50
H279	278	ENBC	3305573	64.00	0.25	0.25	0.50	256.00	8.00	<0.12	<0.12	256.00	2.00	<0.06	0.50
H280	279	ENBC	3305573	>256.00	4.00	8.00	0.25	64.00	256.00	1.00	2.00	>256.00	1.00	<0.06	0.25
H281	280	KLEOX	5255773	>256.00	1.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	1.00	<0.06	0.25
H282	281	ECO	5144552	>256.00	<0.12	<0.12	0.50	2.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H283	282	ENBC	3305573	>256.00	1.00	2.00	8.00	64.00	256.00	0.50	1.00	>256.00	1.00	<0.06	32.00
H284	283	ENBC	3305573	>256.00	1.00	0.50	0.50	64.00	32.00	0.25	0.25	>256.00	2.00	<0.06	0.25
H285	284	MORM	0174000	256.00	<0.12	<0.12	1.00	8.00	16.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H286	285	SERM	5107521	>256.00	2.00	4.00	2.00	32.00	>256.00	0.25	<0.12	>256.00	4.00	0.12	1.00
H287	360	SERM	5307721	64.00	<0.12	0.25	4.00	8.00	64.00	<0.12	<0.12	256.00	1.00	0.12	0.50
H288	286	SER	5105721	256.00	0.50	0.50	2.00	32.00	>256.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H289	287	ENBC	3105573	>256.00	4.00	16.00	16.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00
H290	288	ACIA	0004042	128.00	8.00	32.00	0.50	64.00	64.00	64.00	4.00	256.00	0.50	0.25	0.25
H292	289	ENB	3304173	>256.00	16.00	64.00	4.00	64.00	>256.00	16.00	16.00	>256.00	1.00	<0.06	32.00
H293	290	SER	4305523	>256.00	2.00	2.00	1.00	>256.00	128.00	4.00	4.00	>256.00	1.00	<0.06	1.00
H294	291	ENBC	3305573	>256.00	2.00	4.00	0.50	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H295	292	ECO	5144552	256.00	<0.12	<0.12	0.50	2.00	1.00	<0.12	<0.12	8.00	0.50	<0.06	0.50
H296	293	ENBC	3105573	>256.00	4.00	4.00	8.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00
H297	294	ENBC	3305573	128.00	2.00	2.00	0.50	64.00	16.00	0.50	0.50	128.00	1.00	<0.06	8.00
H298	295	ERWA	1005422	64.00	16.00	16.00	4.00	64.00	32.00	32.00	2.00	256.00	0.50	0.25	1.00
H299	296	ACIA	0004042	128.00	32.00	32.00	2.00	64.00	128.00	64.00	4.00	>256.00	0.50	4.00	1.00
H301	297	ENBC	3305573	128.00	2.00	2.00	0.50	64.00	16.00	0.50	0.50	128.00	0.50	<0.06	8.00
H302	298	ENB	1105573	>256.00	4.00	4.00	16.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H303	299	ACIA	0004042	64.00	16.00	16.00	128.00	64.00	64.00	32.00	16.00	>256.00	1.00	4.00	64.00
H304	300	ACIA	0004042	64.00	16.00	16.00	128.00	64.00	64.00	32.00	8.00	>256.00	1.00	2.00	32.00
H305	301	ECO	5144572	64.00	<0.12	<0.12	0.50	4.00	4.00	0.50	0.25	64.00	0.50	<0.06	0.25
H306	302	ENBC	3305573	64.00	<0.12	<0.12	0.50	64.00	8.00	<0.12	<0.12	64.00	1.00	<0.06	0.50
H307	303	ACIL	0200000	64.00	16.00	16.00	1.00	32.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H308	304	CITF	1444533	>256.00	16.00	8.00	0.25	128.00	64.00	4.00	16.00	>256.00	1.00	<0.06	0.25
H309	305	ECO	5144572	>256.00	<0.12	0.12	0.50	2.00	1.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H310	306	SERM	5306721	>256.00	0.25	0.50	2.00	16.00	>256.00	<0.12	<0.12	>256.00	1.00	<0.06	0.50
H311	307	MORM	0174000	>256.00	0.50	2.00	8.00	8.00	64.00	0.50	2.00	>256.00	2.00	<0.06	32.00
H312	308	KLEOX	5255773	>256.00	1.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	1.00	<0.06	0.50
H313	309	ENBC	3305573	>256.00	2.00	4.00	0.50	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H314	310	ENBC	3305573	>256.00	8.00	8.00	0.50	128.00	>256.00	2.00	2.00	>256.00	1.00	<0.06	0.25
H315	311	CIT	1644513	>256.00	16.00	8.00	0.25	128.00	64.00	4.00	16.00	>256.00	1.00	<0.06	0.25
H316	312	CITF	1004572	>256.00	2.00	2.00	8.00	32.00	64.00	1.00	8.00	>256.00	1.00	<0.06	32.00
H317	313	ENBC	3105573	>256.00	0.50	1.00	0.50	64.00	128.00	0.50	0.25	>256.00	1.00	<0.06	0.25
H318	314	ENBC	3305523	>256.00	0.50	1.00	0.50	256.00	64.00	1.00	0.50	>256.00	4.00	4.00	0.50
H319	315	ENBC	3305573	>256.00	2.00	4.00	0.50	64.00	128.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H320	316	PSEA	2210004	256.00	2.00	4.00	2.00	>256.00	>256.00	1.00	0.50	>256.00	4.00	0.12	2.00
H321	317	MORM	0174000	256.00	<0.12	0.25	1.00	4.00	32.00	<0.12	0.50	>256.00	4.00	<0.06	1.00
H322	318	ENBC	3305573	>256.00	32.00	32.00	0.50	64.00	256.00	8.00	16.00	>256.00	1.00	<0.06	0.25
H323	319	ECO	5144572	8.00	<0.12	<0.12	0.50	2.00	1.00	<0.12	<0.12	32.00	0.50	<0.06	0.25
H326	320	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	4.00	16.00	>256.00	4.00	<0.06	0.25
H327	321	ENBA	4305573	>256.00	2.00	4.00	0.50	64.00	64.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H328	322	ENBA	5305773	>256.00	2.00	4.00	0.50	64.00	64.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H329	323	ENBC	3305573	>256.00	1.00	0.25	8.00	32.00	2.00	1.00	0.25	>256.00	1.00	<0.06	0.25
H330	324	ENBC	3305573	>256.00	8.00	8.00	8.00	256.00	>256.00	4.00	2.00	>256.00	1.00	<0.06	32.00
H331	325	ACIA	0204042	64.00	16.00	16.00	1.00	64.00	32.00	64.00	2.00	128.00	0.50	0.25	0.50
H332	326	ECO	5144572	>256.00	<0.12	<0.12	0.50	2.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H333	327	ENBC	3105573	>256.00	4.00	4.00	16.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	64.00
H334	328	ECO	1144572	>256.00	<0.12	<0.12	0.25	2.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H335	329	ACIA	0004042	64.00	16.00	32.00	128.00	128.00	64.00	64.00	4.00	256.00	1.00	2.00	32.00
H337	331	ENBC	3305573	>256.00	2.00	2.00	0.50	64.00	128.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H338	332	ENBC	3305573	128.00	0.25	0.25	0.25	256.00	8.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H339	333	ACIA	0004002	64.00	16.00	16.00	1.00	64.00	32.00	64.00	2.00	256.00	0.50	0.25	0.50
H340	334	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.25	1.00
H341	335	ENBC	3305573	>256.00	0.50	0.50	0.50	128.00	32.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25
H342	336	SERM	5307721	128.00	<0.12	0.25	0.50	16.00	256.00	<0.12	<0.12	>256.00	2.00	<0.06	0.25
H343	337	ACIA	0004042	64.00	32.00	32.00	128.00	128.00	8.00	64.00	4.00	256.00	1.00	2.00	32.00
H344	338	SERM	5307521	256.00	<0.12	0.25	1.00	32.00	256.00	<0.12	<0.12	>256.00	4.00	0.12	0.50
H345	339	ENB	1105573	64.00	<0.12	<0.12	0.50	64.00	8.00	<0.12	<0.12	128.00	1.00	<0.06	0.25
H346	340	SERM	5307721	>256.00	2.00	2.00	2.00	32.00	>256.00	0.25	<0.12	>256.00	4.00	0.12	0.50
H347	341	ACIA	0004042	64.00	16.00	16.00	2.00	32.00	32.00	32.00	2.00	256.00	0.50	0.50	1.00
H348	342	ECO	5144572	>256.00	<0.12	<0.12	0.50	1.00	2.00	<0.12	<0.12	8.00	0.50	<0.06	0.25
H349	343	CITF	1004572	>256.00	1.00	2.00	8.00	16.00	128.00	1.00	0.50	>256.00	2.00	<0.06	32.00
H350	344	ENBC	3305573	>256.00	16.00	32.00	0.25	64.00	>256.00	8.00	8.00	>256.00	2.00	<0.06	0.25
H351	345	KLEP	5215773	64.00	<0.12	0.25	0.25	32.00	16.00	<0.12	<0.12	32.00	0.50	0.25	0.25
H352	346	ENBC	3305573	>256.00	4.00	4.00	0.25	64.00	128.00	2.00	1.00	>256.00	1.00	<0.06	0.25
H353	347	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	32.00	2.00	256.00	0.50	0.50	1.00
H354	348	SERM	5307721	256.00	2.00	4.00	2.00	64.00	>256.00	<0.12	<0.12	>256.00	1.00	<0.06	1.00
H355	349	ACIA	0004042	32.00	16.00	16.00	0.50	128.00	64.00	32.00	2.00	256.00	0.50	1.00	0.25
H356	350	SERM	5307721	256.00	1.00	2.00	1.00	64.00	>256.00	0.25	<0.12	>256.00	1.00	1.00	0.25
H357	351	SER	5307723	64.00	<0.12	<0.12	2.00	16.00	128.00	<0.12	<0.12	>256.00	0.50	0.12	0.50
H358	352	ENBC	3305573	>256.00	4.00	4.00	0.25	256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H359	353	SERM	5307721	256.00	2.00	4.00	2.00	64.00	>256.00	<0.12	<0.12	>256.00	1.00	0.12	1.00
H360	354	SERM	5306721	>256.00	2.00	2.00	2.00	64.00	>256.00	<0.12	128	>256.00	1.00	<0.06	1.00
H361	355	CITF	1004572	>256.00	2.00	1.00	16.00	32.00	16.00	0.50	0.50	>256.00	1.00	0.12	64.00
H362	356	SER	5307722	64.00	<0.12	<0.12	2.00	32.00	128.00	<0.12	<0.12	>256.00	1.00	0.12	1.00
H363	357	SERM	5107721	64.00	<0.12	<0.12	4.00	32.00	64.00	<0.12	<0.12	256.00	4.00	<0.06	0.50
H364	358	ENBC	3305573	64.00	1.00	2.00	1.00	32.00	16.00	0.50	1.00	256.00	0.50	<0.06	1.00
H365	359	MORM	0174000	128.00	<0.12	<0.12	0.50	8.00	16.00	<0.12	<0.12	>256.00	8.00	<0.06	0.50
H367	361	ENBC	3305573	>256.00	0.50	1.00	0.25	64.00	64.00	0.50	0.50	>256.00	1.00	<0.06	0.25
H368	362	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H369	363	ACIA	0004042	16.00	2.00	4.00	16.00	16.00	16.00	8.00	1.00	64.00	1.00	0.12	0.25
H370	364	ACIA	0004042	64.00	16.00	16.00	0.25	128.00	64.00	32.00	2.00	256.00	0.50	0.12	<0.12
H371	365	ENBC	3305563	32.00	<0.12	<0.12	2.00	256.00	8.00	<0.12	0.25	64.00	1.00	<0.06	0.50
H372	366	ENBC	3305573	>256.00	16.00	16.00	0.25	64.00	>256.00	4.00	4.00	>256.00	4.00	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H373	367	SERM	5307420	>256.00	2.00	2.00	1.00	>256.00	>256.00	0.50	0.50	>256.00	1.00	1.00	0.25
H374	368	SERM	5307721	64.00	16.00	8.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.50	0.25
H375	369	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H376A	370	SERM	5347721	>256.00	16.00	16.00	1.00	64.00	>256.00	4.00	0.50	>256.00	1.00	1.00	0.25
H376B	371	SERM	5106721	>256.00	16.00	16.00	1.00	64.00	>256.00	4.00	0.50	>256.00	1.00	1.00	0.25
H377	372	ECO	5144552	>256.00	0.25	1.00	0.50	16.00	16.00	1.00	1.00	128.00	1.00	<0.06	0.25
H378	373	ENBC	3305573	>256.00	16.00	16.00	0.25	64.00	>256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H379	374	ENBC	3305573	>256.00	8.00	16.00	0.25	64.00	256.00	4.00	4.00	>256.00	2.00	<0.06	0.25
H380	375	ENBC	3305573	>256.00	8.00	16.00	0.50	64.00	>256.00	4.00	0.25	>256.00	1.00	1.00	0.25
H381	376	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H382A	377	ACIA	0005042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.25
H382B	378	ACIA	0005042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.25
H383	379	PSEORZ	0001003	64.00	16.00	16.00	1.00	64.00	64.00	32.00	2.00	256.00	0.50	0.25	0.50
H384	380	ENBC	3305573	>256.00	16.00	8.00	0.25	64.00	128.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H385	381	ENB	1305573	>256.00	4.00	4.00	0.25	>256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H386A	382	ENBC	3305573	>256.00	<0.12	2.00	<0.12	128.00	128.00	1.00	0.50	256.00	2.00	<0.06	<0.12
H386B	383	ENBC	3305573	>256.00	<0.12	0.25	<0.12	128.00	128.00	1.00	0.25	256.00	2.00	<0.06	<0.12
H387A	384	ENB	1305573	>256.00	4.00	4.00	0.25	128.00	>256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H387B	385	ENB	1305573	>256.00	4.00	4.00	0.50	>256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H388	386	ENBC	3305573	>256.00	4.00	8.00	0.25	128.00	256.00	4.00	1.00	>256.00	1.00	<0.06	0.25
H389	387	ECO	5144572	>256.00	0.25	0.50	0.25	8.00	16.00	1.00	0.50	128.00	0.50	<0.06	0.25
H390	388	ENBC	3305573	>256.00	16.00	32.00	0.50	128.00	256.00	8.00	4.00	>256.00	2.00	<0.06	0.25
H391	389	ENBC	3305573	>256.00	32.00	32.00	0.25	256.00	256.00	16.00	16.00	>256.00	1.00	<0.06	0.25
H392	390	ENBC	3305573	>256.00	1.00	1.00	0.25	128.00	32.00	<0.12	0.25	>256.00	2.00	<0.06	0.25
H393	391	ACIA	0004042	128.00	16.00	16.00	128.00	256.00	64.00	64.00	8.00	256.00	1.00	2.00	64.00
H394	392	ENBC	3305573	>256.00	4.00	4.00	0.50	128.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H395	393	ENBC	3305573	>256.00	<0.12	<0.12	0.50	128.00	8.00	<0.12	<0.12	256.00	1.00	<0.06	16.00
H396	394	ECO	5144572	128.00	<0.12	0.25	0.25	8.00	16.00	0.50	0.50	128.00	1.00	<0.06	0.25
H397	395	ENBC	3305573	>256.00	16.00	32.00	0.50	128.00	256.00	4.00	4.00	>256.00	2.00	<0.06	0.25
H398	396	ENBC	3305533	>256.00	2.00	4.00	0.25	128.00	256.00	0.50	0.50	>256.00	2.00	<0.06	0.25
H399	397	PSE	1553773	128.00	8.00	16.00	1.00	256.00	64.00	32.00	2.00	256.00	0.50	0.25	0.50
H400	398	ENBC	3305533	>256.00	8.00	8.00	0.25	128.00	128.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H401	399	MORM	0104000	128.00	<0.12	<0.12	0.50	4.00	16.00	<0.12	<0.12	256.00	1.00	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H402	400	ENBC	3305573	>256.00	<0.12	0.25	2.00	64.00	4.00	<0.12	<0.12	64.00	2.00	<0.06	16.00
H403	330	ENBC	3305573	16.00	0.50	1.00	0.50	16.00	16.00	<0.12	0.25	64.00	0.50	<0.06	0.50
H404	216	ACIA	0200042	64.00	16.00	32.00	0.50	128.00	1.00	32.00	2.00	256.00	0.50	0.50	0.25
H405	112	ENBC	3305573	>256.00	2.00	4.00	4.00	128.00	128.00	1.00	1.00	>256.00	1.00	<0.06	1.00

Appendix II

MICs of 200 strains of Gram negative, oxidase negative, aerobic bacilli, with CXM MICs of $\geq$32 mg/L, to 12 antimicrobial agents.

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H010	10	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	2.00	256.00	0.50	0.25	1.00
H017	17	ACIA	0004042	32.00	32.00	32.00	0.50	256.00	64.00	64.00	4.00	256.00	1.00	1.00	0.25
H018	18	ACIA	0004042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H026	26	ACIA	0004042	64.00	16.00	16.00	8.00	128.00	64.00	32.00	8.00	>256.00	1.00	1.00	32.00
H032	32	ACIA	0004042	64.00	16.00	16.00	8.00	64.00	32.00	32.00	4.00	256.00	1.00	1.00	32.00
H033	33	ACIA	0004042	64.00	8.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	1.00	0.50	16.00
H040	40	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.50	1.00
H048A	48	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	32.00	4.00	256.00	0.50	0.50	1.00
H063	65	ACIA	0004042	128.00	64.00	32.00	2.00	128.00	128.00	128.00	4.00	>256.00	0.50	1.00	1.00
H068	70	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	8.00	256.00	0.50	0.25	1.00
H075	76	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	1.00
H076	77	ACIA	0004042	256.00	1.00	1.00	4.00	16.00	>256.00	0.25	<0.12	>256.00	0.50	0.12	1.00
H101	99	ACIA	0005042	>256.00	16.00	32.00	8.00	128.00	64.00	64.00	4.00	>256.00	1.00	1.00	128.00
H117A	115	ACIA	0005042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.25	1.00
H124	123	ACIA	0005042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	4.00	256.00	0.50	0.50	1.00
H126	125	ACIL	0000071NE	128.00	32.00	64.00	0.25	>256.00	64.00	>256.00	64.00	>256.00	0.50	0.50	0.25
H128	127	ACIA	0200042	32.00	8.00	8.00	1.00	32.00	32.00	16.00	2.00	128.00	0.50	0.25	1.00
H131	130	ACIL	0200000	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	256.00	1.00	0.50	1.00
H136	135	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	64.00	4.00	256.00	0.50	0.50	1.00
H140	139	ACIA	0004042	64.00	16.00	16.00	0.25	64.00	32.00	32.00	2.00	256.00	0.50	0.12	0.25
H141	140	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	8.00	256.00	0.50	0.50	1.00
H146	145	ACIA	0205042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	4.00	256.00	0.50	0.50	1.00
H154	154	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	32.00	32.00	2.00	256.00	0.50	0.50	1.00
H159	159	ACIA	0205042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H162	162	ACIA	0004042	32.00	16.00	32.00	0.50	256.00	64.00	64.00	8.00	256.00	0.50	0.50	0.25
H253	253	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.25	1.00
H255	255	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	4.00	>256.00	0.50	0.50	1.00
H256	256	ACIA	0004042	64.00	16.00	16.00	4.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	1.00
H259	259	ACIA	0004042	64.00	16.00	32.00	0.50	128.00	64.00	32.00	2.00	>256.00	0.50	0.25	0.25
H268	267	ACIA	0004042	64.00	32.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	1.00
H278	277	ACIA	0004042	64.00	16.00	16.00	1.00	64.00	64.00	64.00	2.00	256.00	0.50	0.50	0.50
H290	288	ACIA	0004042	128.00	8.00	32.00	0.50	64.00	64.00	64.00	4.00	256.00	0.50	0.25	0.25
H299	296	ACIA	0004042	128.00	32.00	32.00	2.00	64.00	128.00	64.00	4.00	>256.00	0.50	4.00	1.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H307	303	ACIL	0200000	64.00	16.00	16.00	1.00	32.00	32.00	32.00	2.00	128.00	0.50	0.25	0.50
H331	325	ACIA	0204042	64.00	16.00	16.00	1.00	64.00	32.00	64.00	2.00	128.00	0.50	0.25	0.50
H335	329	ACIA	0004042	64.00	16.00	32.00	128.00	128.00	64.00	64.00	4.00	256.00	1.00	2.00	32.00
H339	333	ACIA	0004002	64.00	16.00	16.00	1.00	64.00	32.00	64.00	2.00	256.00	0.50	0.25	0.50
H340	334	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	64.00	2.00	256.00	0.50	0.25	1.00
H353	347	ACIA	0004042	64.00	16.00	16.00	2.00	64.00	64.00	32.00	2.00	256.00	0.50	0.50	1.00
H355	349	ACIA	0004042	32.00	16.00	16.00	0.50	128.00	64.00	32.00	2.00	256.00	0.50	1.00	0.25
H370	364	ACIA	0004042	64.00	16.00	16.00	0.25	128.00	64.00	32.00	2.00	256.00	0.50	0.12	<0.12
H382A	377	ACIA	0005042	64.00	16.00	16.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.25	0.25
H393	391	ACIA	0004042	128.00	16.00	16.00	128.00	256.00	64.00	64.00	8.00	256.00	1.00	2.00	64.00
H044	44	CITF	1604573	>256.00	8.00	4.00	0.50	256.00	64.00	4.00	32.00	>256.00	1.00	0.12	0.50
H129	128	CITD	3344552	>256.00	8.00	4.00	0.50	4.00	256.00	16.00	0.50	128.00	0.50	<0.06	0.50
H138	137	CITD	3344573	>256.00	4.00	2.00	0.50	>256.00	256.00	8.00	0.50	64.00	1.00	<0.06	0.50
H161	161	CITF	1604773	>256.00	<0.12	<0.12	8.00	64.00	64.00	<0.12	<0.12	64.00	1.00	<0.06	32.00
H257	257	CITF	3544772	>256.00	4.00	4.00	1.00	128.00	64.00	4.00	4.00	>256.00	0.50	<0.06	1.00
H308	304	CITF	1444533	>256.00	16.00	8.00	0.25	128.00	64.00	4.00	16.00	>256.00	1.00	<0.06	0.25
H315	311	CIT	1644513	>256.00	16.00	8.00	0.25	128.00	64.00	4.00	16.00	>256.00	1.00	<0.06	0.25
H316	312	CITF	1004572	>256.00	2.00	2.00	8.00	32.00	64.00	1.00	8.00	>256.00	1.00	<0.06	32.00
H349	343	CITF	1004572	>256.00	1.00	2.00	8.00	16.00	128.00	1.00	0.50	>256.00	2.00	<0.06	32.00
H037	37	ECO	5144572	>256.00	0.50	2.00	0.25	64.00	64.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H084	85	ECO	5044572	32.00	<0.12	0.50	0.50	64.00	32.00	0.25	0.25	128.00	0.50	<0.06	0.50
H175	174	ECO	5044572	32.00	<0.12	<0.12	0.25	2.00	256.00	<0.12	<0.12	32.00	2.00	<0.06	0.25
H002	2	ENB	3306573	>256.00	8.00	32.00	0.25	256.00	>256.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H005	5	ENBC	3305573	256.00	2.00	4.00	0.50	>256.00	64.00	4.00	1.00	>256.00	0.50	<0.06	0.50
H008	8	ENBC	3305573	>256.00	4.00	8.00	8.00	128.00	256.00	4.00	8.00	>256.00	1.00	0.12	32.00
H014	14	ENBC	3305573	>256.00	2.00	4.00	8.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	32.00
H015	15	ENBC	3305573	>256.00	8.00	16.00	8.00	64.00	256.00	4.00	8.00	>256.00	1.00	<0.06	32.00
H016	16	ENBC	3305573	>256.00	8.00	16.00	0.25	64.00	256.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H021	21	ENBC	3305573	>256.00	0.50	1.00	0.25	128.00	32.00	0.25	0.25	>256.00	4.00	0.12	0.25
H022	22	ENBC	3305573	>256.00	1.00	1.00	<0.12	64.00	64.00	0.25	0.25	>256.00	2.00	0.12	<0.12
H030	30	ENBC	3305573	>256.00	4.00	8.00	0.25	256.00	256.00	4.00	4.00	>256.00	0.50	0.12	0.25
H031	31	ENBC	3305573	>256.00	4.00	8.00	0.25	256.00	>256.00	4.00	4.00	>256.00	0.50	0.12	0.25
H038	38	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	4.00	>256.00	2.00	<0.06	8.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H039	39	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	4.00	>256.00	2.00	<0.06	8.00
H042	42	ENBC	3305573	>256.00	16.00	32.00	8.00	128.00	>256.00	8.00	8.00	>256.00	1.00	<0.06	64.00
H043	43	ENBC	3105573	>256.00	0.50	1.00	0.25	>256.00	32.00	0.25	0.50	>256.00	0.50	0.12	0.25
H047	47	ENBC	3305773	>256.00	1.00	2.00	0.50	256.00	64.00	1.00	1.00	>256.00	0.50	<0.06	0.50
H048B	49	ENBC	3305573	>256.00	1.00	2.00	0.50	256.00	64.00	1.00	1.00	>256.00	1.00	<0.06	0.50
H051	52	ENBC	3305573	>256.00	16.00	32.00	0.25	256.00	>256.00	8.00	8.00	>256.00	1.00	<0.06	8.00
H053	54	ENBC	3305573	>256.00	1.00	1.00	0.25	>256.00	64.00	1.00	0.50	>256.00	0.50	<0.06	0.25
H054	55	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	8.00	8.00	>256.00	1.00	0.12	8.00
H055	56	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	256.00	8.00	8.00	>256.00	1.00	<0.06	8.00
H057	58	ENBC	3305573	256.00	1.00	1.00	0.50	>256.00	64.00	1.00	0.50	>256.00	0.50	<0.06	0.25
H058	59	ENB	3304573	>256.00	8.00	8.00	0.25	>256.00	128.00	4.00	4.00	>256.00	0.50	0.12	8.00
H059	60	ENBC	3305573	>256.00	<0.12	0.25	0.50	4.00	32.00	<0.12	<0.12	32.00	0.50	0.12	8.00
H060	61	ENBC	3305573	>256.00	4.00	16.00	0.50	>256.00	128.00	4.00	4.00	>256.00	0.50	0.12	0.25
H061A	62	ENBC	3105573	>256.00	4.00	8.00	8.00	64.00	128.00	4.00	4.00	>256.00	0.50	<0.06	32.00
H064	66	ENBC	3305573	>256.00	2.00	4.00	8.00	256.00	>256.00	0.50	0.50	>256.00	2.00	<0.06	32.00
H066	68	ENBC	3305533	>256.00	2.00	2.00	8.00	128.00	256.00	0.50	0.50	>256.00	1.00	<0.06	32.00
H067	69	ENBC	3305573	>256.00	8.00	16.00	0.50	64.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H073	74	ENB	3304573	>256.00	2.00	4.00	8.00	64.00	64.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H077	78	ENBC	3305573	>256.00	1.00	2.00	0.50	>256.00	32.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H083	84	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	8.00	8.00	>256.00	2.00	<0.06	8.00
H092	91	ENBC	3305573	>256.00	64.00	64.00	0.50	128.00	>256.00	16.00	16.00	>256.00	2.00	<0.06	0.25
H093	92	ENB/CI	3104573	>256.00	16.00	64.00	0.25	64.00	>256.00	8.00	8.00	>256.00	0.50	<0.06	0.25
H099	97	ENBC	3105573	>256.00	0.50	1.00	0.25	>256.00	64.00	0.50	0.50	>256.00	0.50	0.12	0.25
H103	101	ENBC	3305573	>256.00	4.00	16.00	8.00	128.00	>256.00	4.00	4.00	>256.00	4.00	<0.06	32.00
H104	102	ENBC	3305573	>256.00	4.00	8.00	8.00	128.00	256.00	4.00	2.00	>256.00	2.00	<0.06	32.00
H105	103	ENBC	3305573	>256.00	8.00	16.00	0.25	>256.00	>256.00	4.00	4.00	>256.00	2.00	<0.06	<0.12
H106	104	ENBC	3305573	>256.00	16.00	16.00	0.25	128.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H108	106	ENBC	3305573	>256.00	4.00	8.00	0.25	128.00	256.00	4.00	2.00	<256.00	0.50	<0.06	0.25
H109	107	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	256.00	4.00	2.00	>256.00	2.00	<0.06	64.00
H118	117	ENBC	3305573	>256.00	16.00	8.00	0.25	128.00	256.00	4.00	2.00	>256.00	0.50	<0.06	0.25
H119	118	ENBA	5304773	>256.00	4.00	2.00	1.00	32.00	>256.00	0.50	8.00	>256.00	4.00	0.12	0.50
H122	121	ENBC	3305573	>256.00	4.00	4.00	8.00	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H127	126	ENBC	3305573	>256.00	1.00	4.00	0.25	128.00	256.00	0.25	0.25	>256.00	0.50	<0.06	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H132	131	ENBC	3305573	>256.00	4.00	4.00	8.00	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H137	136	ENBC	3305573	>256.00	16.00	32.00	0.25	>256.00	>256.00	8.00	8.00	>256.00	8.00	<0.06	0.25
H150	149	ENBC	3305773	>256.00	2.00	2.00	0.50	256.00	64.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H153A	152	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	128.00	2.00	2.00	>256.00	1.00	<0.06	64.00
H155	155	ENBA	5305773	>256.00	2.00	4.00	0.50	256.00	64.00	1.00	1.00	>256.00	0.50	<0.06	0.25
H156	156	ENBC	3305573	>256.00	2.00	1.00	8.00	256.00	128.00	0.50	0.25	>256.00	1.00	<0.06	64.00
H164	163	ENBC	3305573	>256.00	8.00	16.00	8.00	64.00	128.00	4.00	4.00	>256.00	2.00	<0.06	32.00
H166	165	ENBC	3305573	>256.00	1.00	1.00	8.00	256.00	32.00	0.50	<0.12	>256.00	2.00	<0.06	32.00
H182	182	ENBC	3305573	>256.00	8.00	32.00	0.25	32.00	128.00	4.00	8.00	>256.00	1.00	<0.06	0.25
H190	192	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H191	193	ENBC	3305533	>256.00	128.00	128.00	8.00	128.00	256.00	16.00	32.00	>256.00	1.00	<0.06	32.00
H192	194	ENBC	3305573	>256.00	2.00	8.00	0.50	64.00	64.00	0.50	1.00	>256.00	1.00	<0.06	0.25
H193	195	ENBC	3305573	256.00	4.00	8.00	4.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	32.00
H197	198	ENBC	3305573	>256.00	32.00	32.00	0.25	128.00	>256.00	16.00	8.00	>256.00	4.00	<0.06	0.25
H251	251	ENBC	3305573	>256.00	4.00	8.00	8.00	64.00	256.00	2.00	2.00	>256.00	1.00	<0.06	64.00
H252	252	ENBC	3305533	>256.00	4.00	4.00	0.50	256.00	256.00	1.00	1.00	>256.00	2.00	<0.06	0.25
H262	261	ENBA	5305773	>256.00	4.00	4.00	0.25	>256.00	256.00	4.00	4.00	>256.00	4.00	<0.06	0.25
H267	266	ENB	2305573	256.00	8.00	8.00	0.25	64.00	256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H272	271	ENB	3304573	256.00	1.00	1.00	0.25	128.00	64.00	1.00	0.50	>256.00	1.00	<0.06	0.25
H280	279	ENBC	3305573	>256.00	4.00	8.00	0.25	64.00	256.00	1.00	2.00	>256.00	1.00	<0.06	0.25
H283	282	ENBC	3305573	>256.00	1.00	2.00	8.00	64.00	256.00	0.50	1.00	>256.00	1.00	<0.06	32.00
H289	287	ENBC	3105573	>256.00	4.00	16.00	16.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00
H292	289	ENB	3304173	>256.00	16.00	64.00	4.00	64.00	>256.00	16.00	16.00	>256.00	1.00	<0.06	32.00
H294	291	ENBC	3305573	>256.00	2.00	4.00	0.50	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H296	293	ENBC	3105573	>256.00	4.00	4.00	8.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00
H302	298	ENB	1105573	>256.00	4.00	4.00	16.00	64.00	128.00	1.00	2.00	>256.00	1.00	<0.06	64.00
H314	310	ENBC	3305573	>256.00	8.00	8.00	0.50	128.00	>256.00	2.00	2.00	>256.00	1.00	<0.06	0.25
H317	313	ENBC	3105573	>256.00	0.50	1.00	0.50	64.00	128.00	0.50	0.25	>256.00	1.00	<0.06	0.25
H318	314	ENBC	3305523	>256.00	0.50	1.00	0.50	256.00	64.00	1.00	0.50	>256.00	4.00	4.00	0.50
H322	318	ENBC	3305573	>256.00	32.00	32.00	0.50	64.00	256.00	8.00	16.00	>256.00	1.00	<0.06	0.25
H326	320	ENBC	3305573	>256.00	16.00	32.00	0.25	128.00	>256.00	4.00	16.00	>256.00	4.00	<0.06	0.25
H327	321	ENBA	4305573	>256.00	2.00	4.00	0.50	64.00	64.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H330	324	ENBC	3305573	>256.00	8.00	8.00	8.00	256.00	>256.00	4.00	2.00	>256.00	1.00	<0.06	32.00

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H333	327	ENBC	3105573	>256.00	4.00	4.00	16.00	64.00	128.00	1.00	1.00	>256.00	1.00	<0.06	64.00
H341	335	ENBC	3305573	>256.00	0.50	0.50	0.50	128.00	32.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25
H350	344	ENBC	3305573	>256.00	16.00	32.00	0.25	64.00	>256.00	8.00	8.00	>256.00	2.00	<0.06	0.25
H352	346	ENBC	3305573	>256.00	4.00	4.00	0.25	64.00	128.00	2.00	1.00	>256.00	1.00	<0.06	0.25
H358	352	ENBC	3305573	>256.00	4.00	4.00	0.25	256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H367	361	ENBC	3305573	>256.00	0.50	1.00	0.25	64.00	64.00	0.50	0.50	>256.00	1.00	<0.06	0.25
H368	362	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H372	366	ENBC	3305573	>256.00	16.00	16.00	0.25	64.00	>256.00	4.00	4.00	>256.00	4.00	<0.06	0.25
H378	373	ENBC	3305573	>256.00	16.00	16.00	0.25	64.00	>256.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H379	374	ENBC	3305573	>256.00	8.00	16.00	0.25	64.00	256.00	4.00	4.00	>256.00	2.00	<0.06	0.25
H380	375	ENBC	3305573	>256.00	8.00	16.00	0.50	64.00	>256.00	4.00	0.25	>256.00	1.00	1.00	0.25
H381	376	ENBC	3305573	>256.00	2.00	4.00	0.25	64.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H384	380	ENBC	3305573	>256.00	16.00	8.00	0.25	64.00	128.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H385	381	ENB	1305573	>256.00	4.00	4.00	0.25	>256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H386A	382	ENBC	3305573	>256.00	<0.12	2.00	<0.12	128.00	128.00	1.00	0.50	256.00	2.00	<0.06	<0.12
H387B	385	ENB	1305573	>256.00	4.00	4.00	0.50	>256.00	128.00	4.00	2.00	>256.00	1.00	<0.06	0.25
H390	388	ENBC	3305573	>256.00	16.00	32.00	0.50	128.00	256.00	8.00	4.00	>256.00	2.00	<0.06	0.25
H392	390	ENBC	3305573	>256.00	1.00	1.00	0.25	128.00	32.00	<0.12	0.25	>256.00	2.00	<0.06	0.25
H394	392	ENBC	3305573	>256.00	4.00	4.00	0.50	128.00	256.00	1.00	1.00	>256.00	1.00	<0.06	0.25
H397	395	ENBC	3305573	>256.00	16.00	32.00	0.50	128.00	256.00	4.00	4.00	>256.00	2.00	<0.06	0.25
H398	396	ENBC	3305533	>256.00	2.00	4.00	0.25	128.00	256.00	0.50	0.50	>256.00	2.00	<0.06	0.25
H400	398	ENBC	3305533	>256.00	8.00	8.00	0.25	128.00	128.00	4.00	4.00	>256.00	1.00	<0.06	0.25
H405	112	ENBC	3305573	>256.00	2.00	4.00	4.00	128.00	128.00	1.00	1.00	>256.00	1.00	<0.06	1.00
H298	295	ERWA	1005422	64.00	16.00	16.00	4.00	64.00	32.00	32.00	2.00	256.00	0.50	0.25	1.00
H120	119	HAFA	5104112	128.00	8.00	4.00	0.25	8.00	64.00	0.50	8.00	>256.00	1.00	<0.06	<0.12
H123	122	HAFA	5305112	128.00	4.00	4.00	<0.12	8.00	64.00	1.00	16.00	256.00	1.00	<0.06	0.25
H019	19	KLEOX	5254733	>256.00	4.00	1.00	8.00	8.00	128.00	32.00	0.12	128.00	1.00	<0.06	32.00
H020	20	KLEP	5215773	>256.00	32.00	64.00	8.00	64.00	>256.00	8.00	32.00	>256.00	1.00	<0.06	32.00
H023	23	KLEOX	5255773	>256.00	0.50	<0.12	8.00	1.00	64.00	2.00	<0.12	64.00	1.00	0.12	64.00
H052	53	KLEB	5245773	>256.00	1.00	0.25	16.00	1.00	64.00	4.00	<0.12	64.00	0.50	<0.06	64.00
H062	64	KLEB	5245773	>256.00	1.00	0.25	16.00	1.00	64.00	4.00	<0.12	256.00	1.00	<0.06	64.00
H139	138	KLEP	1215773	>256.00	0.25	0.25	4.00	32.00	32.00	0.25	0.50	64.00	0.50	0.25	16.00
H199	200	KLEOX	5045773	>256.00	1.00	0.25	1.00	0.50	64.00	4.00	<0.12	64.00	0.25	<0.06	0.50

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H249	249	KLEOX	0004353	64.00	16.00	16.00	2.00	64.00	64.00	64.00	4.00	256.00	0.50	0.50	1.00
H276	275	KLEOX	0255773	>256.00	2.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	0.50	<0.06	0.25
H281	280	KLEOX	5255773	>256.00	1.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	1.00	<0.06	0.25
H312	308	KLEOX	5255773	>256.00	1.00	0.25	0.50	1.00	64.00	4.00	<0.12	64.00	1.00	<0.06	0.50
H082	83	MORM	0174000	256.00	0.50	2.00	0.50	8.00	32.00	0.50	2.00	>256.00	2.00	<0.06	1.00
H088	87	MORM	0174000	>256.00	0.25	0.50	0.50	256.00	>256.00	0.50	1.00	>256.00	4.00	0.12	0.50
H181	181	MORM	0174000	>256.00	0.50	2.00	4.00	4.00	64.00	0.50	4.00	>256.00	4.00	<0.06	64.00
H250	250	MORM	0374000	256.00	0.25	2.00	0.50	4.00	32.00	0.50	2.00	>256.00	4.00	<0.06	0.50
H007	7	PROSTU	0254000	>256.00	0.50	4.00	0.50	128.00	256.00	2.00	4.00	>256.00	4.00	0.12	0.50
H024	24	PROVU	0070020	64.00	<0.12	<0.12	<0.12	1.00	64.00	<0.12	<0.12	128.00	4.00	<0.06	<0.12
H269	268	PROP	0036020	>256.00	0.25	<0.12	<0.12	2.00	256.00	<0.12	<0.12	256.00	8.00	<0.06	<0.12
H011	11	SERM	5347761	128.00	<0.12	<0.12	2.00	8.00	64.00	<0.12	<0.12	>256.00	1.00	<0.06	0.25
H013	13	SER	5207763	256.00	<0.12	0.50	1.00	16.00	>256.00	<0.12	<0.12	>256.00	4.00	<0.06	0.50
H029	29	SERL	1106723	32.00	<0.12	<0.12	0.25	8.00	32.00	<0.12	<0.12	128.00	1.00	<0.06	<0.12
H034	34	SERM	4106721	64.00	0.12	<0.12	2.00	16.00	128.00	<0.12	<0.12	256.00	0.50	0.12	0.50
H049	50	SERM	4106763	128.00	<0.12	0.25	4.00	16.00	32.00	<0.12	<0.12	>256.00	4.00	0.12	0.25
H087	86	SERL	5107768	16.00	<0.12	0.25	2.00	16.00	32.00	<0.12	<0.12	256.00	1.00	0.12	1.00
H148	147	SERO	5346773	8.00	0.25	0.25	1.00	8.00	32.00	<0.12	<0.12	64.00	1.00	0.12	1.00
H171	170	SERM	4117521	64.00	<0.12	<0.12	2.00	32.00	256.00	0.50	<0.12	>256.00	1.00	0.25	0.25
H178B	178	SERM	5107761	>256.00	<0.12	<0.12	2.00	16.00	256.00	<0.12	<0.12	256.00	2.00	0.25	16.00
H198	199	SERM	5307720	>256.00	1.00	1.00	2.00	32.00	>256.00	0.25	<0.12	>256.00	4.00	0.12	0.50
H277	276	SERL	5304723	32.00	<0.12	<0.12	0.25	8.00	64.00	<0.12	<0.12	128.00	1.00	<0.06	<0.12
H288	286	SER	5105721	256.00	0.50	0.50	2.00	32.00	>256.00	<0.12	<0.12	>256.00	4.00	<0.06	1.00
H310	306	SERM	5306721	>256.00	0.25	0.50	2.00	16.00	>256.00	<0.12	<0.12	>256.00	1.00	<0.06	0.50
H342	336	SERM	5307721	128.00	<0.12	0.25	0.50	16.00	256.00	<0.12	<0.12	>256.00	2.00	<0.06	0.25
H344	338	SERM	5307521	256.00	<0.12	0.25	1.00	32.00	256.00	<0.12	<0.12	>256.00	4.00	0.12	0.50
H346	340	SERM	5307721	>256.00	2.00	2.00	2.00	32.00	>256.00	0.25	<0.12	>256.00	4.00	0.12	0.50
H356	350	SERM	5307721	256.00	1.00	2.00	1.00	64.00	>256.00	0.25	<0.12	>256.00	1.00	1.00	0.25
H357	351	SER	5307723	64.00	<0.12	<0.12	2.00	16.00	128.00	<0.12	<0.12	>256.00	0.50	0.12	0.50
H359	353	SERM	5307721	256.00	2.00	4.00	2.00	64.00	>256.00	<0.12	<0.12	>256.00	1.00	0.12	1.00
H360	354	SERM	5306721	>256.00	2.00	2.00	2.00	64.00	>256.00	<0.12	<0.12	>256.00	1.00	<0.06	1.00
H363	357	SERM	5107721	64.00	<0.12	<0.12	4.00	32.00	64.00	<0.12	<0.12	256.00	4.00	<0.06	0.50
H373	367	SERM	5307420	>256.00	2.00	2.00	1.00	>256.00	>256.00	0.50	0.50	>256.00	1.00	1.00	0.25

HOOD_N	LAB_N	ORGANIS	API_PRO	AMP	ROC	CTX	NET	MEF	CXM	AZT	CAZ	CRD	IMP	CIP	GEN
H374	368	SERM	5307721	64.00	16.00	8.00	1.00	64.00	32.00	32.00	2.00	128.00	0.50	0.50	0.25
H376A	370	SERM	5347721	>256.00	16.00	16.00	1.00	64.00	>256.00	4.00	0.50	>256.00	1.00	1.00	0.25

Appendix III

β-lactamase profiles of the 200 cefuroxime resistant strains.

HOOD_NO	LAB_NO	ORGANISM	CXMMIC	β-lactamase
H010	10	ACIA	32.00	C
H017	17	ACIA	64.00	C
H018	18	ACIA	32.00	C
H026	26	ACIA	64.00	C
H032	32	ACIA	32.00	C
H033	33	ACIA	32.00	C
H040	40	ACIA	32.00	C
H048A	48	ACIA	64.00	C
H063	65	ACIA	128.00	C
H068	70	ACIA	32.00	C
H075	76	ACIA	64.00	C
H076	77	ACIA	>256.00	C
H101	99	ACIA	64.00	C
H117A	115	ACIA	32.00	C
H124	123	ACIA	32.00	C
H126	125	ACIL	64.00	C
H128	127	ACIA	32.00	C
H131	130	ACIL	32.00	C
H136	135	ACIA	32.00	C
H140	139	ACIA	32.00	C
H141	140	ACIA	64.00	C
H146	145	ACIA	64.00	C
H154	154	ACIA	32.00	C
H159	159	ACIA	32.00	C
H162	162	ACIA	64.00	C
H253	253	ACIA	64.00	C
H255	255	ACIA	64.00	C
H256	256	ACIA	32.00	C
H259	259	ACIA	64.00	C
H268	267	ACIA	64.00	C
H278	277	ACIA	64.00	C
H290	288	ACIA	64.00	C
H299	296	ACIA	128.00	C
H307	303	ACIL	32.00	C
H331	325	ACIA	32.00	C
H335	329	ACIA	64.00	C
H339	333	ACIA	32.00	C
H340	334	ACIA	64.00	C
H353	347	ACIA	64.00	C
H355	349	ACIA	64.00	C
H370	364	ACIA	64.00	C
H382A	377	ACIA	32.00	C
H393	391	ACIA	64.00	C
H044	44	CITF	64.00	C
H129	128	CITD	256.00	C
H138	137	CITD	256.00	C
H161	161	CITF	64.00	T-1
H257	257	CITF	64.00	C
H308	304	CITF	64.00	C
H315	311	CIT	64.00	C
H316	312	CITF	64.00	C

HOOD_NO	LAB_NO	ORGANISM	CXMMIC	β-lactamase
H349	343	CITF	128.00	T-1
H037	37	ECO	64.00	C
H084	85	ECO	32.00	C
H175	174	ECO	256.00	C
H002	2	ENB	>256.00	P99+T-1
H005	5	ENBC	64.00	>P99
H008	8	ENBC	256.00	P99+T-1
H014	14	ENBC	128.00	P99+T-1
H015	15	ENBC	256.00	P99+T-1
H022	22	ENBC	64.00	T-1
H030	30	ENBC	256.00	P99+T-1
H031	31	ENBC	>256.00	P99+T-1
H038	38	ENBC	>256.00	P99+T-1
H043	43	ENBC	32.00	>P99+T-1
H051	52	ENBC	>256.00	P99+T-1
H053	54	ENBC	64.00	>P99
H054	55	ENBC	>256.00	P99+T-1
H055	56	ENBC	256.00	P99+T-1
H057	58	ENBC	64.00	P99
H058	59	ENB	128.00	P99
H059	60	ENBC	32.00	P99+T-1
H060	61	ENBC	128.00	P99
H061A	62	ENBC	128.00	P99
H064	66	ENBC	>256.00	T-1
H066	68	ENBC	256.00	P99+T-1
H067	69	ENBC	256.00	<P99
H073	74	ENB	64.00	P99+T-1
H077	78	ENBC	32.00	>P99
H083	84	ENBC	>256.00	P99+T-1
H092	91	ENBC	>256.00	>P99
H093	92	ENB/CI	>256.00	P99
H099	97	ENBC	64.00	P+>P+T-1
H103	101	ENBC	>256.00	P99+T-1
H104	102	ENBC	256.00	P99+T-1
H105	103	ENBC	>256.00	P99+T-1
H106	104	ENBC	256.00	P+>P+T-1
H108	106	ENBC	256.00	P99
H109	107	ENBC	256.00	P99+T-1
H118	117	ENBC	256.00	P99
H119	118	ENBA	>256.00	P+>P
H122	121	ENBC	256.00	P99+T-1
H127	126	ENBC	256.00	>P99
H132	131	ENBC	256.00	P99+T-1
H137	136	ENBC	>256.00	P99+T-1
H150	149	ENBC	64.00	>P99
H153A	152	ENBC	128.00	P99+T-1
H155	155	ENBA	64.00	>P99
H156	156	ENBC	128.00	>P99+T-1
H164	163	ENBC	128.00	P99+T-1

HOOD_NO	LAB_NO	ORGANISM	CXMMIC	β-lactamase
H166	165	ENBC	32.00	P99+T-1
H182	182	ENBC	128.00	P99
H190	192	ENBC	128.00	P99+T-1
H191	193	ENBC	256.00	>P99+T-1
H192	194	ENBC	64.00	P99
H193	195	ENBC	128.00	P99+T-1
H197	198	ENBC	>256.00	>P99+T-1
H251	251	ENBC	256.00	P99+T-1
H252	252	ENBC	256.00	P99+T-1
H262	261	ENBA	256.00	>P99
H267	266	ENB	256.00	P99
H272	271	ENB	64.00	P99
H280	279	ENBC	256.00	T-1
H283	282	ENBC	256.00	P99+T-1
H289	287	ENBC	128.00	P99+T-1
H292	289	ENB	>256.00	P99+T-1
H294	291	ENBC	128.00	P99
H296	293	ENBC	128.00	P99+T-1
H302	298	ENB	128.00	P99+T-1
H314	310	ENBC	>256.00	>P99
H322	318	ENBC	256.00	>P99
H326	320	ENBC	>256.00	P99
H327	321	ENBA	64.00	>P99
H330	324	ENBC	>256.00	P99+T-1
H333	327	ENBC	128.00	P99+T-1
H341	335	ENBC	32.00	>P99
H358	352	ENBC	128.00	P99
H367	361	ENBC	64.00	P99
H368	362	ENBC	256.00	P99
H372	366	ENBC	>256.00	P99+T-1
H380	375	ENBC	>256.00	>P99
H381	376	ENBC	256.00	P99
H384	380	ENBC	128.00	>P99
H385	381	ENB	128.00	>P99
H386A	382	ENBC	128.00	>P99
H387B	385	ENB	128.00	>P99
H390	388	ENBC	256.00	>P99
H392	390	ENBC	32.00	>P99+T-1
H394	392	ENBC	256.00	P99
H397	395	ENBC	256.00	P99+T-1
H398	396	ENBC	256.00	P99
H400	398	ENBC	128.00	>P99
H405	112	ENBC	128.00	P99
H298	295	ERWA	32.00	C
H120	119	HAFA	64.00	C+T-1
H123	122	HAFA	64.00	C+SHV1
H019	19	KLEOX	128.00	K1+T-1
H020	20	KLEP	>256.00	C+T-1
H023	23	KLEOX	64.00	K14
H052	53	KLEB	64.00	K14

HOOD_NO	LAB_NO	ORGANISM	CXMMIC	β-lactamase
H062	64	KLEB	64.00	K14
H139	138	KLEP	32.00	T-1
H199	200	KLEOX	64.00	K14
H249	249	KLEOX	64.00	C
H276	275	KLEOX	64.00	K14
H281	280	KLEOX	64.00	K14
H312	308	KLEOX	64.00	K14
H082	83	MORM	32.00	C
H088	87	MORM	>256.00	C
H181	181	MORM	64.00	C+T-1
H250	250	MORM	32.00	C
H007	7	PROSTU	256.00	C
H024	24	PROVU	64.00	C
H269	268	PROP	256.00	C
H011	11	SERM	64.00	C
H013	13	SER	>256.00	C
H029	29	SERL	32.00	C
H034	34	SERM	128.00	C
H049	50	SERM	32.00	C
H087	86	SERL	32.00	C
H148	147	SERO	32.00	C
H171	170	SERM	256.00	C
H178B	178	SERM	256.00	C+T-1
H198	199	SERM	>256.00	C
H277	276	SERL	64.00	C
H288	286	SER	>256.00	C
H310	306	SERM	>256.00	C
H342	336	SERM	256.00	C
H344	338	SERM	256.00	C
H346	340	SERM	>256.00	C
H359	353	SERM	>256.00	C
H360	354	SERM	>256.00	C
H363	357	SERM	64.00	C
H373	367	SERM	>256.00	C
H374	368	SERM	32.00	C
H376A	370	SERM	>256.00	C

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A novel method for the identification and distinction of the beta-lactamases of the genus *Acinetobacter*

J. HOOD* & S.G.B. AMYES *Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK*

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The characterization of the chromosomal beta-lactamases of *Acinetobacter* has proved difficult because of the poor focusing of these enzymes in conventional isoelectric focusing on polyacrylamide gels. We describe a novel isoelectric focusing method, which employs an agarose gel incorporating a detergent with sorbitol and urea, to examine the beta-lactamases produced by eight clinical strains of *Acinetobacter calcoaceticus*; we have identified four different beta-lactamases. The molecular masses of each of the beta-lactamases was estimated and most of them ranged from 600 000 to >1 000 000. These are the largest beta-lactamases so far described and their size is likely to be one reason for their poor solubility in conventional polyacrylamide systems.

Acinetobacter calcoaceticus is an increasingly important cause of nosocomial infection (Bergogne-Bérézin *et al.* 1987) and one of the particular attributes of strains isolated in hospitals is resistance to many antimicrobial agents. The beta-lactam resistance of these strains probably results from the production of beta-lactamases (Joly-Guillou *et al.* 1987).

The exact nature and characterization of the (presumed) chromosomal cephalosporinases of *Acinetobacter* spp. is still not clear (Joly-Guillou & Bergogne-Bérézin 1986; Joly-Guillou *et al.* 1987, 1988). Morohoshi & Saito (1977) described a beta-lactamase in one strain of *Acinetobacter* as an enzyme with a preference for cephalosporins rather than penicillins and found that it had a mol. mass 30 000. Medeiros (1984) described the chromosomal beta-lactamases of *Acinetobacter anitratum* as inducible enzymes whereas Baurenfeind (1986) described them as either inducible or constitutive cephalosporinases. More recently, Joly-Guillou *et al.* (1987) studied 100 clinical strains of *Acinetobacter* and found that most of them (67) possessed a TEM-1 plasmid-mediated beta-lactamase. On the other hand, 30 had a chromosomal enzyme with a pI > 8 which was presumed to be similar to that described by Morohoshi & Saito (1977). No satisfactory enzyme identification technique was available to prove this point. Of these strains 7% had constitutive production and therefore high level resistance and 23 had both TEM-1 and the chromosomal beta-lactamases. There were nine strains that had a novel carbenicillin-hydrolysing enzyme (presumably plasmid encoded), which they called CARB-5. The same group of workers (Joly-Guillou *et al.* 1988) now describe a cephalosporinase activity in 41 of 100 clinical strains and state that beta-lactamase inducibility in *Acinetobacter* spp. has not been clearly demonstrated. These workers attempted to classify *Acinetobacter calcoaceticus* into four phenotypes based on the distribution of beta-lactamases and their MIC values to 14 beta-lactam antibiotics.

In the course of a study into beta-lactam resistance in Gram-negative aerobic bacteria in our hospital we found that a high proportion (22%) were *Acinetobacter* spp. Most of the

* Corresponding author.

strains were *var. anitratus* although a few were *var. lwoffi*. We now describe a novel method which allows isoelectric focusing of high molecular weight beta-lactamases and their distinction from one another.

Materials and Methods

BACTERIAL STRAINS

Eight strains of *Acinetobacter* spp. with MICs to aztreonam of ≥ 16 mg/l were collected from clinical sources in the Royal Infirmary Edinburgh. They were identified by either API 20E or API 20NE (API System. S.A., France). (see Table 1).

PREPARATION OF CRUDE BETA-LACTAMASES

Cells were grown in 4·5 ml of nutrient broth overnight at 37°C and used to inoculate a 1 l culture of nutrient broth and this was shaken (200 osc/min) at 37°C for 18 h. Cells were harvested at 5000 **g** for 15 min at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments) and the pellet was washed in 500 ml of 25 mmol/l sodium phosphate buffer (pH 7·0) and re-centrifuged at 5000 **g** for 15 min. The pellet was then resuspended in 3 ml of sodium phosphate buffer (pH 7·0) and the cells were disrupted by ultrasonication with constant cooling (8 μm, 1 min × 3, MSE Soniprep 150, MSE Instruments, Crawley). The cell debris was removed by centrifugation at 38 000 **g** for 15 min at 4°C (Sorvall).

PREPARATION OF PARTIALLY PURIFIED BETA-LACTAMASE

One ml amounts of crude enzyme was initially applied to a G-75 Sephadex gel filtration column (2 cm^2 × 90 cm) (Pharmacia, Uppsala, Sweden). The beta-lactamase peak was first identified by testing each volume for activity with the chromogenic cephalosporin, nitrocephin. Thirty μl of each volume were added to 100 μl of nitrocephin solution (50 mg/l) in a microtitre plate. Those which showed a rapid colour change (<120 s) from yellow to red indicated the presence of beta-lactamase activity.

These were then further assayed by the spectrophotometric assay method of O'Callaghan *et al.* (1972) to determine the peak beta-lactamase activity. If the activity appeared in the void volume another 1 ml of crude preparation was added to a similar column containing Sephacryl S-300 (Pharmacia) and the peak fractions determined as before. The peak fractions were pooled and then used to load the isoelectric focusing gels.

BETA-LACTAMASE MOLECULAR MASS DETERMINATIONS

This was carried out using the method of Andrews (1964). Appropriate proteins were applied to both the G-75 Sephadex and the Sephacryl S-300 columns. The position of the peaks of the protein markers was established by measuring the optical density of the fractions by the method of Waddell (1956). Beta-lactamase samples could then be applied to the calibrated columns and their mol. mass determined from the standard curves.

IEF POLYACRYLAMIDE GELS

The gels were prepared by the method of Matthew *et al.* (1975) using pH 3·5–10·6 ampholines (LKB). Samples of the enzymes (up to 100 μl) were applied, about 2 cm from the anode, on to the gel surface. The amount added (in μl) being equal to the time taken (in s) for a mixture of 30 μl of beta-lactamase preparation and 100 μl of nitrocephin solution (50 mg/l) to change from yellow to red.

Isoelectric focusing was carried out at 4°C at 1 W (constant), 550 V (limiting) and 20 mA (limiting) for 18 h. Five μl of isoelectric point (pI) standard markers (BDH) were also spotted on the gel to quantify the pI gradient of the gel.

1% AGAROSE, 4.3 mol/l UREA, 10% SORBITOL (AUS) GELS

AUS gels were prepared by melting 377 mg of agarose (agarose for isoelectric focusing type VIII, Sigma) in 15 ml of distilled water in a microwave oven (550 W) for 30–40 s. This was then allowed to cool to 60°C in a waterbath. Four g of D-sorbitol (BDH) were added to 9·6 ml of distilled water and heated to 60°C in a water bath. Similarly 10·2 ml of 10% (w/v) Triton X-100 (BDH) were added to 10 g of urea (BDH) and placed in the 60°C waterbath. The sorbitol solution and urea in Triton X-100 were

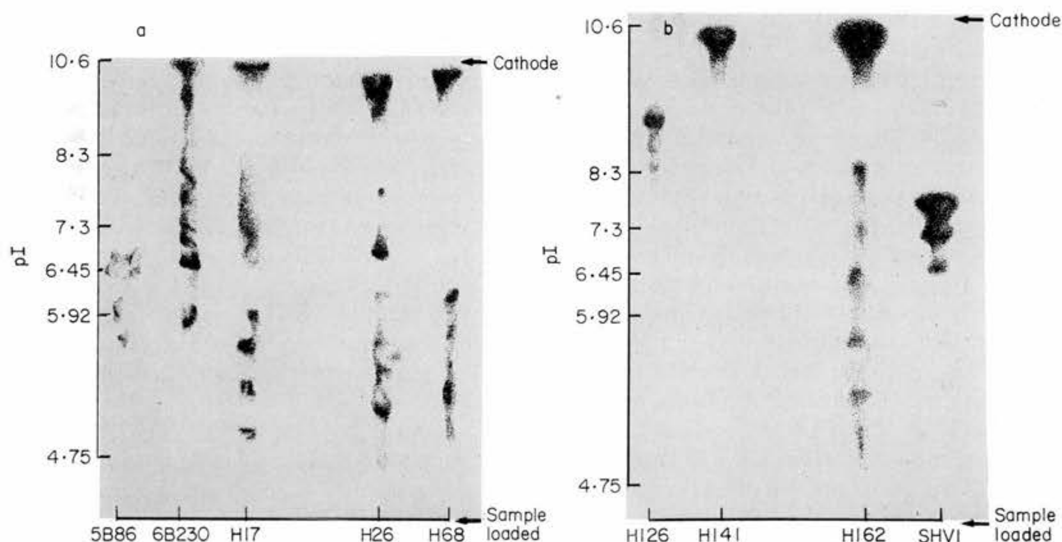

Fig. 1. Conventional polyacrylamide IEF gels of *Acinetobacter* chromosomal beta-lactamases and the plasmid beta-lactamase SHV1. Enzyme activity identified with nitrocephin.

then added and mixed. Just before incorporation of this mixture into the agarose, 2·5 ml of 3·5–10·6 ampholines (LKB) and 0·5 ml beta-mercaptoethanol (Sigma) were added. The final mixture of 1% agarose, 4·3 mol/l urea and 10% sorbitol was then gently stirred at 60°C and drawn up into a preheated glass pipette. The solution was then pipetted on the hydrophobic surface of a GelBond sheet (LKB) which was placed on the LKB 2217 ultrophor electrofocusing unit. This gel gave approximately a 1 mm overall thickness. The gel was left overnight (with the lid firmly down to prevent the gel drying out) to achieve mechanical strength.

SAMPLE PREPARATION

Sample size (in μl) was equalized with time, as described above. However, it was found that to aid the solubilization of the enzymes in the system: (a) a sample concentrate was added to each sample in the ratio of 1 μl of sample concentrate for each 5 μl of beta-lactamase in an Eppendorf tube. The sample concentrate consisted of (μl): LKB ampholines 3·5–10·6, 200; beta-mercaptoethanol, 100; Triton X-100 10% (v/w), 200; and (b) just before application onto this gel the sample mixture was saturated with urea (BDH). The excess urea was pelleted by centrifugation (30 s at 2874 **g**, MSE Microcentaur).

The samples (and isoelectric point markers) were applied to the surface of the gel but near the middle rather than 2 cm from the anode as with the polyacrylamide gels. Electrode wicks (LKB) were applied to the gel, the anode soaked in 0·1 N phosphoric acid (BDH Analar) and the cathode soaked in 0·1 N NaOH (BDH Analar). The electrofocusing unit was maintained at 15–20°C and a constant power was maintained at 30 W, setting the voltage and current at the limiting values of 1500 V and 30 mA respectively. The gel was electrophoresed until the pI markers had focused (i.e. for around 2 h 30 min).

STAINING

Polyacrylamide gels and AUS gels were stained by repeatedly overlaying the surface with a sheet of Whatman No 54 paper which had been dipped in nitrocephin solution (500 mg/l). The focused bands of beta-lactamase activity appear red on a yellow background.

Results

Eight different strains of *Acinetobacter* were chosen at random from the beta-lactam resistant population in the Royal Infirmary Edinburgh. Cell-free extracts were obtained from each and they were studied for the presence of

beta-lactamases. The eight cell extracts were first examined by the polyacrylamide IEF system (Fig. 1). It was immediately obvious that 7/8 beta-lactamase preparations did not focus satisfactorily. Streaks of enzyme activity were seen between the anode and cathode but most ended up against the cathode. In one case, however, H126, there was poor focusing of the enzyme with a pI of about 9·1. These poor results were not an artefact of the system used as the plasmid-mediated beta-lactamase SHV-1 was included as a control to show normal focusing in this gel system. Faced with this problem, we attempted to classify *Acinetobacter* beta-lactamases by a sodium dodecyl sulphate-free polyacrylamide gel system (i.e. differentiating them by molecular weight). The results of this were equally disappointing. In a similar manner to the polyacrylamide IEF the *Acinetobacter* enzymes migrated rapidly to the cathode during electrophoresis while the plasmid-encoded TEM-1, TEM-2 and OXA-2 beta-lactamase controls migrated to their expected positions (Hood and Amyes, unpublished data).

These results suggested to us that there was an inherent problem in the use of polyacrylamide as a separation matrix, perhaps as a result of the mol. mass of the enzymes, so we determined the mol. mass of each of these beta-lactamases by gel filtration and these are shown in Table 1. Most of these enzymes were very large indeed, in the region of 600 000 to greater than 1 000 000. Thus their inability to migrate satisfactorily, in the two polyacrylamide systems described, suggested that the problem resulted from poor enzyme solubility and it seemed necessary to employ a system that promoted the solubility of the large enzymes. Thus an agarose, urea and sorbitol gel system was devised. By this technique, we were able to focus the partially purified beta-lactamases from the eight clinical strains (see Fig. 2). At least, four different focusing patterns were formed. Five of the eight enzymes have very similar focusing patterns; the beta-lactamases from strains H17, H26, H68, H141 and 6B230 all had major bands at pI 8·6 and pI 8·8. However, the enzyme from H26, which was run on both Gel 2a and Gel 2b as an internal control, had additional minor bands at pI 9·8 and 10·1 on Gel 2b. H17 also had a minor band at pI 10·3. The other three enzymes were completely different, H162 had a single band at pI 8·7; H126 has a major band at pI 8·2 and a minor band at pI 8·1 and 5B86 has a major band at pI 7·3 and a minor band at pI 7·7.

Discussion

SEPARATION BY ISOELECTRIC FOCUSING

The separation of the beta lactamases of *Acinetobacter* (that are presumed to be chromosomal) by IEF on conventional polyacrylamide gels has proved totally unsuitable because of the inability of most of the enzymes to focus. Recently Joly-Guillou *et al.* (1988) described IEF findings of nine strains with 'a cephalosporinase type' enzyme as having 'variable bands of beta-lactamase activity located at pH > 8'.

In our series, in seven of eight preparations tested, streaks of beta-lactamase activity

Table 1. Properties of eight aztreonam-resistant strains of *Acinetobacter*

Acinetobacter calcoaceticus strain details				Beta-lactamase details	
Strain no.	Subspecies	Specimen type	Az* MIC	Mol. mass†	ACE‡ type
5B86	*var lwoffi*	blood	16	>1 000 000	4
6B230	*var anitratus*	blood	32	>1 000 000	1
H17	*var anitratus*	urine	64	640 000	1
H26	*var anitratus*	sinus swab	32	>1 000 000	1
H68	*var anitratus*	urine	64	>1 000 000	1
H126	*var lwoffi*	wound swab	>256	32 500	3
H141	*var anitratus*	wound swab	64	>1 000 000	1
H162	*var anitratus*	wound swab	64	60 500	2

* Az, aztreonam MIC in mg/l.
† Mol. mass, molecular mass.
‡ ACE, *Acinetobacter* chromosomal enzyme.

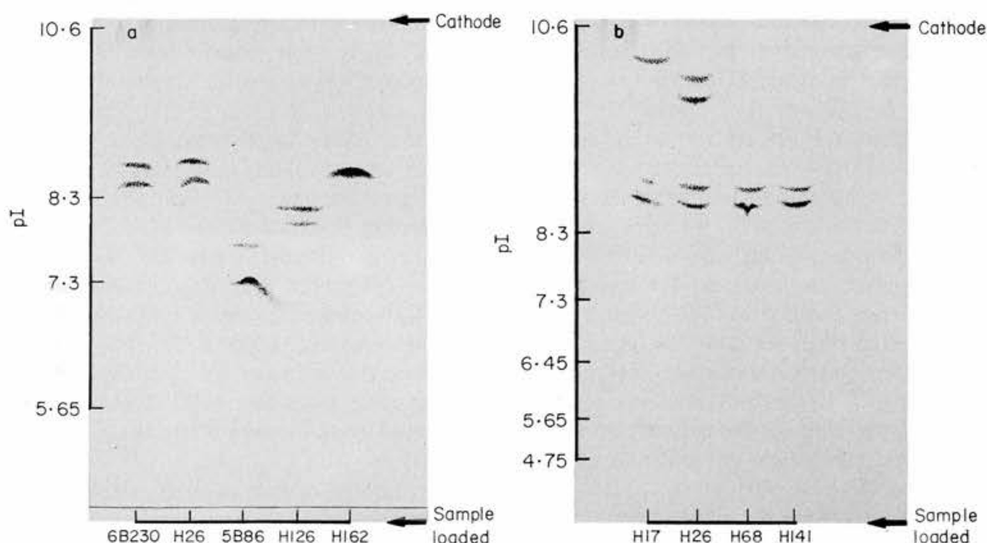

Fig. 2. Agarose, urea and sorbitol (AUS) IEF gels. Focused bands of *Acinetobacter* chromosomal beta-lactamase activity identified with nitrocephin. (a) 6B230, ACE 1; H26, ACE 1; 5B86, ACE 4; H126, ACE 3; H162, ACE 2. (b) H17, ACE 1; H26, ACE 1; H68, ACE 1; H141, ACE 1.

occurred from the point at which the enzymes were placed on the gel, to the cathode. Two facts led us to believe that the problem in this system was one of solubility. Firstly the mol. mass of these beta-lactamases varied from 32 500 to >1 000 000, and this would result in considerable sieving effects in the polyacrylamide gel. Secondly, when the enzymes were applied to a sodium dodecyl sulphate-free polyacrylamide gel system for separation on their molecular size, all the *Acinetobacter* beta-lactamases behaved in exactly the same way. They migrated directly to the cathode whereas control enzymes of TEM-1, TEM-2 and OXA-2 migrated as their size would predict.

The use of thin-layer IEF gels containing agarose has previously been described for the resolution of proteins with a mol. mass of greater than 2×10^6 (Saravis & Zamcheck 1979). Vecoli *et al.* (1983) compared the use of an agarose gel IEF with that of conventional polyacrylamide gel IEF in characterizing low molecular weight beta-lactamases and showed good correlation with the isoelectric points in most samples tested. Olsson & Lååს (1981) described a method which incorporated urea, agarose and sorbitol into a gel allowing complex mixtures of poorly soluble proteins to focus, e.g. cheese and soya bean meal. They noted the problems associated with urea and agarose gels i.e. (1) the agarose matrix is disturbed, leading to lower mechanical strength which results in a longer gelling time; and (2) urea and ammonium cyanide form an equilibrium pair leading to a risk of carbamylation. The use of sorbitol and beta-mercaptoethanol, however, minimized this problem.

Olsson & Lååს (1981) also noted that the presence of urea may affect the pI of some proteins, they gave the example of two isomers of lentil lectin which have pIs of 8·15 and 8·65, without urea, and pIs of 5·0 and 5·5 in 8 mol/l urea. Our findings show the pI of the beta-lactamase of H126 to be around 9·1 without urea and 8·1 in 4·3 mol/l urea. We would emphasize, however, that our technique highlights the differences in pIs in these beta-lactamases under the same preparation and running conditions rather than giving their absolute values.

In this paper, we describe a modification of the above technique that has allowed us to distinguish at least four different *Acinetobacter* beta-lactamases. We have designated the common beta-lactamase found in H17, H26, H68, H141 and 6B230 as ACE1. We believe that the extra minor banding found in H26 and H17 in Gel 2b may also be present in the others since in Gel 2a (in which H26 also appears) poor

focusing occurred at the pI 9–10·6 range with the 10·6 pI marker itself focusing badly. The enzyme present in strain H162 we have designated ACE2, and we have called the beta-lactamase in strain H126, ACE3 and the enzyme in strain 5B86, ACE4 (see Fig. 2).

We have found this technique to be reproducible. In its development, we tried incorporating urea into a polyacrylamide IEF gel with the same sample preparations. Electrophoresis with this system resulted in four different beta-lactamase patterns (in the same ascending order of pI) but there was poorer focusing and greater gel distortion. This presumably also resulted from the sieving effects of the polyacrylamide on the large molecular weight molecules (Hood and Amyes, unpublished data).

We have also found that the bands take longer to appear when the nitrocephin solution is added (red on yellow background) than in conventional polyacrylamide IEF gels. The problem can be exacerbated by the need to use 1N NaOH as the cathode buffer. This alkali hydrolyses the nitrocephin causing the gel in the cathodic region to turn red. This may make it difficult to visualize the beta-lactamase staining bands near the cathode. However, we have found that the problem can be partially overcome by overlaying this area with a weak acid before staining with nitrocephin.

SEPARATION BY MOLECULAR MASS

The mol. mass of most of these beta-lactamases is surprisingly large. Morohoshi & Saito (1977) studied an *Acinetobacter* chromosomal beta-lactamase and estimated its molecular weight as being 30 000. We have one beta-lactamase, H126, with a similar mol. mass. Sawai *et al.* (1982) looked at the molecular weights of cephalosporinases of Gram-negative bacteria and found molecular sizes ranging from 29 800 to 38 000 in *Proteus vulgaris, P. morganii, Citrobacter freundii, Enterobacter cloacae, Serratia marcescens* and *Escherichia coli.*

The largest beta-lactamase so far described is the L1 beta-lactamase of *Pseudomonas maltophilia.* Bicknell *et al.* (1985) estimated its mol. mass as 123 000 existing as a tetramer with subunits of 31 600 whereas Saino *et al.* (1982) estimated that its mol. mass was 118 000 with 26 000 mol. mass subunits. The gene encoding this beta-lactamase was located in the bacterial chromosomal DNA (Dufresne *et al.* 1988). It seems likely that these *Acinetobacter* beta-lactamases are coded for in the chromosomal DNA and exist in subunit form. However, their size may seem large because of covalent or strong non-covalent linkage with other cell envelope components. Therefore further work is required to elucidate the above speculations.

The two lowest molecular weight beta-lactamases, H126 and H162, focus best. The beta-lactamase from strain H126 (ACE-3) with a mol. mass of 32 500, is the only enzyme to focus on the ordinary polyacrylamide IEF and the enzyme from strain H162 (ACE-2) is the only enzyme to focus into one band on the AUS gel IEF.

The finding of four different beta-lactamases, which are almost certainly chromosomally mediated in the genus *Acinetobacter* may not be surprising. In this instance there are two different enzymes in both the *var anitratus* and in the *var lwoffi* strains. Recently, Bakken *et al.* (1988) have described at least four different inducible (presumably chromosomal) beta-lactamases in *Aeromonas* spp. and Matthew & Harris (1976) noted there were several species of bacteria with more than one beta-lactamase type.

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

D.J. Payne, J. Hood, M.S. Marriott [1] and S.G.B. Amyes

Department of Bacteriology, The Medical School, University of Edinburgh, Edinburgh, and [1] Department of Chemotherapy, Glaxo Group Research Ltd, Greenford, Middlesex, U.K.

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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

Correspondence to: S.G.B. Amyes, Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, U.K.

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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

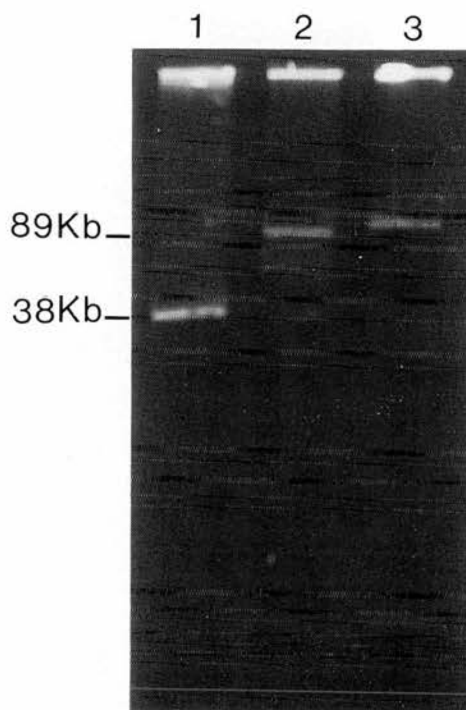

Fig. 1. Agarose gel electrophoresis of plasmid DNA from the *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 along with standard size plasmids. 1. R6K; 2. R1; 3. Plasmid isolated from *E. coli* transconjugant of *K. pneumoniae* 8825.

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).

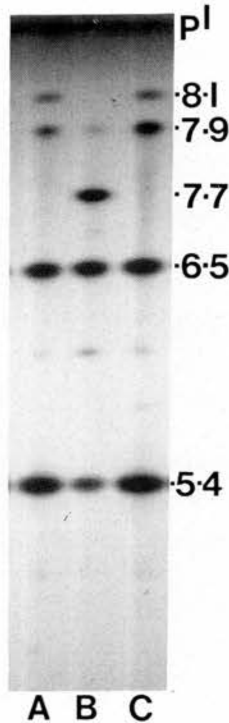

Fig. 2. IEF of β-lactamases produced by *K. pneumoniae* 8825 and its *E. coli* J53-2 transconjugant. A. β-lactamases produced by the *E. coli* transconjugant of *K. pneumoniae* 8825 selected on ceftazidime and rifampicin; B. β-lactamases produced by *K. pneumoniae* 8825; C. β-lactamases produced by the *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.

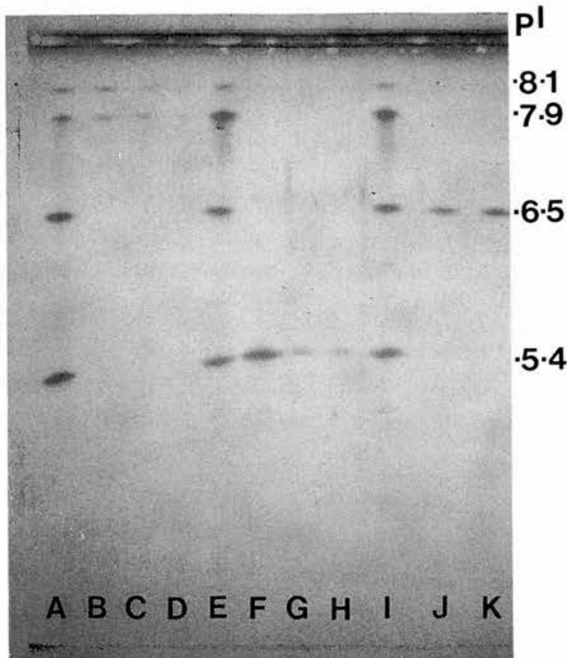

Fig. 3. IEF gel of β-lactamases separated by FPLC System. A. β-lactamases produced by *E. coli* J53-2 transconjugant of *K. pneumoniae* 8825 purified by G-75 Sephadex gel filtration; B. Fraction 2 eluted from FPLC System; C. Fraction 3; D. Fraction 4; E. Crude β-lactamase preparation of *E. coli* J53-2 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.

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THE CHROMOSOMAL ß-LACTAMASES OF THE GENUS *ACINETOBACTER:* ENZYMES WHICH CHALLENGE OUR IMAGINATION

J. Hood[a] and S.G.B. Amyes[b]

[a]University Department of Bacteriology
Royal Infirmary
Glasgow G4 OSF, UK

[b]Department of Bacteriology, The Medical School
University of Edinburgh
Edinburgh EH8 9AG, UK

INTRODUCTION

At first sight it would appear that the properties of the presumed chromosomal ß-lactamases of the genus *Acinetobacter* have been fully characterised. Isoelectric points (pI) in polyacrylamide gel systems were first described by Matthew and Harris (1976) and reiterated by Sykes and Matthew (1976). Other workers since then have described the pIs of *Acinetobacter* ß-lactamases as ranging from 7.5 to >10 (Medeiros et al., 1985); >8 (Joly-Guillou et al., 1987) and 9.9 (Hikida et al., 1989). Similarly, the molecular mass (M_r) of *Acinetobacter* ß-lactamase has been described as 30,000 by Morohoshi and Saito (1977) and as 38,000 by Hikida et al. (1989).

The kinetics of hydrolysis to various ß-lactam antibiotics have been most fully described by Morohoshi and Saito (1977) and, subsequently, to newer agents by Hikida et al. (1989). These authors also examined the effect of a variety of ß-lactamase inhibitors. There is little doubt, however, from these data that the *Acinetobacter* ß-lactamases are cephalosporinases (Sykes and Matthew, 1976; Morohoshi and Saito, 1977; Medeiros, 1984; Bauernfeind, 1986; Neu, 1986; Joly-Guillou et al., 1987; Hikida et al., 1989).

The Biology of Acinetobacter, Edited by K. J. Towner *et al.*
Plenum Press, New York, 1991

The inducibility of these cephalosporinases by specific ß-lactam inducers has been far from clear. They have been described as being inducible by Morohoshi and Saito (1977) and quoted as inducible by Sykes and Matthew (1976), Medeiros (1984), Neu (1986) and Hikida et al. (1989). Bauernfeind (1986) classified them as inducible or constitutive, whereas Joly-Guillou et al. (1988) stated that ß-lactamase inducibility in Acinetobacter had not yet been clearly demonstrated. On the basis of the work by Morohoshi and Saito (1977), Bush (1989) classified the *Acinetobacter* chromosomal ß-lactamase in Group One, i.e. a cephalosporinase not inhibited by clavulanic acid (CEP-N). Subsequent substrate profile and inhibition studies by Hikida et al. (1989) suggested that this classification was correct.

This article presents evidence that the true character of the chromosomal ß-lactamase of the genus *Acinetobacter* is not as simple as has been proposed. Indeed there appears to be considerable heterogeneity, with at least four distinct ß-lactamases produced by this genus.

ISOELECTRIC POINTS ON POLYACRYLAMIDE ELECTROPHORESIS

Matthew and Harris (1976) listed pIs of around 8.6 for two ß-lactamases produced by strains of *Acinetobacter: A. inotti* 1786E and *Acinetobacter* sp. 1787E. These same two strains appear in Sykes and Matthew (1976) as *A. inotti* (1786E) pI 8.6 and *A. mallei* (1787E) pI 8.7. The latter strain *(A. mallei)* 1787E was not an *Acinetobacter* at all, but a *Pseudomonas mallei*. This organism had somewhat controversially been placed in the genus *Acinetobacter* by Cowan and Steel (1965) - it became *P. mallei* in subsequent editions (Cowan and Steel, 1974). Therefore, this enzyme can no longer be considered as one produced by the genus *Acinetobacter*.

The former strain *(A. inotti)* 1786E is equally interesting. An extensive taxonomic literature search has failed to find any previous or subsequent mention of the specific epithet 'inotti'. This strain is part of the Glaxo Research Laboratories' collection, and one of the original authors (Miss A.M. Harris) kindly sent us strain 1786E for further study. The freeze-dried vial was still marked as *A. inotti*, but it was identified by the API 20NE (API System. S.A. France) as *A. lwoffii* (new specific epithet: *A. junii*). On reflection, we suggest that 'lwoffi', when written, could easily have been wrongly transcribed to 'inotti' and perhaps this error took place somewhere between its original entry in the strain book and the subsequent publication.

Medeiros et al. (1985) were the first to mention that not all of the chromosomal ß-lactamases of *Acinetobacter* focused on conventional polyacrylamide isoelectric focusing (IEF) systems. Of 14 strains of *A. calcoaceticus*, the pI was stated to be 8.8 for one enzyme, 9.0 for one, 9.7 for one, 10.0 for four, > 10 for three and a 'blur' for four. Joly-Guillou et al.

(1987) described 30 strains of *Acinetobacter* with a chromosomal cephalosporinase of pI >8. This again suggests poor focusing of these enzymes on conventional polyacrylamide IEF systems. More recently, Hikida et al. (1989) described a pI of 9.9 for a purified cephalosporinase from *A. calcoaceticus* (ML 4961). However, these authors employed broad range ampholines of pH 3.5-10.0 in their IEF gel. This suggests that the enzyme had migrated to, or almost to, the cathode. Unfortunately, there was no photograph of the IEF gel.

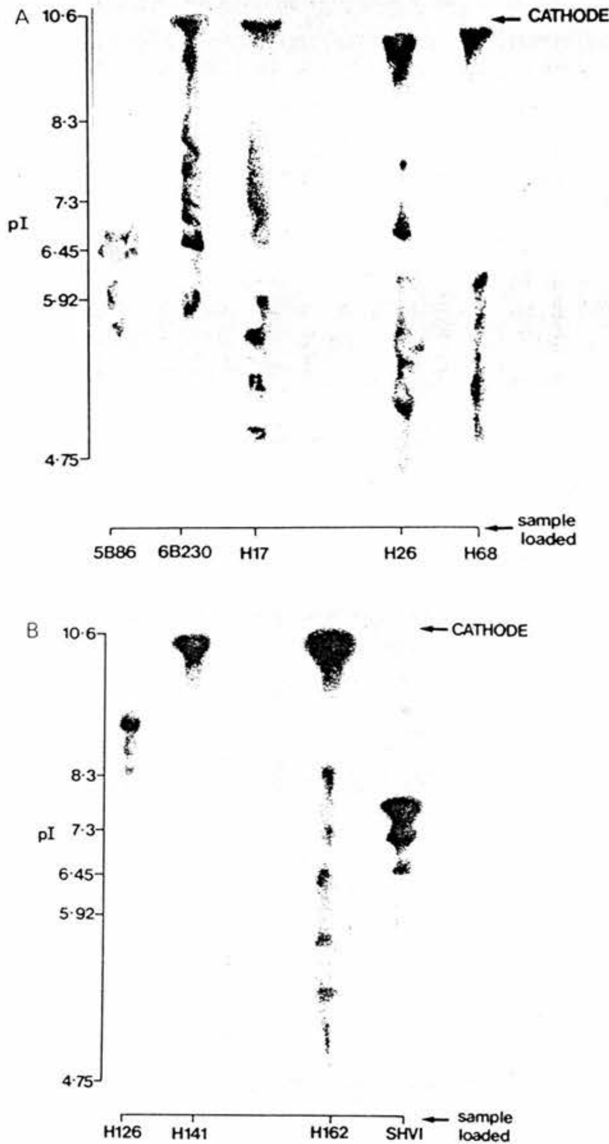


Fig. 1. Conventional polyacrylamide IEF gels of *Acinetobacter* chromosomal ß-lactamases and the plasmid ß-lactamase SHV-1. Enzyme activity identified with nitrocephin

Table 1. Properties of eight aztreonam-resistant strains of Acinetobacter

Acinetobacter strains					β-lactamase properties	
Strain No.	sub species	new species	Specimen type	Az[a] MIC	M_r[b]	ACE type[c]
5B86	*var. lwoffii*	*A. lwoffii*	blood	16	>1,000,000	4
6B230	*var. anitratus*	*A. junii*	blood	32	>1,000,000	1
H17	*var. anitratus*	*A. baumannii*	urine	64	640,000	1
H26	*var. anitratus*	*A. baumannii*	sinus swab	32	>1,000,000	1
H68	*var. anitratus*	*A. baumannii*	urine	64	>1,000,000	1
H126	*var. lwoffii*	*A. junii*	wound swab	>256	32,500	3
H141	*var. anitratus*	*A. baumannii*	wound swab	64	>1,000,000	1
H162	*var. anitratus*	*A. baumannii*	wound swab	64	60,500	2

[a] Az = aztreonam MIC in mg/l

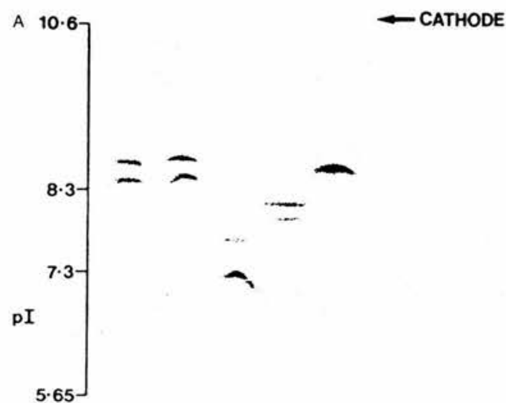
[b] M_r = molecular mass

[c] ACE = *Acinetobacter* chromosomal enzyme

We have studied eight partially purified ß-lactamases of *Acinetobacter*, obtained from aztreonam-resistant strains collected in the Royal Infirmary, Edinburgh (Table 1; Hood and Amyes, 1989). These ß-lactamases were applied to a conventional IEF system (Fig. 1), but seven of these enzymes did not focus in this system. Only the ß-lactamase from strain H126 *(A. lwoffii*, now *A. junii)* focused, with a pI of about 9.1. We postulated that the failure of these enzymes to focus resulted from two factors. Firstly, the basic nature of the enzyme (high pI), and secondly, their high molecular masses (see Table 1 and subsequent discussion on molecular masses). An agarose, urea and sorbitol (AUS) gel system was therefore devised for isoelectric focusing of these enzymes. This combination allowed the focusing of these basic enzymes (Fig. 2) and their classification into four groups, termed ACE-1 to ACE-4. ß-Lactamases from five of the eight strains studied were placed in the ACE-1 group. Four of these ACE-1 enzymes were derived from *A.baumannii* - the most prevalent *Acinetobacter* species isolated from clinical specimens (Bouvet and Grimont, 1987). A complete description of the AUS gel technique and further explanation of the IEF patterns has been published elsewhere (Hood and Amyes, 1989). Further confirmation of these groupings has been obtained from preliminary studies employing two additional techniques. Crude enzyme preparations from all eight strains were applied to either a MONO S or a MONO Q high performance ion-exchange column connected to a Fast Protein Liquid Chromatography (FPLC$^{(R)}$) system (Pharmacia, Uppsala, Sweden).

The FPLC method has been described fully elsewhere (Payne et al., 1990). Peaks of ß-lactamase activity obtained on the columns were detected by the nitrocephin spot test (Table 2) and demonstrated the clear differences between ACE-1, ACE-3 and ACE-4, while suggesting that ACE-2 is similar to ACE-1. In addition, the results suggest that there may be two subgroups within the ACE-1 group: one with a retention volume of 15-18 ml, the other with a retention volume of 12-14 ml. However, further work is required to verify these sub-groupings.

The purified enzymes obtained from the FPLC/ion exchange were also applied to a gel system on the PhastSystem$^{(R)}$ (Pharmacia, Uppsala, Sweden). This gel system was devised to resolve basic proteins on a polyacrylamide (PAGE) minigel. The exact sample preparation and running conditions were those described by Olsson and Tooke (1988). A Phastgel Homogeneous 20 (i.e. sodium dodecyl sulphate-free) was run with the reversed polarity electrodes. After electrophoresis, the enzymes were visualised by nitrocephin solution (500 mg/l). ACE-1 (e.g. H141) and ACE-2 (e.g. H162) preparations migrated similar distances from the cathode, whereas ACE-3 (H126) migrated slightly less far. Insufficient ACE-4 (5B86) was available to test on this system. Control preparations of the plasmid-mediated ß-lactamases SHV-1/TEM-1 and OXA-2/TEM-2 migrated to appropriate positions in this gel system, i.e. TEM-1 and TEM-2 stayed at the cathode, while SHV-1 and OXA-2 migrated to a point just behind the ACE enzymes. Further experimentation employing this technique is required. If it proves to be reproducible with crude ß-lactamase preparations, it would be an



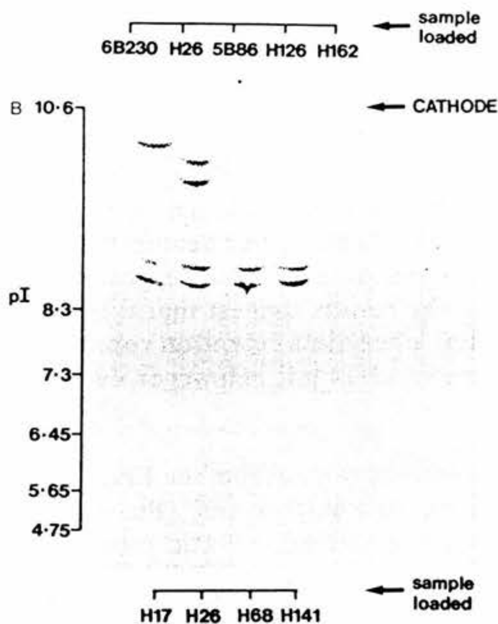


Fig. 2. Agarose, urea and sorbitol (AUS) IEF gels. Focused bands of *Acinetobacter* chromosomal ß-lactamase activity identified with nitrocephin

A. 6B230 - ACE-1
H26 - ACE-1
5B86 - ACE-4
H126 - ACE-3
H162 - ACE-2

B. H17 - ACE-1
H26 - ACE-1
H68 - ACE-1
H141 - ACE-1

Table 2. FPLC(R) of crude enzyme preparations on high performance ion exchange

Strain no.	Species	ACE type	Retention volume of major activity (ml)
Mono S (cation exchange) in 50 mM phosphate buffer pH7			
6B230	*A. junii*	1	15-16
H17	*A. baumannii*	1	17-18
H26	*A. baumannii*	1	18
H162	*A. baumannii*	2	15-16
H68	*A. baumannii*	1	12-13
H141	*A. baumannii*	1	13-14
Mono Q (anion exchange) in 25 mM Tris/HCl buffer pH8			
H126	*A. junii*	3	13
5B86	*A. lwoffii*	4	29

extremely useful rapid technique for the study of ß-lactamases obtained from clinical isolates of *Acinetobacter*.

MOLECULAR MASS ESTIMATIONS

Morohoshi and Saito (1977) gave the M_r of their cephalosporinase from *A. anitratum* NCTC 7844 as 30,000 when measured by gel filtration on a Sephadex G-75 column. Hikida et al. (1989) gave the M_r of their cephalosporinase from *A.calcoaceticus* ML4961 as 38,000. They estimated this M_r by SDS gel electrophoresis after purification of the enzyme with strong cation exchange and gel filtration.

We estimated the M_r of the ß-lactamases in our eight strains of *Acinetobacter* by gel filtration, initially on Sephadex G-75 and then, if required, on Sephacryl S-300 (Hood and Amyes, 1989). The results are listed in Table 1. One enzyme (ACE-3 from strain H126) had a similar M_r (35,000) to that described by Morohoshi and Saito (1977) and Hikida et al. (1989). The ACE-2 ß-lactamase from strain H162 had an M_r of 60,500, but the other six enzymes ranged from 640,000 to > 1,000,000. The results suggested that some of the ß-lactamases of *Acinetobacter* are either very large, or seem large because they are linked to other cell envelope components and the enzyme purification techniques used were incapable of removing these components. This raises the exciting hypothesis that these enzymes could be an intermediate step between penicillin binding proteins and the ß-lactamases. Alternatively, these enzymes may exist in multiple subunit form. Further experiments are being undertaken to examine these possibilities.

BIOCHEMISTRY

The specific activities of the eight ACE enzymes were measured for a selection of cephalosporins, penicillins and a monobactam (Table 3). The enzyme preparations were partially purified by gel filtration (Hood and Amyes, 1989). The spectrophotometric assay employed was that of O'Callaghan et al.(1969) and was carried out on a Pye-Unicam SP1800 UV/VIS spectrophotometer. Cephalosporins and the monobactam were used at concentrations of either 100 μM or 1000 μM (Table 3) and the penicillins at a concentration of 1000 μM. The specific activities of the eight ACE enzymes (Table 3) show clearly that these enzymes are cephalosporinases, although most of them also have some activity against the penicillins tested. The apparent detection of weak activity against cefuroxime in half the strains and weak activity against ampicillin and carbenicillin in one strain may be dismissed since the spectrophotometer was working at the limit of its sensitivity and, therefore, activity against these substrates may also be possible in the other strains. The only clear difference was the specific activity of ACE-4 obtained from strain 5B86, which was considerably lower than the others. Interestingly, ACE-1 from strain 6B230 and ACE-3 from H126 seemed similar - they are the only *A.junii* strains in the study.

Table 3. Specific activities[a] of ACE enzymes against a selection of cephalosporins and a monobactam (100 μM or 1000 μM) and penicillins (100 μM)

Strain no.	ACE type	AZ[b]	CAZ[b]	CTX[b]	CXM[b]	CER	CED	PENG	AMP	CARB
5B86	4	-	-	-	-	25	-	-	-	-
6B230	1	-	-	-	0.014	330	56	2.8	-	-
H17	1	-	-	-	-	93	69	4.7	-	-
H26	1	-	-	-	-	93	33	6.7	-	-
H68	1	-	-	-	0.13	31	19	6.0	0.7	0.46
H126	3	-	-	-	-	230	46	84	-	-
H141	1	-	-	-	0.16	49	17	4.7	-	-
H162	2	-	-	-	0.12	93	17	5.8	-	-

[a] μmoles of substrate hydrolysed/min/mg protein

[b] specific activities also measured with high substrate concentration (1000 μM)

\- no detectable activity

AZ, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CXM, cefuroxime; CER, cephaloridine; CED, cephradine; PENG, penicillin; AMP, ampicillin; CARB, carbenicillin.

Table 4. MIC values (mg/l) for ACE-producing strains of a selection of cephalosporins, penicillins, and a monobactam

Strain no.	ACE type	AZ	CAZ	CTX	CXM	CER	CED	PENG	AMP	CARB
5B86	4	16	2	4	8	8	16	16	8	16
6B230	1	32	8	4	16	64	128	>32	128	64
H17	1	64	4	32	64	64	256	>32	32	32
H26	1	32	8	32	64	256	>256	>32	64	32
H68	1	64	8	16	32	64	256	>32	32	32
H126	3	>256	64	64	64	128	256	>32	128	128
H141	1	64	8	16	64	64	256	>32	64	32
H162	2	64	8	32	64	64	256	>32	32	32

Abbreviations as in Table 3.

Table 4 shows the MIC values of the substrates used in Table 3 for these strains. The results showed that all the *Acinetobacter* strains studied had a high degree of resistance to penicillins and first, second and third generation cephalosporins. There was some correlation between the resistance levels and the enzyme produced. All the strains encoding the ACE-1 enzyme had very similar MICs of all the drugs tested. H162, which encoded ACE-2, could not be distinguished from the ACE-1 strains on its resistance profile. However, strain H126, which encoded ACE-3, was generally more resistant to the drugs tested than the ACE-1 or ACE-2 producers. Specifically, H126 was more resistant to the third generation cephalosporins and the monobactam, aztreonam. On the other hand, strain 5B86, which encoded ACE-4, was generally less resistant than the other ACE producers. It was particularly less resistant to the penicillins and the first generation cephalosporins tested.

Table 5 shows the kinetics of hydrolysis for each of the enzymes, i.e. K_m values with nitrocephin and cephaloridine as substrates. This value was obtained by measuring the rate of hydrolysis at limiting substrate concentrations and Lineweaver-Burk plots. The K_m values obtained with both nitrocephin and cephaloridine were broadly similar to each other (Table 5). The K_m values to cephaloridine ranged from 150 μM to 710 μM and were similar to the values found by Morohoshi and Saito (1977) and Hikida et al. (1989) of 250 μM and 511 μM respectively.

These results show that all four ACE enzymes have moderate affinity for cephaloridine and nitrocephin. All the K_m values were within the same order of magnitude and could not convincingly be distinguished from one

Table 5. Kinetic data for ACE enzymes

Strain no.	ACE type	$K_m{}^a$ (μm) Nitrocephin	$K_m{}^a$ (μM) Cephaloridine
5B86	4	220	710
6B230	1	280	150
H17	1	550	160
H26	1	250	150
H68	1	280	180
H126	3	150	380
H141	1	220	210
H162	2	250	260

[a] K_m, this value was obtained by measuring rates of hydrolysis at limiting substrate concentrations and determined by Lineweaver-Burk plots

Table 6. ID_{50}[a] of ACE enzymes with nitrocephin as substrate (μM)

Strain no.	ACE type	Aztreonam	Cloxacillin	Clavulanate
5B86	4	>100	0.18	>100
6B230	1	28	0.012	>100
H17	1	8	0.005	>100
H26	1	5	0.022	>100
H68	1	9	0.1	>100
H126	3	0.08	0.003	>100
H141	1	1.8	0.02	>100
H162	2	23	0.022	>100

[a]ID_{50} = amount of inhibitor required for 50% inhibition of nitrocephin hydrolysis

another. Thus none of the ACE enzymes demonstrated greater substrate affinity than the others and K_m values were a poor discriminator.

Table 6 shows the effect of the ß-lactamase inhibitors aztreonam, cloxacillin and clavulanic acid, expressed as the concentration required for 50% inhibition (ID_{50}) of enzyme activity. The enzymes from all the strains were readily inhibited by cloxacillin. Most were inhibited within the range of 0.003-0.022 μM. However, two enzymes required at least 0.1 μM cloxacillin for 50% inhibition. One of these enzymes was ACE-1, but this level of cloxacillin is still considered to be very low. Thus cloxacillin inhibition also seems to be a poor discriminator of the ACE enzymes, although a characteristic feature of them all is that they are cloxacillin sensitive. Similarly, the enzymes from all the strains were extremely resistant to clavulanic acid inhibition. The inability of any of these enzymes to be inhibited significantly by 0.1 mM clavulanic acid shows that clavulanic acid resistance is a characteristic feature of all these enzymes, but is incapable of being used as a discriminator.

Differences in inhibition were, however, identified when aztreonam was used as an inhibitor. There was little difference in the ID_{50} for the ACE-1 and ACE-2 enzymes, which were in the range of 1.8 to 28 μM (Table 6). The ACE-3 enzyme from strain H126 was much more sensitive to aztreonam inhibition than the others (ID_{50} = 0.08 μM), while the ACE-4 enzyme from strain 5B86 was much more resistant (ID_{50} = >100 μM). We believe that the inhibition profiles with aztreonam provide good discrimination of the ACE-3 and ACE-4 enzymes.

Table 7 shows the effect of EDTA, $HgCl_2$ and *p*CMB. All the enzymes were inhibited to the same extent by a fixed concentration of $HgCl_2$, but were

Table 7. Effect of inhibitors and metal ions on ACE enzymes with nitrocephin as substrate

Strain no.	ACE type	Percentage inhibition		
		EDTA[a]	$HgCl_2$[b]	pCMB[c]
5B86	4	<20	89	0
6B230	1	<20	98	0
H17	1	<20	96	0
H26	1	<20	97	0
H68	1	<20	95	0
H126	3	<20	95	0
H141	1	<20	98	0
H162	2	<20	98	0

[a] 1 mM EDTA (inhibition variable: see text)
[b] 1 mM $HgCl_2$
[c] 0.1 mM *p*-chloromercuribenzoate

virtually unaffected by moderately high levels of *p*CMB. Thus no discrimination of these enzymes could be obtained from these profiles.

The effect of 1 mM EDTA on the enzymes with either nitrocephin or cephaloridine as the substrate was variable, although generally below 20%. Hikida et al. (1989) found that EDTA had no effect on their enzyme's activity. Since many of the enzymes described here are of large M_r and, therefore, may exist in subunit form, it is possible that metal ions play a role in their function. This may explain the variable effects of EDTA on their activities. However, further experiments with more purified enzyme will be required to elucidate these findings.

The above biochemical data (EDTA apart) place all of these ß-lactamases firmly in Bush Group One (Bush, 1989).

INDUCTION EXPERIMENTS

Morohoshi and Saito (1977) described how the enzyme activities of five strains of *A. anitratum* "increased by about five to ten-fold" following treatment with 100 μg of benzylpenicillin, cephaloridine or 6-aminopenicillanic acid for 1 h at 37°C. Hikida et al. (1989) quote Morohoshi and Saito (1977) as having shown that *Acinetobacter* cephalosporinases are inducible, but they did not carry out induction experiments themselves.

• We have carried out induction experiments with all eight strains, employing cefoxitin as the inducer at one quarter the MIC value for the culture. The method employed was that described by Minami et al. (1980). No discernible ß-lactamase induction was found with any of the strains studied. In discussions with D. Livermore and C.C. Sanders (personal communications) it was stated that evidence of induction had not yet been found in any strain of *Acinetobacter* tested. This includes the use of cefoxitin, imipenem and penicillin as inducers. Much more convincing evidence would therefore be required before it can be claimed that these enzymes are inducible, since current evidence suggests they are not.

ROLE OF PRESUMED CHROMOSOMAL ß-LACTAMASES OF ACINETOBACTER IN ß-LACTAM RESISTANCE

Although it seems likely that these enzymes are largely responsible for the observed resistance to ß-lactam drugs, it is also probable that other factors, e.g. altered permeability or alterations in the penicillin binding proteins,may be equally important in some strains. The ultimate test must involve the cloning of the ß-lactamase gene from each of these strains into a suitable recipient, thereby allowing further study of the resistance mechanism. This would also enable their place on the bacterial chromosome, rather than on a plasmid, to be confirmed.

CONCLUSIONS

As one would expect from a genus that is very heterogeneous, with at least 17 genospecies (Grimont and Bouvet, this volume), the presumed chromosomal ß-lactamases are also heterogenous. At least four different ACE enzymes have been described in *Acinetobacter* on the basis of IEF in AUS gels, molecular mass and biochemical properties. As has been outlined above, these are very basic proteins with pIs probably greater than 10. So basic, in fact, that it is difficult to explain how the cell might secrete them into the periplasmic space; thus they may be bound to the cell membrane. As a group, they generally do not focus well, or at all, in conventional polyacrylamide IEF systems. It is possible that further different chromosomal enzymes exist, since we describe ACE-1 to ACE-4 in only three of the possible 17 genospecies, i.e. in *A. baumannii, A. junii* and *A. lwoffii*.

Further work is needed to explain the large M_r of the enzymes found in six of the eight strains. This may then shed light on the possible evolutionary place of these interesting enzymes. Future work on the eight enzymes should include further purification, perhaps by FPLC. This would allow closer comparison with the biochemical kinetic data obtained by Hikida et al. (1989) for their enzyme. It would be interesting to know the exact genospecies of both the Hikida and Morohoshi strains. It is clear, however, that the enzymes described in this article, together with those described by

Morohoshi and Saito (1977) and Hikida et al. (1989), are all cephalosporinases, not inhibited by clavulanic acid, and can be placed in Bush Group One (CEP-N).

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NOSOCOMIAL INFECTIONS IN A CARDIOTHORACIC INTENSIVE THERAPY UNIT:DEVICES AND DESIGN

HOOD,J.and ANDERSON,Felicity A.

University Department of Bacteriology
Royal Infirmary,GLASGOW,G4 OSF.

Department of Bacteriology
University of Edinburgh
Teviot Place EDINBURGH,EH8 9AG

The regional cardiothoracic unit of the Royal Infirmary Edinburgh has a 12 bedded intensive therapy unit.We describe two separate episodes of nosocomial infection,firstly 20 cases of infection/colonisation with a multiresistant Enterobacter.This outbreak was related to poor handwashing facilities and major design faults in the waste disposal area,particularly the lack of a "slop hopper".This resulted in the misuse and malfunction of the bedpan washer.This in turn led to contamination of the hands of the nursing staff with resultant cross infection.Appropriate control measures are described.
Secondly we describe the sudden increased isolation of Acinetobacter spp.from arterial line and CVP tips.14 cases were infected,11 of which were first on their operating list.This coincided with a new "monitor" technician and a lack of experienced nursing staff.Therefore the transducer sets and lines were primed with fluid the evening prior to a list (and used if an emergency case arose that evening).Acinetobacter was grown from the preparation area,transducer heads,domes and tubing.Measures taken included the priming of devices as required without storage.
These outbreaks illustrate the problems of infection acquired in the ITU with respect to unit design,device and patient handling.

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Session No.15 Oral Session *12 December 1990 ; 11:48 a.m.*
Mabtapud Room

LACK OF TRANSFERABLE THIRD GENERATION CEPHALOSPORIN RESISTANCE IN SCOTLAND

D.J. Payne, J. Hood, R. Paton & S.G.B. Amyes

Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, Scotland

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 4 mg/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.

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Mycobacterial antigens studied because they cause antibody responses in the BALB/c mice used to raise monoclonal antibodies, may not be relevant to T cell-mediated responses in man. Moreover, mice are immunised with killed bacilli which lack secreted antigens, though these are likely to be important for rapid recognition of infecting organisms, and, following diffusion to the spleen where they can be presented to T cells in the absence of adjuvant effects of cell wall components, they may induce suppression. We have used ^{35}S-methionine-labelled supernates of 4–7-day cultures of *Mycobacterium tuberculosis* and BCG to identify secreted antigens. These antigens, characterised by SDS-PAGE, β-scanning, and polyclonal and monoclonal antibodies, constitute a small subset of those present in long-term supernates, when leakage from dead organisms may be significant. Abundant 30- and 31-Kda secreted antigens of *M. tuberculosis* and BCG bind strongly to fibronectin. Moreover, leprosy patients have high levels of antibody to them suggesting that antigenically related molecules are also important antigens of this non-cultivable species. In view of the regulatory roles of fibronectin in macrophage and lymphocyte function, these 30–31-Kda antigens are likely to play a role in the pathogenesis of human mycobacterial disease.

Mycobacterial lipoarabinomannan is a potent trigger of release of tumour necrosis factor from macrophages

C. Moreno, Janice Taverne*, Angela Mehlert, C. Bate*, R. Brealey†, J. Playfair* and G. Rook†

*MRC Tuberculosis and Related Infections Unit, RPMS, Hammersmith Hospital, London W12 and Departments of *Immunology and †Medical Microbiology, University College and Middlesex School of Medicine, London W1*

The fever, weight loss, necrosis, acute phase responses and raised agalactosyl IgG in patients with tuberculosis are probably due to release of cytokines, including Tumour Necrosis Factor (TNF), from macrophages activated by exposure to γ interferon, and further activated following autocrine stimulation by 1,25-(OH)2 vitamin D3. However cytokine release requires not only activation, but also a subsequent trigger. We have shown that the lipoarabinomannan (LAM) from *Mycobacterium tuberculosis* can trigger the release of TNF from human monocytes and activated mouse peritoneal macrophages *in vitro*. Release is enhanced by pre-incubation in the presence of indomethacin or γ interferon. Lipomannan is not active. LAM also causes release of TNF *in vivo* in mice pretreated with *Propionibacterium acnes*, with a potency comparable to that of the lipopolysaccharide (LPS) from gram-negative organisms. We have shown that the effect is not due to contaminating LPS by affinity chromatography, and by a treatment with dilute alkakli which inactivates LAM but not LPS. Moreover when LAM was subjected to SDS-PAGE, and then electrophoretically blotted on to nitrocellulose, the TNF triggering activity was localised in the part of the blot where LAM could be demonstrated with specific monoclonal antibody.

Clonal origin of multiresistant serotype O12 *Pseudomonas aeruginosa* in Europe

T. L. Pitt, D. Livermore*, D. Pitcher, A. Vatopoulos† and N. J. Legakis†

*Division of Hospital Infection and National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, *Department of Medical Microbiology, The London Hospital Medical College, Turner Street, London E1 2AD and †Department of Microbiology, National University of Athens, PO Box 8540, 100 10 Athens, Greece*

Antibiotic multiresistant *Pseudomonas aeruginosa* strains are rare in clinical specimens but in recent years a number of reports have documented outbreaks of multiresistant strains of an uncommon serotype, O12, in Greece, Italy and Belgium. Two hundred and eight epidemiologically distinct strains were identified from 10 countries by typing, 48 of which were resistant to carbenicillin (MIC$>$128 mg/L) and gentamicin ($>$4 mg/L). One quarter of the resistant strains were serotype O12 and these orginated from 8 countries. A panel of O12 strains, which included the sensitive serotype strains, was examined to determine the homogeneity of the strains. All strains were indistinguishable by phage and pyocin typing but lysogenic phage profiles indicated that one of the English isolates was distinct. Electrophoretic characterisation of outer-membrane proteins, lipopolysaccharides and esterase enzymes supported the results of traditional methods. Restriction fragment length polymorphism of DNA fragments hybridised with a cDNA probe copy of rRNA from the O12 serotype strain provided proof of the genetic relatedness of the strains. All clinical O12 isolates produced PSE-1 β-lactamase which conferred high level resistance to azlocillin, carbenicillin, cefsulodin and cefotaxime but not ceftazidime. Plasmids were found in only 5 strains. We propose that the similarity of resistance mechanism, phenotype and genotype of O12 strains in Europe suggests a clonal origin of the strain.

A novel method to identify and distinguish *Acinetobacter* chromosomal β-lactamases

J. Hood and S. G. B. Amyes

Department of Bacteriology, Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG

Acinetobacter strains are an increasingly important cause of nosocomial infection. An outbreak of acineto-

bacter infection associated with arterial pressure transducers was described. A study in our hospital of cefuroxime resistance in gram-negative aerobes found that 22% of this resistance resulted from *Acinetobacter* strains (42 of 192). Eight of these organisms (6 var. *anitratus* and 2 var. *lwoffi*) were studied further. All of them were found to produce a cephalosporinase (presumably chromosomal). The molecular sizes of these enzymes were determined by gel filtration and they were studied by: (a) a novel isoelectric focusing technique incorporating urea and sorbitol in agarose gels; (b) measuring their specific activities to a selection of β-lactam drugs; (c) estimating their ID50s to aztreonam; (d) determining their Kms to nitrocefin and cephaloride. The molecular weight of six of these enzymes was found to be high—in the range 500,000–>1,000,000. These are the highest molecular weights described so far for β-lactamases. It has not previously been possible to use the standard polyacrylamide gel IEF to distinguish acinetobacter β-lactamases because of their insolubility in this system. Their high molecular weights are partly responsible for this. Our novel IEF system clearly distinguishes at least four different acinetobacter β-lactamases.

CHARACTERISATION OF *NEISSERIA GONORRHOEAE* FROM BAHRAIN

K. M. Bindayna and Catherine A. Ison

Department of Medical Microbiology, St Mary's Hospital Medical School, Paddington, London W2 1PG

Sixty one strains of *Neisseria gonorrhoeae* were collected between February 1988 and August 1988 from the main hospital in Bahrain (Salmanyia Medical Center) which is located in the heart of the capital city Manama. Most of the gonococcal strains were collected from men attending different small medical centers throughout the country and sent to the main hospital. These strains were characterised by serotyping, auxotyping and antibiotic susceptibility testing. In the total population only 7 serovars were found; 34 (56%) of the strains belonged to serovar IB-5/7, 9 (14%) to IB-1 and 18 (30%) belonged to other serovars (IB-3, IB-4, IB-8, IB-22 and IA-6). Only two auxotypes, prototrophic and proline requiring, were identified. Eleven (18%) of the strains were penicillinase-producing *N. gonorrhoeae* (PPNG). Among non-PPNG, 36 (72%) of the 50 strains were resistant to penicillin (MIC ≥ 1 mg/L) and 22 (44%) showed increased resistance to cefuroxime (MIC ≥ 0·5 mg/L). This compares with 41 serovars and a level of penicillin resistance of 5–10% in gonococci isolated in London. The level of antibiotic resistance in these strains was unexpectedly high. This could be due to the uncontrolled use of antibiotics which may have applied a selective pressure, or these strains may have been imported from the Far East which has a history of antibiotic resistance.

THE USE OF GENE PROBES FOR THE DETECTION OF TOXIGENIC *ESCHERICHIA COLI*

M. J. Woodward, C. Wray and P. J. Carroll

Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB

Gene probes to LT, STpa, STpb, VT1 and VT2 were used to examine 1031 strains (339 from cattle and 692 from pigs) of *Escherichia coli* isolated from cases of enteric disease. Of the bovine strains, 60 hybridised with the STpa probe and the presence of this gene correlated with the possession of either the K99 (F5) or F41 adhesin. Five bovine strains possessed STbp genes and five produced either VT1 or VT2. Thirty-six percent of the porcine strains hybridised with the gene probes. Most of the 160 K88 (F4)-positive strains produced both LT and STpb, although some cultures were detected which produced LT or STpb alone; both STpa and STpb were found in four cultures. Strains possessing the K99 (F5) adhesin, with the exception of one LT producer, possessed STpa, STpb or VT2 genes alone, or in combination. Strains belonging to serogroup O138:K81 were more heterogeneous as to their toxin genes and many combinations of toxin genes were found. Thus, of the 52 strains, eight produced only VT2; 32 VT2 STpa STpb; one LT VT2 STpa STpb; 2 STpb; 4 STpa STpb; one LT VT2; 2 LT, STpa. Gene probes were found to provide a rapid means of toxin detection in *E. coli* and a replacement for animal experimentation.

RESISTANCE TO A VETERINARY AMINOGLYCOSIDE ANTIBIOTIC (APRAMYCIN) CAUSING CROSS RESISTANCE TO GENTAMICIN IN HUMAN CLINICAL ISOLATES OF ENTEROBACTERIACEAE

A. P. Johnson, N. Woodford, E. J. Threlfall* and R. C. George

*Antibiotic Reference Laboratory, Division of Hospital Infection and *Division of Enteric Pathogens, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT*

Apramycin is an aminoglycoside antibiotic used extensively but exclusively in veterinary medicine. Apramycin resistance mediated by production of the modifying enzyme AAC (3) IV encoded on transferable plasmids has been reported in animal isolates of *Salmonella* and *Escherichia coli*. The AAC (3) IV enzyme also confers resistance to gentamicin and other aminoglycosides used to treat serious human infections. We have screened a large number of gentamicin-resistant Enterobacteriaceae for cross-resistance to apramycin in order to investigate the occurrence of this resistance pattern in human isolates. Six strains of *E. coli* and one of *Klebsiella pneumoniae* isolated from human infections have now been shown to be resistant to both apramycin (MIC > 16 mg/L) and gentamicin (MIC > 4 mg/L). All the strains contain large plasmids of molecular weight 40–90 Mda, with four of the *E. coli* strains also harbouring plasmids in the range 1·0–4·7 Mda. The larger plasmids were

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endocarditis in susceptible groups. A questionnaire was included in an issue of the *British Dental Journal* and circulated to 16,500 dental surgeons. Questionnaires were also sent nationwide to 650 cardiologists and 1390 members of four infection societies and 2500 general physicians through the Royal College of Physicians of Edinburgh. The response rate overall was 17% providing 3544 questionnaires for analysis. Preliminary results of the survey were presented. It appears from the questionnaires returned that there is now widespread acceptance of the BSAC recommendations; >90% of respondents indicated that they would prescribe amoxycillin 3 g less than 2 h before the dental procedure. Erythromycin was the most popular alternative in penicillin-allergic patients with 95% choosing this option although often with suboptimal dosage. Dental procedures considered for prophylaxis included extractions (99·9%), scaling (97·6%), root canal work (78%) and cavity filling (35%). Only 12% of respondents used topical oral antiseptics with chlorhexidine being chosen in most cases (79%).

Cefuroxime and gentamicin resistance in non-pseudomonal gram-negative aerobic bacilli isolated from different units within a large teaching hospital

J. Hood and S. G. B. Amyes

Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG

The Royal Infirmary of Edinburgh is a large teaching hospital of 1000 beds containing most regional and supraregional specialities. Over 8000 clinical isolates of non-pseudomonal gram-negative aerobes were collected over a 36-month period (March 1986–March 1989). Resistance to cefuroxime and gentamicin was estimated by the Stoke's method. Overall resistance to cefuroxime was 13% while resistance to gentamicin was only 2·6%. The resistance to cefuroxime was related to specific ward areas: General medical, 8·2%; Haematology, 12·7%; General surgery, 13·7% (range 7·0–20·9%); Urology, 15·9%; Orthopaedics, 11·7%; Cardiothoracic ITU, 34·9%; General ITU, 38·0%; Head injury unit, 24·6%; Acute renal dialysis unit, 22·0%; Special Care Baby Unit, 16·9%. The incidence of cefuroxime resistance was related to the overall use of β-lactam drugs. The highest resistance was found in areas where cefuroxime was the main treatment or prophylactic agent. Gentamicin resistance was generally lower: Surgical hospital, 1·9% (range 0·5–5%); Medical hospital, 3·1% (range 1·0–6·1%); Intensive care areas, 4·5% (range 1·5–6·7%). The resistance to gentamicin was low even in areas of high use, e.g., 4·1% in the Special Care Baby Unit. The extra care exercised in the use of these potentially more toxic drugs may be one reason for the lower incidence of resistance.

Use of a DNA hybridisation technique to study the effect of quinolone antibiotics on plasmid curing in an experimental model of urinary tract infection

A. C. Phillips and K. J. Towner

Department of Microbiology and PHLS Laboratory, University Hospital, Nottingham NG7 2UH

The determination of plasmid copy number by conventional methodology presents considerable logistical difficulties when applied to large numbers of samples. In an attempt to overcome these problems, a biotin-labelled DNA probe was used in dot-blot tests to detect the presence of bacteria carrying plasmid DNA in an experimental model simulating infection of the urinary tract. The colour intensity generated in positive hybridisation tests was measured using a computer-controlled image analysis system and was shown to be proportional to the number of plasmid copies present. When the system was used to investigate the effect of quinolone antibiotics on plasmid carriage by bacteria it was demonstrated that ciprofloxacin, used at a concentration of half the MIC, was effective at reducing plasmid copy number, while nalidixic acid, used at a similar concentration, had a considerably reduced effect. Up to 96 samples, e.g. taken at different time intervals with varying concentrations of antibiotic, can be screened simultaneously. The method can be used to investigate plasmid curing and copy numbers in any system for which a suitable DNA probe is available.

A new method of measuring opsonophagocytosis using luminescent bacteria

H. Kenny, C. A. Webster, R. C. B. Slack and J. P. Arbuthnott

Department of Microbiology and PHLS Laboratory, Queen's Medical Centre, Nottingham NG7 2UH

Conventional direct methods of assessing phagocytosis usually involve labour intensive and poorly reproducible methods of viable counting. We have used the recombinant *Escherichia coli* strain HB101 (pSB100) carrying the *lux* AB gene sequences cloned from *Vibrio harveyi** to compare luminescence with conventional plate counts and turbidimetry. Bacteria containing the *lux* AB gene emit light when long chain aldehydes, e.g., decanal are added. Human and casein-induced murine polymorphonuclear neutrophils (PMNL) were used with both immune and non-immune serum for opsonisation. The results of the calibrations showed a good correlation between luminescence and conventional methods. The reproducible lower limit of detection with a luminometer was 10^6 bacteria/ml; with a scintillation counter it was 10^2 bacteria/ml. The luminescence method allowed assessment of phagocytosis and intra-cellular killing within the time scale of the experiment whereas the conventional methods only measured intra-cellular killing and required

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THE RELATIONSHIP BETWEEN ANTIMICROBIAL PRESCRIBING PATTERNS AND THE RESISTANCE PROFILES IN GRAM NEGATIVE AEROBIC BACILLI WITHIN SPECIFIC UNITS OF A LARGE SCOTTISH TEACHING HOSPITAL.

J. Hood* and S.G.B. Amyes, Department of Bacteriology, The Royal Infirmary and University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, Scotland

The Royal Infirmary of Edinburgh is a 1000 bedded teaching hospital containing most regional and supraregional specialities. The study consisted of 5890 clinical isolates of, non-pseudomonal, gram negative aerobes. They were collected over a 21 month period in 1986-1987. Their resistance to cefuroxime was estimated by the Stoke's method. Overall resistance to cefuroxime was found to be 12.3%. The resistance to cefuroxime was related to specific ward areas. General medical : 7-9%; Haematology : 12%; General surgery : 13% (range 5.5 -> 19.8%); Urology : 16%; Orthopaedic surgery : 10.4%; Cardiothoracic surgery : 33%; General intensive care area : 32%; Head and spinal injury unit : 25.7%; Acute renal dialysis unit : 17.6%; Special care baby unit : 13.1%. The incidence of cefuroxime resistance was related to the overall use of beta-lactam drugs in a given area; the highest resistance was found in areas where cefuroxime was the main treatment/prophylactic agent. The lowest incidence was found in areas where aminoglycosides were the main treatment/prophylactic agent. Resistance was also much greater in the intensive care areas. A clearly resistant subgroup of these bacteria with MIC > 32 mg/l cefuroxime was studied further. The predominant species involved were Enterobacter (45%) and Acinetobacter (22%). MIC_{90}'s (in mg/L) were: ceftazidime = 8; cefotaxime = 64; aztreonam = 64; imipenem = 4; gentamicin = 32; netilmicin = 8; ciprofloxacin = 0.5. Amongst the cefuroxime resistant strains a slightly higher proportion were resistant to aztreonam than ceftazidime and almost the same proportion were resistant to cefotaxime but there was generally a lower proportion of resistance to the aminoglycosides.

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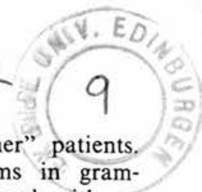

HOSPITAL LEGIONNAIRES' DISEASE: VIRULENCE AMONG STRAINS OF *LEGIONELLA PNEUMOPHILA* SEROGROUP 1

A. Colville, R. C. B. Slack and J. L. Crowley

Public Health Laboratory, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH

An outbreak of Legionnaires' disease occurred in a large teaching hospital during 1988–89. The hospital has four interconnected blocks, each with its own domestic hot water system supplied by a common main. Three of the blocks house ward areas. After an extensive case finding exercise, 12 episodes of Legionnaires' disease were identified, which may have been acquired nosocomially. All these cases were associated with one block, but several wards on different levels were implicated. *Legionella pneumophila* was isolated from one patient and from several points in the domestic hot water supply in two of the blocks. The clinical and environmental isolates were typed by monoclonal antibody and restriction fragment length polymorphism (RFLP) techniques. The only strain isolated from the block where cases occurred was Benidorm, RFLP type 14, which was identical to the clinical isolate. In other areas, in which susceptible patients were cared for, several different strains of *L. pneumophila* serogroup 1 were isolated, but not the Benidorm RFLP type 14. In addition, *L. pneumophila* serogroup 1 was found in the hospital during the earlier PHLS survey of hospital and hotel water supplies, yet no nosocomial cases were identified until 1988. Epidemiological evidence suggests that the outbreak was due to a single strain. As this was not identified in the first survey, it is suggested that nosocomial disease may have occurred only when a "virulent" strain of *L. pneumophila* colonised the domestic hot water system in one block of this large building. Further investigation of possible virulence mechanisms is in progress.

QUANTITATION OF BACTERIA IN PATIENTS WITH POSITIVE BLOOD CULTURES

P. S. Rice and R. C. Spencer

Department of Bacteriology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF

A 3-year review of blood cultures showed that 122 and 279 clinically significant positive peripheral blood cultures were received from patients with and without ("others") haematological malignancy respectively. Using a pour plate containing 2 ml of blood, colonies were counted and stratified into five groups—0, 1–10, 11–100, >100 but <1000, and >1000 colonies per plate. In "other" patients colony counts per plate were: 0, 60·9%, 1–10, 21·8%; 11–100, 10·5%; >100<1000, 3·9%; and >1000, 2·9%. By comparison, counts in haematology patients were: 0, 37·7%; 1–10, 25·4%; 11–100, 21·3%; >100<1000, 6·6% and >1000, 9%. When only gram-negative septicaemias are examined, colony counts >100 were found in 7·4% of haematology patients compared with 0·72% in "other" patients. Mean numbers of circulating organisms in gram-negative septicaemia show a similar trend with an average of 8 organisms/ml in "others" compared with 49 organisms/ml in haematology patients. Thus the bacterial load appears to be greater in haematology patients and the difference is amplified in gram-negative sepsis. This may, in part, be due to the role of endotoxin in the stimulation of neutrophil activity.

PASTEURELLA MULTOCIDA MENINGITIS IN INFANCY—A RARE AND PREVENTABLE DISEASE

B. I. Duerden and C. A. MacKenzie*

*Departments of Microbiology and *Paediatrics, Children's Hospital, Western Bank, Sheffield*

Pasteurella multocida, a commensal of the mouth and upper respiratory tract of dogs and other animals, causes infected animal bites; systemic infection is uncommon in man. *P. multocida* meningitis is very rare, but may occur in infants in close contact with animals. A 3-week-old girl developed signs of meningitis and septicaemia. CSF examination showed 140 white cells/cm^3 (95% polymorphs), glucose 2·9 mmol, protein 3·2 g/L; organisms were not seen by microscopy. A gram-negative cocco-bacillus with bipolar staining was isolated; initially thought to be *Haemophilus influenzae*, it was identified 24 h later as *P. multocida*. The same organism was isolated from blood cultures. Agglutinating antibodies were demonstrated in convalescent serum samples but not in a sample taken on admission. Initial treatment with ceftazidime was effective for 72 h; benzyl penicillin was then added. The baby made a good recovery and left hospital after 2 weeks. Close contact with two dogs at home was confirmed. The dogs were encouraged to "get to know" the baby (and *vice versa*) and had been known to lick her, including her face. The presumed route of infection was either the nasal or upper respiratory tract mucosa, or the conjunctiva. Such close contact between neonates or infants and animals should be avoided.

LARGE OR SMALL? THE MOLECULAR MASS OF THE CHROMOSOMAL β-LACTAMASE OF *ACINETOBACTER BAUMANI* ESTIMATED BY FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC^R)

J. Hood and S. G. B. Amyes*

*University Department of Bacteriology, Royal Infirmary, Glasgow G4 0SF and *Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG*

The combination of FPLC^R for separation and nitrocephin for detection is the most powerful tool yet devised for the purification of β-lactamases. Employing this method, we purified crude enzyme derived from an ACE-1 producing strain (H63) of *Acinetobacter bauman*, which had a molecular mass (M_r) previously estimated as 580 × 10^3 by conventional gel filtration on Sephacryl-300. Three

peaks (one major and two minor) were found after FPLC[R] with a strong cation exchanger (MonoS). These peaks were then applied, employing FPLC[R], on to a previously calibrated gel filtration column (Superose 12). The major peak of activity eluted with an M_r of about $(11–12) \times 10^3$. This was then concentrated and applied to a sodium dodecyl sulphate-polyacrylamide electrophoresis gel (stained with silver) on a PhastSystem[R]. A major band was found corresponding to an M_r of about 14×10^3. The other minor peaks of activity on the ion exchange step produced peaks corresponding to M_rs of 500×10^3 and $(35–40) \times 10^3$. These results show that this enzyme, through apparently large on conventional gel filtration, actually exists in several ionic forms with a subunit size of about $(10–15) \times 10^3$. These β-lactamases are clearly novel and may well be an evolutionary intermediate between penicillin binding proteins and conventional β-lactamases.

A METHOD FOR TYPING *NEISSERIA MENINGITIDIS* BY ANALYSIS OF RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

D. M. Jones, A. J. Fox, S. J. Gray and N. S. Saunders*

*Public Health Laboratory, Withington Hospital, Manchester M20 8LR and *Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT*

A typing technique for *Neisseria meningitidis* which employs restriction fragment length polymorphisms (RFLPs) has been developed. A library of *N. meningitidis* *Eco*R1 fragments in the vector λgtWES. λB was prepared and the recombinant λphage DNAs from a number of clones were assessed for use as probes. One such probe (λNM3) was found to give excellent discrimination between unrelated strains of *N. meningitidis*. Southern blots of meningococcal DNA digested with the restriction endonuclease *Ava*I revealed 1–10 bands following hybridisation of biotinylated λNM3 and subsequent detection with a streptavidin-alkaline phosphatase conjugate. Preliminary results demonstrated a high degree of typability and reproducibility (*c.* 100%). When applied to epidemiologically unrelated strains the method has a high numerical index of discrimination (0·92) which is of the same order as phenotyping (0·94) and isoenzyme analysis (0·95) of the same strains. Repeated isolates from long term carriers and isolates from multiple sites of single individuals give identical typing patterns. Strains of the phenotype B15P1.16, regarded as clonally related, show some limited heterogeneity and the different typing patterns are constant in strains from different parts of the country. The method may throw some new light on the epidemiology of meningococcal infection.

POLYPEPTIDE COMPOSITION OF FLAGELLA OF *HELICOBACTER PYLORI*

C. J. Luke, E. Kubiak,* A. Cockayne, T. S. J. Elliot* and C. W. Penn

*School of Biological Sciences, University of Birmingham, Birmingham B15 2TT and *Department of Clinical Microbiology, Queen Elizabeth Hospital, Birmingham B15 2TH*

Flagella of *Helicobacter pylori* were removed from intact organisms by shearing and collected by differential centrifugation. The isolated flagella were treated with detergent, and the polypeptide composition of native and detergent-treated flagella was compared by electronmicroscopy and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). While native flagella were covered by a sheath of membranous material, detergent-treated flagella appeared devoid of this covering. By SDS-PAGE, the detergent-treated flagella contained a single major polypeptide of 54 Kda while native flagella contained this polypeptide as well as others of 26, 30 and 66 Kda. The latter are thus putative components of the flagellar sheath, and if, as has been postulated, there is a relationship between the sheath, a membranous structure, and the outer membrane, these proteins may also represent possible outer membrane proteins. Immunogold electronmicroscopy with a polyclonal mouse antiserum, monospecific to the 54 Kda polypeptide by Western blot, showed that detergent-treated, but not native flagella were reactive with the antibody, thus confirming the location of the 54 Kda polypeptide on the flagellar core and the absence of exposure of this antigen on native flagella.

A NOVEL METHOD TO PREVENT CATHETER-ASSOCIATED INFECTIONS

T. S. J. Elliot, J. Holford, P. Sisson* and P. Byrne*

*Department of Clinical Microbiology, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH and *Department of Microbiology and Public Health Laboratory, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE*

Intravenous catheter-associated infections continue to cause clinical problems, ranging from localised phlebitis to bacteraemia and septicaemia. In an attempt to overcome these infections, we have used a novel approach which has involved electrical charge. Bacteria have an overall negative electrical charge. In order to repel microorganisms, catheters have been developed with a modified negative charge. The electrical charge with certain physical properties has been produced by a small generating unit. A suitable catheter material which will conduct electricity has also been developed. A model test system was subsequently designed to evaluate several different electrical charges under variable conditions. The electrical system has now been refined to produce maximum bacterial repulsion. The development of the test system, together with the electrical fields, has now resulted in a device suitable for clinical trials. This novel approach to catheter-associated infections, including the preliminary in-vitro findings, were presented.

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CEFUROXIME AND GENTAMICIN RESISTANCE IN GRAM NEGATIVE AEROBIC BACILLI ISOLATED FROM 18 UNITS WITHIN A LARGE SCOTTISH TEACHING HOSPITAL.

J.HOOD[1], A.B.HARRIS[2] AND S.G.B.AMYES[2]. University Department of Bacteriology[1], Glasgow Royal Infirmary, Glasgow, G4 OSF and Department of Medical Microbiology[2], University of Edinburgh, EH8 9AG, SCOTLAND.

The study investigated the resistance patterns of gentamicin and cefuroxime within non-pseudomonal Gram negative bacilli isolated in 18 distinct units of a large teaching hospital, over a 5 year period(1986-1991). 13,000 (non-duplicate) isolates had their sensitivities estimated by the Stokes Method. Over a 12 month period (1989-1990) all antimicrobial use within these areas was calculated and related to the throughput of patients. Resistance was then related to actual antimicrobial usage. Cefuroxime resistance varied from unit to unit. Surgery, overall:12.8% (range:4.6% in gynaecology to 23.7% in vascular). Medicine, overall:11.8% (range:6.8% in general medicine to 20.5% in CAPD/renal).The ITUs had a significantly greater cefuroxime resistance, overall:33% (range:18.8% in special care baby unit to 42.7% in the general ITU). Gentamicin resistance was much lower. Surgery, overall:1.7% (range:0.7% in gynaecology to 4% in urology). Medicine,overall:2.6%(range:1% in general medicine to 4.7% in CAPD/renal). ITU,overall:4%(range:2.3% in neurosurgery to 6.6% in the renal ITU). Conclusions: cefuroxime resistance is much higher than gentamicin resistance; there is considerable variation in resistance from unit to unit;cefuroxime resistance is increasing whereas gentamicin resistance appears stable; cefuroxime resistance is not simply related to beta-lactam use but also to the nature of the patient's underlying condition.

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CHARACTERIZATION OF AMINOGLYCOSIDE RESISTANCE IN ITALY

G.Molinari, G.C.Schito, R.H.Hare, G.H.Miller. Institute of Microbiology, University of Genoa, Italy. Schering-Plough Research, Bloomfield, NJ, USA.

A total of 286 aminoglycoside-resistant gram-negative bacilli were collected in 9 institutions evenly dispersed in Italy. The pathogens analyzed included: 66 Serratia spp., 41 Enterobacter spp., 35 P.mirabilis, 34 Klebsiella spp., 33 E.coli, 28 P.aeruginosa and 49 organisms belonging to other species. Of these strains, 87% were resistant to tobramycin and gentamicin, 78% to netilmicin, 27% to amikacin and 18% to isepamicin. The aminoglycoside resistance patterns (AGRPs) were determined by disk testing and DNA hybridization. The most common enzymes found were AAC(3)-V (65%), AAC(6')-I (30%) and ANT(2") (14%). Permeability barriers were detected in 10% of the strains. Combinations of resistance mechanisms were present in 81 organisms (28%). The most frequent AGRP in Serratia was AAC(3)-V (29%), followed by AAC(6')-I alone or combined either with AAC(3)-I or ANT(2") or both (23%). The association AAC(3)-V + AAC(6')-I was found in 21% of the Serratia and, in combination with two more enzymes, AAC(3)-I + ANT(2"), in 12 strains (18%). Permeability accounted for 36% of E.coli resistance mechanisms while 18% of the strains contained AAC(3)-V and another 18% possessed ANT(2"). In the remaining Enterobacteriaceae, AAC(3)-V alone (64%) or combined with AAC(6')-I (10%) was the prevalent pattern of resistance. Five strains produced AAC(3)-IV, a rare enzyme. Of the Pseudomonas 29% had AAC(6')-II, 21% AAC(6')-I and 11% ANT(2") or AAC(3)-Ia. Like in other southern European studies, ACC(3)-V predominated in all institutions and the prevalence of this mechanism might explain the higher resistance rates found for netilmicin in the italian strains.

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CLINICAL IMPACT OF INCREASING ISOLATION RATE AND MULTIRESISTANCE OF KLEBSIELLA SPP: THE GREEK EXPERIENCE

M.Lelekis*, S.Evangelou, A.Strouza, D.Petropoulou, P.Tsouroulas, P.Gargalianos, E.Karakatsani, J.Kosmidis.
First Dept of Medicine and Dept of Microbiology, The General Hospital of Athens, Athens, Greece

Due to a recent increase in the isolation rate of Klebsiella spp and the rates of their resistance to newer antibiotics in our hospital, we performed a prospective study of the clinical importance of these organisms, involving all patients(pts) from whom one of these organisms had been isolated since January 1,1991. In 29 of the first 38 pts(34 K.pneumoniae, 4 K.oxytoca) the bacterium was isolated from urine and in 9 from bronchial secretions. In 27 pts the pathogen was considered responsible for a true infection(15 hospital-acquired, 12 community-acquired). Empiric treatment was given to all these 27 pts and this was later proven inappropriate in 5 cases. 19 out of 22 with appropriate but only 1 out of 5 with inappropriate treatment were cured. 3 pts with UTI died despite treatment: 1 with appropriate treatment for a multiresistant hospital-acquired strain and 2 with inappropriate treatment(1 sensitive, 1 multiresistant strain, both community-acquired).

In conclusion, increase in both isolation rate of Klebsiellae and their resistance percentages was responsible for a high treatment failure due to inappropriate empiric therapy.

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CONTRIBUTION OF BETA-LACTAMASES TO RESISTANCE IN XANTHOMONAS MALTOPHILIA.

M. Akova*, G. Bonfiglio, D. M. Livermore. Hacettepe University School of Medicine, Ankara, Turkey, and London Hospital Medical College, London, UK.

X.maltophilia produces two inducible beta-lactamases, L1 and L2, and resists beta-lactams, including carbapenems. L1 is a zinc-metalloenzyme with carbapenemase activity, L2 is an unusual cephalosporinase. We derived mutants with high-(stably derepressed) and low-level constitutive (basal) beta-lactamase expression from 3 reference strains. With a single exception the mutants had altered expression of both enzymes, indicating shared regulatory components. The exception was a mutant that became basal for L1 enzyme but remained inducible for L2. A parent strain with low inducibility was more susceptible to penicillins, cephalosporins and carbapenems than those where higher enzyme levels were inducible. Mutational beta-lactamase derepression increased resistance to ureidopenicillins and newer cephalosporins. Basal mutants were more susceptible than inducible strains to these drugs, indicating that inducible expression was also partly protective. Inducible and derepressed organisms were similarly resistant to meropenem and imipenem, but basal mutants were more susceptible to meropenem than imipenem. MICs of meropenem, penicillins and cephalosporins, but not imipenem, were greater on Mueller-Hinton than on Isosensitest and Diagnostic Sensitivity Test agars. This behavior was independent of beta-lactamase inducibility, and may reflect permeability differences between cells grown in different media.

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DECREASED SUSCEPTIBILITY OF PENICILLIN-RESISTANT PNEUMOCOCCI TO BETALACTAM ANTIBIOTICS.

J. Liñares, T. Alonso, JL Pérez, J. Ayats, A. Domínguez, R. Pallarés, R. Martín. Hospital de Bellvitge, Universidad de Barcelona, Spain.

During a 12-year period (1979-90), 1492 pneumococci recovered from clinical sources were tested for antibiotic susceptibility; 270 (18%) of them showed intermediate penicillin resistance (IPR) and 215 (14.4%) were highly resistant (R) to penicillin. Multiple resistance (≥3 antibiotics) was observed in 73 % of these IPR and R strains.
To compare the *in vitro* activity of 16 betalactam antibiotics, we tested three groups of pneumococci by agar dilution method: 100 penicillin susceptible (S), 100 IPR, and 100 R strains.
Minimal inhibitory concentrations, µg/ml, (MICs-90 of S/IPR/R), from greater to lesser active antimicrobial, were as follows: imipenem (0.01/0.25/0.5), meropenem (0.01/0.5/1), cefotaxime (0.01/0.5/1), ceftriaxone (0.01/0.5/1), cefpirome (0.01/0.5/1), penicillin (0.03/1/4), ampicillin (0.03/1/8), cefuroxime (0.06/2/16), cephalotin (0.25/8/32), cefamandole (0.25/8/32), cefixime (0.5/16/32), ceftizoxime (0.5/16/32) cefaclor (0.5/32/64), ceftazidime (0.5/32/64), cefoxitin (1/32/64), and cefonicid (1/32/64).
Our results show that IPR and R pneumococcal strains had decreased sensitivity to other betalactam agents. However, some of these agents such as imipenem, meropenem, cefotaxime, ceftriaxone, and cefpirome, with MICs 2 to 8 times lower than penicillin, could be useful for the treatment of infections due to resistant strains. The remaining cephalosporins should not be used in empirical treatment of pneumococcal infections in our area, where 50 % of clinical isolates were resistant to penicillin in 1990.

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EVALUATION OF A RAPID METHOD AND AGAR DIFFUSION FOR DETECTION OF BETALACTAM RESISTANCE PHENOTYPES.

T.Fosse[1], F.Daoulas[2], et ML.Garrigues[3]. Hôpital Pasteur-Nice(1), Hôpital Necker-Paris(2), et Diagnostics Pasteur(3).

A rapid method in liquid medium, SIR *ENTEROBACTERIACEAE* 4H, was compared to agar diffusion (AD) on 409 wild strains; discrepant results were analysed first by comparing data obtained in the two sites of evaluation: inter-center variability was found dependent on careful control of inoculum density and incubation time. Discrepant strains have been tested again by determining MIC for ampicillin, amoxicillin/clavulanic acid, piperacillin, cephalothin, cefuroxim and cefotaxim. Results of both methods were then confronted to MIC and to each type of betalactamase, which was deducted from betalactam resistance phenotype. Both method have been tested also with reference strains (Collection 1 and 2, Unity of Antibacterial Agents, Institut Pasteur), and analysed in the same way.
Global correlation with MIC resulted in 71.1 and 72.2 % concordance for AD and SIR 4H respectively. Discordances expressed as minor (24.4% and 20%), major (2.2 and 2.2), and very major (2.2 and 5.5%) were also closely related but were dependent essentially on different phenotypes: compared to AD, SIR 4H detected most high level penicillinases, but could hardly identify some inducible cefalosporinases. Very major errors with cefuroxim observed in this case could be corrected with cephalothin results: this inter-antibiotic variability reflected variable expression of resistance mechanisms.
We conclude that as for diffusion, SIR *ENTEROBACTERIACEAE* 4H results have to be interpreted, and confronted with identification, especially for inducible resistance detection.