THE EFFECT OF GROWTH CONDITIONS ON β-LACTAM RESISTANCE IN *ENTEROBACTER CLOACAE*

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The effect of growth conditions on β-lactam resistance in *Enterobacter cloacae*

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The nutritional requirements of *Enterobacter cloacae* E7 (a clinical isolate) were quantitatively defined using a chemically defined medium (CDM). *E.cloacae* strains E12, E13, E14 and E16 (also clinical isolates) grew in this medium at similar growth rates to E7.

Antibiotic sensitivity patterns were determined for these five strains of *E.cloacae*. Bacteria grown in Iso-sensitest broth, urine and serum were generally 2-8 fold less sensitive than those grown in nutrient deprived CDM.

The effect of growth medium and rate on β-lactamase production and on induction of β-lactamase by cefotaxime was examined. Growth medium and rate had no significant effect upon β-lactamase production in the absence of β-lactam antibiotics. However growth rate profoundly affected β-lactamase induction by cefotaxime. Faster growing bacteria produced greater levels of β-lactamase activity in significantly less time than slower growing bacteria.

Expression of porin proteins and iron regulated membrane proteins (IRMPs), in different media, was subject to variation both within and between strains of *E.cloacae*. However there was no correlation between expression of these outer membrane proteins and growth medium, nor was there any correlation between expression of porin proteins and antibiotic susceptibility. Growth in iron-deprived CDM, urine and serum and growth in phosphate deprived CDM (CDM-P) induced the expression of IRMPs in *E.cloacae*.

Penicillin-binding proteins (PBPs) 5 and 6 of *E.cloacae* were sensitive to changes in the growth medium, particularly in serum and CDM-P. Further investigations are required to determine the effect of growth medium on the higher molecular weight PBPs.

Key words: *Enterobacter cloacae*, growth environment, β-lactamase, porin, penicillin-binding protein.

To my Mam and Dad

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ABBREVIATIONS

APS	ammonium persulphate
BIS	N,N'-methylene-bis acrylamide
cAMP	cyclic adenosine monophosphate
CDM	chemically defined medium
CDM+Fe	iron-replete CDM
CDM-Fe	iron-deprived CDM
CDM-K	potassium-deprived CDM
CDM-Mg	magnesium-deprived CDM
CDM-P	phosphate-deprived CDM
CF	cystic fibrosis
CFU	colony forming unit
cm	centimetre
CTX	cefotaxime
CXM	cefuroxime
°C	degrees centigrade
d	daltons
DMSO	dimethyl sulphoxide
DNA	deoxy-ribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
FOX	cefoxitin
g	gramme
h	hour
Hu	human serum
Hr	horse serum
IEF	isoelectric focusing
IRMP	iron-regulated membrane protein
Kd	kilodaltons
l	litre
M	moles per litre
MEZ	mezlocillin
µg	microgramme
mg	milligramme
MIC	minimum inhibitory concentration
min	minute
µl	microlitre
ml	millilitre
mm	millimetre
MOPs	3-(N-morpholino)propane sulphonic acid
Mr	molecular weight
NCTC	National Collection of Type Cultures
nm	nanometre
OD	optical density
OD_{420}	optical density at 420nm
OM	outer membrane
OMP	outer membrane protein
PAGE	polyacrylamide-gel electrophoresis
PBP	penicillin-binding protein
pI	isoelectric point
psi	pounds per square inch
R_f	retardation factor
s	seconds
SDS	sodium-dodecyl sulphate
TEMED	N,N,N',N-tetramethylethylene diamine
t_d	doubling time
TRIS	tris(hydroxymethyl)aminoethane
$t_{1/2}$	half time

UR	normal pooled urine
UR+Fe	iron-supplemented normal pooled urine
UTI	urinary tract infection
v/v	volume per volume
w/v	weight per volume

1. INTRODUCTION

1.1. Introductory notes.

The development of new antibiotics is a dynamic process, constantly being challenged by the emergence of new mechanisms of resistance in bacteria. An important step forward in the development of β-lactam antibiotics was the introduction of the "β-lactamase stable" second generation cephalosporins in the mid-1970's (Neu 1974), closely followed by the expanded spectrum third generation " β-lactamase stable" cephalosporins in the early 1980's (Jones and Thornsberry 1982, Neu and Labthavikul 1982a, O'Callaghan 1979, Richards *et al* 1984). These newer cephalosporins improved the possibility of effectively treating infections caused by many of the non-fastidious Gram-negative bacilli: species such as *Enterobacter*, *Citrobacter*, *Serratia*, indole positive *Proteus*, *Providencia*, *Acinetobacter*, *Morganella* and *Pseudomonas* previously not included in the antibacterial spectra of the β-lactam antibiotics. Shortly after the introduction of the second generation cephalosporins, however, reports of emergence of resistance began to appear associated with therapeutic failure or relapse of infection (Beckwith and Jahre 1980, Levine and M^{C}Cain 1978, Olson *et al* 1983, Perkins *et al* 1978, Sanders *et al* 1982a).

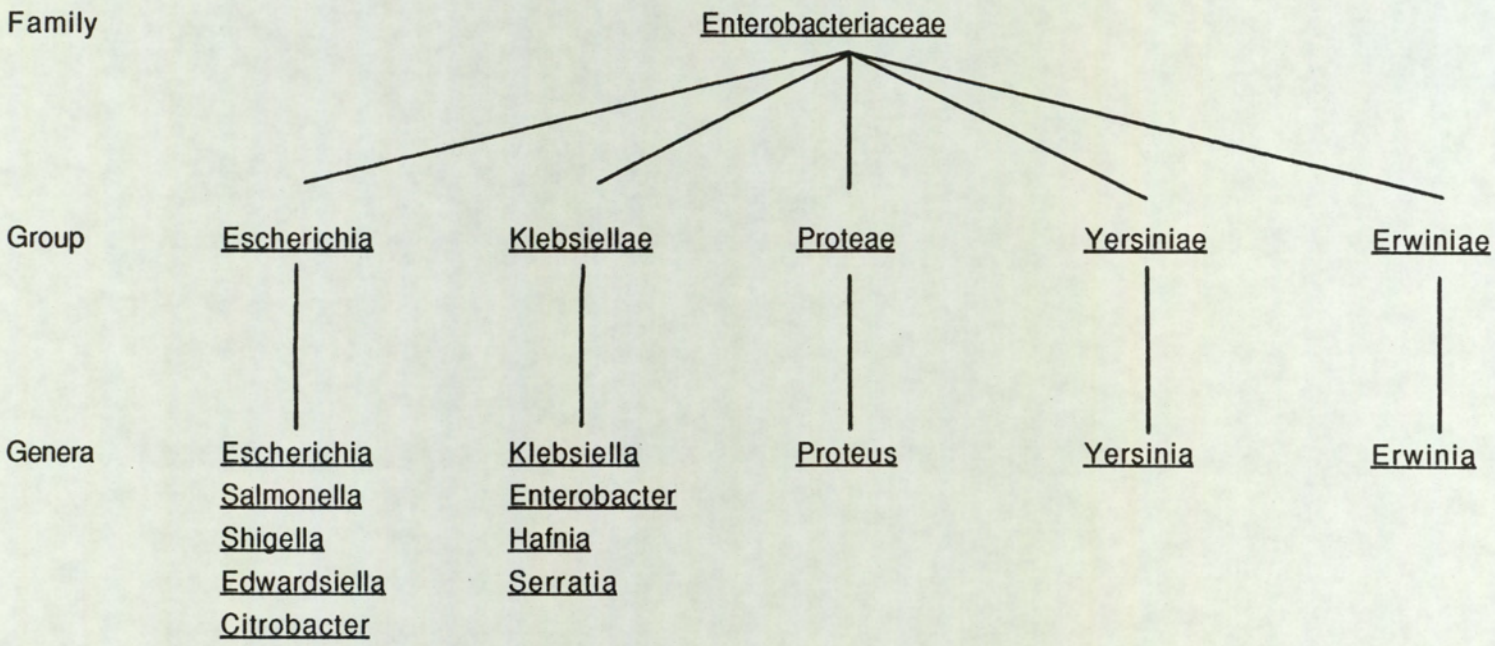
It is the great adaptability of bacteria to their environment which enables their survival and consequently contributes to the emergence of resistance to newer antibiotics. The resistance mechanisms to β-lactam antibiotics operate in the Gram-negative envelope - the prime

structure through which the bacterium interacts with its environment. There are three possible mechanisms of resistance: reduced penetration of the drug to the target enzymes (through porin channels), mutation of the target enzymes (penicillin-binding proteins; PBPs) and enzymatic inactivation of the β-lactam antibiotic. Specific nutrient deprivation and growth rate influence the Gram-negative envelope (Brown and Williams 1985a,b) and also extracellular enzyme production (Ombaka et al 1983). The purpose of this study is to examine the effect of these growth conditions upon porin, PBP and β-lactamase production in E.cloacae and to correlate the importance of these effects on the development of β-lactam resistance.

1.2. Nomenclature.

E.cloacae is a motile Gram-negative bacillus belonging to the genus Enterobacter, which with the genera Klebsiella, Serratia and Hafnia form the group Klebsiellae. This group is part of the Enterobacteriaceae family (see Figure 1). The genus Enterobacter, formerly called Cloaca and Aerobacter (Edmondson and Sanford 1967), is composed of a number of species including: E.cloacae, E.aerogenes, E.agglomerans, E.gergoviae, E.sakasakii, E.amnigenus, E.intermedium and E.taylorae many of which have only recently been identified (Brenner et al 1980, Farmer et al 1980, 1985a,b). From a clinical viewpoint the two most important species of this genus are E.cloacae and E.aerogenes (John et al 1982).

FIGURE 1. The Enterobacteriaceae family



Data from (Taussig 1984)

1.3. Occurrence of E.cloacae in infection.

Patients infected with E.cloacae are generally elderly (or extremely young) and immunocompromised by major illness and/or surgery. Such patients have multiple risk factors which may include: compromised host defences, respiratory therapy support, vascular access devices, urinary tract abnormality or catheterisation, prolonged hospital stay and heavy exposure to antibiotics (Follath et al 1987, John et al 1982, Sanders et al 1982a, Steinhauer et al 1966). Reports of patients infected with E.cloacae have included: cardiac surgery patients (Olson et al 1983, Weinstein 1985, 1986), patients with haematological disorders (Follath et al 1987), burns patients (John et al 1982, Quinn et al 1987), patients in neonatal intensive care units (Anderson and Heiber 1983, Bryan et al 1985, Jarvis et al 1983) and motor accident patients (Sanders et al 1982a). Therefore the infections caused by E.cloacae are generally secondary to the major cause of illness. Nevertheless these secondary infections become important to the clinical illness and may be the cause of death in such patients (Follath et al 1987). The infections most commonly reported include: pneumonia, septicaemia, wound infections and urinary tract infections (Follath et al 1987, John et al 1982, Sanders et al 1982a, Weinstein 1986).

E.cloacae may be endemic in certain hospital environments, for example, intensive care units and burn centres, where patients are highly susceptible to infections caused by this organism. However E.cloacae is not endemic in every hospital: where it is endemic, there is usually an

association of heavy use of the newer cephalosporins (Sanders and Sanders 1985). This point is well illustrated by a recent outbreak of infections due to cefotaxime resistant E.cloacae in a neonatal intensive care unit. Ten weeks previous to this outbreak cefotaxime was substituted for gentamicin therapy in suspected sepsis. The outbreak was duly stopped by a return to gentamicin therapy (Bryan et al 1985). The use of the newer cephalosporins in therapy (including prophylactic use) may be closely followed (within a few days) by colonization of resistant isolates of E.cloacae or related organisms, in the upper respiratory tract, faeces or urine (Follath et al 1987). The patients who are immunocompromised by therapy or disease may fail to handle an assault by these small resistant populations of bacteria selected by the newer cephalosporins. In some of these patients no recognizable clinical consequences will arise, whereas in others, the resistant organisms will enter the blood stream and are carried to the lung or other organs where they cause acute rapidly progressing infections or, less frequently, delayed localized infections (Follath et al 1987). It can be reasonably assumed that clinical problems or infection with resistant strains will arise less frequently in patients with normal host defence mechanisms.

The spread of E.cloacae infections in a hospital environment may occur by the hands of hospital personnel. In such instances where organisms are multiply resistant, barrier precautions are recommended, with frequent handwashing (Weinstein and Kabins 1981). A number of outbreaks of E.cloacae infections have occurred through the

contamination of a variety of medical products, for example: intravenous fluid (Maki et al 1976), ice (Newsom 1968), platelets (Buchholz et al 1971), pressure-monitoring devices (Weinstein et al 1976), lipid emulsion (Jarvis et al 1983) and haemodialysis (Buxton et al 1978). It is important in such cases to determine the reservoir of the infecting organism.

Studies to determine the incidence of E.cloacae in Gram-negative bacteraemia have been carried out both in Europe and the United States (Centers of Disease Control 1984; European Study Group on Antibiotic Resistance 1987; Haley et al 1985). In 1984 in Europe the proportion of Enterobacter spp causing Gram-negative septicaemia was 7%, the highest incidence being 10-15% in France, Greece and Italy. Similar figures were reported for the United States in a review by John et al (1982). The incidence of Gram-negative bacteraemia caused by E.cloacae was 4-12% at that time. The Study on the Efficacy of Nosocomial Infection Control (SENIC) project report in 1984 estimated that as many as four million nosocomial infections may be occurring per year in United States hospitals and nursing homes. Data from the 1983 National Nosocomial Infections Study (NNIS) show that Pseudomonas aeruginosa, Enterobacter spp and Serratia spp accounted for 19% of all nosocomial isolates in the participating hospitals. These organisms were responsible for 15.8% of the nosocomial bacteraemias encountered that year. A calculation by Sanders and Sanders (1987) on these data estimated that 760,000 patients per year have nosocomially acquired infections due to one of these three organisms and of these

31,600 could have bacteraemia. Although these are rough estimates, they serve to emphasize the point that E.cloacae may be frequently encountered as the aetiologic agent of nosocomially acquired infections.

1.4. Influence of the environment on the Gram-negative cell envelope.

The envelope of E.cloacae, like other Gram-negative bacteria, is a complex structure conferring shape and rigidity. Most data on the structure and biosynthesis of the envelope has been obtained from studies using Escherichia coli and Salmonella typhimurium, and is the subject of a number of comprehensive reviews (Beveridge 1981, Brown 1977, Costerton et al 1974, Hammond et al 1984, Lugtenberg and van Alphen 1983, Nikaido and Nakae 1979). Few studies have examined the structure of the envelope of E.cloacae in detail. However, data obtained from E.coli and S.typhimurium are usually applicable to most Enterobacteriaceae, including E.cloacae.

The Gram-negative envelope is the prime structure through which a bacterium interacts with its environment. Since the environment is constantly changing, the survival and pathogenicity of the bacterium will depend upon the adaptability of the envelope to these changes (Smith 1977). There is much evidence demonstrating that the composition and biological properties of the bacterial surface are largely determined by the growth environment (Brown and Williams 1985b, Ellwood and Tempest 1972, Holme 1972). In addition to

the well documented influences of pH, temperature and osmolarity, such phenotypic variations may be induced by: specific nutrient deprivation (Brown and Williams 1985b, Dalhoff 1985, Ellwood and Tempest 1972, Holme 1972), the rate of growth (Gilbert and Brown 1978,1980, Lodge *et al* 1986, Taylor 1984), growth in biofilms (Costerton *et al* 1981) and the presence of subinhibitory concentrations of antibiotics (Brown and Williams 1985b, Kadurugamuwa *et al* 1985a,b). These changes may in turn influence susceptibility to: antimicrobial agents (Boggis *et al* 1979, Brown 1977, Brown *et al* 1979, Brown and Williams 1985a, Finch and Brown 1975, Turnowsky *et al* 1983) and host defences (Brown and Williams 1985b).

Conditions *in vivo* are substantially different from *in vitro* culture conditions (Brown and Williams 1985a, b, Dalhoff 1985, Gilbert *et al* 1987). Thus it is not surprising that bacteria growing *in vivo* in an infection possess significantly different characteristics to those grown *in vitro* (Brown and Williams 1985b). However, although there is much information on the chemical and physical composition of the *in vivo* environment, apart from the immunological components, there is little information about which aspects are most critical in influencing the pathogen (Brown and Williams 1985b). Bacteria recovered directly from infections, without subculture, provide valuable information on their structure and physiology in these environments. This may lead to the design of *in vitro* culture conditions more relevant to the *in vivo* situation.

1.4.1. Specific nutrient deprivation.

Iron.

Iron is an essential element for almost all living cells, including bacteria (Neilands 1974). This mundane, but biologically precious metal, plays a fundamental role in a wide variety of biochemical processes in microorganisms (Griffiths 1987a, Neilands 1974). Although iron is an essential element, it is also potentially toxic, since its reactions involving oxygen may lead to free radical formation (Griffiths 1987a). In most biological fluids near pH7 and atmospheric oxygen tension, iron forms insoluble hydroxide complexes and hence is biologically unavailable (Aisen 1977). As a result of this property many iron-binding proteins have evolved *in vivo* to store and transport iron in a soluble form (Jacobs and Worwood 1974). In addition while iron is tightly bound to these proteins, it is unavailable for the reactions leading to free radical production (Griffiths 1987a). In the vertebrate host myoglobin, haemoglobin, haemosiderin and ferritin bind iron intracellularly, while the high affinity binding proteins transferrin and lactoferrin bind iron extracellularly (Bullen *et al* 1978). Transferrin and lactoferrin have association constants for iron in the region of $10^{36}M^{-1}$ and are normally only partly saturated (30-40%), therefore the amount of free iron in equilibrium with iron-binding proteins is approximately $10^{-18}M$ (Bullen 1981). Thus although there is normally an abundance of iron present in body fluids, the amount of free iron that is readily available to bacteria is far too small to sustain their

growth (Bullen 1981, Bullen *et al* 1978). The withholding of iron from pathogenic bacteria by the vertebrate host, referred to as "nutritional immunity" constitutes an important component of host defences (Bullen 1981, Finkelstein *et al* 1983, Griffiths 1983, Weinberg 1978, 1984). The host also further confronts the invading microorganism during infection by reducing the total amount of iron bound to serum transferrin (i.e." hypoferraemia of infection"), (Cartwright *et al* 1946). In response to *in vivo* iron deprivation many Gram-negative bacteria synthesize high-affinity iron-uptake systems to scavenge iron in the host. This is an essential component of their virulence factors and pathogenic properties. These systems consist of two components; low molecular weight iron-chelating agents (siderophores) and high molecular weight outer membrane protein (OMP) receptors (Bullen 1981, Finkelstein *et al* 1983, Griffiths 1983, Neilands 1982, Weinberg 1984). The siderophores produced by Gram-negative bacteria fall into two classes: phenolate- and hydroxamate-type siderophores. *Enterobacter* spp produce two siderophores which have been identified as enterochelin (phenolate-type) and aerobactin (hydroxamate-type) in *E.aerogenes* (Gibson and Magrath 1969, O'Brien and Gibson 1970). The same two siderophores are thought to be produced by *E.cloacae* (Krone *et al* 1985, van Tiel-Menkveld *et al* 1981). Both enterochelin and aerobactin are secreted into the growth medium under conditions of iron restriction (Gibson and Magrath 1969, Rogers 1973). Although enterochelin has a higher association constant for iron than aerobactin ($10^{52}M^{-1}$ compared to $10^{23}M^{-1}$ respectively) it

appears that conditions *in vivo* favour iron uptake through the aerobactin system (Griffiths 1987b). An important difference between the two systems is that aerobactin is recycled while enterochelin is only used once (O'Brien *et al* 1971, Braun *et al* 1984), other factors which may limit the effectiveness of the enterochelin system *in vivo* include serum binding (Konopka and Neilands 1984) and the presence of antibodies in serum directed against enterochelin (Moore *et al* 1980) and the ferric-enterochelin receptor (Griffiths *et al* 1985).

In addition to the aerobactin and enterochelin iron-uptake systems, certain members of the *Enterobacteriaceae* are capable of utilising hydroxamate siderophores, including ferrichrome and ferrioxamine B, produced by other organisms (Koninsky 1979, Neilands 1981). Citrate is another compound that can supply iron to certain strains of *E.coli*, but this system does not appear to be widely distributed among the *Enterobacteriaceae* (Griffiths 1987b).

The use of siderophores as the carriers for antibiotics has been attempted. These inhibitory compounds, sideromycins, are structural analogues of the hydroxamate-type siderophores (Zahner *et al* 1977). More recently a catechol-substituted cephalosporin (E-0702) has been described (Watanabe *et al* 1987). Thus the iron transport system may be exploited for antimicrobial therapy.

The receptors and enzymes induced in the outer membrane (OM) as part of the high-affinity iron-uptake system are involved in the uptake and release of iron from the iron-chelator complexes (Neilands 1982). Griffiths (1987b)

suggests that initial adsorption of the iron-loaded siderophore to the surface receptors is required to accumulate iron relative to the concentration in the growth medium. These receptors have apparent molecular weights in the region 70-90Kd and have been studied mostly in E.coli. E.coli K12 may express six species of iron-regulated membrane proteins (IRMPs): an 83Kd protein of unknown function, an 81Kd protein (FepA) which acts as a receptor for ferric-enterochelin, an 80.5Kd protein (FecC) which acts as a receptor for ferric-citrate, a 78Kd protein (FhuA) which acts as a receptor for ferrichrome, a 74Kd protein (IutA) which acts as a receptor for ferric-aerobactin, this protein comigrates with another 74Kd protein (Cir) whose function is unclear (Griffiths 1987b). Recently Chart et al (1988) have demonstrated considerable differences between the patterns of IRMPs exhibited by different strains of E.coli. These workers suggest the possibility of using these different patterns as well as the siderophores produced, as a new set of markers to characterise groups of pathogenic E.coli. In E.cloacae the IRMPs have not been studied in such detail, however an 85Kd protein has been found to fulfil the functions of the E.coli IutA 74Kd protein (Oudega et al 1979).

Evidence that the high-affinity iron-uptake systems exist in vivo was provided initially by Griffiths et al (1983). These workers recovered pathogenic E.coli directly, without subculture, from the peritonea of lethally infected guinea pigs. The E.coli recovered expressed IRMPs, in addition enterochelin along with its degradation products was found in the peritoneal washings. Subsequently the OMP

profiles of four strains of Vibrio cholerae infecting the intestinal tracts of infant rabbits were also found to express IRMPs (Sciortino and Finkelstein 1983). The first direct evidence that bacteria growing in human infections are iron-restricted was reported by Brown et al (1984). Pseudomonas aeruginosa was recovered, without subculture, from the sputum of a cystic fibrosis (CF) patient, the OMP profile of which revealed three IRMPs. Subsequently Lam et al (1984) reported that 5/12 strains of enteric bacteria isolated directly from urinary tract infections (UTIs) expressed two or more IRMPs. Similar results were obtained with Klebsiella pneumoniae and Proteus mirabilis isolated directly from UTIs by Shand et al (1985). Interestingly in K.pneumoniae three IRMPs were expressed in vivo, whereas in vitro, in iron-depleted media Klebsiella species have been shown to produce six IRMPs (Williams et al 1984a). It seems, therefore, that other factors besides iron may influence the regulation of these proteins in vivo. Chart et al (1986) have shown in vitro, that the expression of IRMPs may be influenced by the nature of the iron chelator used, it is possible that other ions play a role in the induction of IRMPs.

In addition to expressing IRMPs and producing siderophores, iron-deprivation causes other changes in bacteria as they adapt and grow under this condition. Under iron-stress, E.coli has been shown to produce a modified tRNA which may be involved in the control of enterochelin production (Griffiths and Humphreys 1976, 1978). Reduced activity of bacterial ribosomes has also been reported under

iron-stress (Iorio and Plocke 1981). Certain bacterial toxins, for example diphtheria toxin, are expressed optimally only during iron restricted growth (Crosa 1987). The hydrolytic enzymes of Pseudomonas are produced in optimal amounts only in a low iron environment (Bjorn et al 1978, 1979, Ombaka et al 1983).

Iron restriction may also affect the sensitivity of microorganisms to antimicrobial agents. In the case of both mucoid and non-mucoid strains of P.aeruginosa it is known that both growth rate and iron limitation profoundly affect the production of PBPs. This alters their sensitivity to the action of β-lactam antibiotics (Brown and Williams 1985b, Turnowsky et al 1983).

Nutrients other than iron.

In comparison to the data available on iron deprivation in vitro and in vivo, relatively little information is available on the deprivation of other nutrients. It is known that transferrins are capable of binding other metal cations besides iron in vitro (Valsberg and Flanagan 1983). It is possible that unsaturated transferrin molecules may be able to chelate cations in addition to iron in vivo, even though affinity constants for these cations are lower (Brown and Williams 1985b). Weinberg (1974) has reported that patients infected with Gram-negative organisms reduce the levels of phosphate in their plasma, to levels that are suboptimal for growth of bacteria. This situation is somewhat similar to the hypoferraemia of infection and was also suggested to occur with zinc (Weinberg 1984).

As with iron, under specific nutrient deprivation certain OMPs are induced, which may be involved in uptake mechanisms for that specific nutrient (Brown and Williams 1985a). Although the effect of specific nutrient deprivation other than iron has not yet been studied in E.cloacae, observations have been made with other enterobacteria. OMPs have been induced by carbon, nitrogen, phosphate, sulphate and potassium limitation in both E.coli and K.pneumoniae (Lugtenberg and van Alphen 1983, Overbeeke and Lugtenberg 1980, Robinson and Tempest 1973, Sterkenberg et al 1984). Those proteins whose uptake functions have been shown include a 36Kd protein, PhoE, of E.coli which is induced by phosphate limitation (Overbeeke and Lugtenberg 1980). This protein is a porin, which produces channels selective for negatively charged molecules, especially phosphorylated compounds (Lugtenberg and van Alphen 1983). In addition nitrogen limited E.coli and K.pneumoniae derepress proteins involved in their high affinity ammonium-uptake sytems. Induction of these nutrient uptake proteins may result in increased susceptibility to antibiotics (Ringrose 1983, Sterkenberg and Wouters 1981).

Apart from changes in the OMP profile, nutrient deprivation can affect other components of the cell wall. The phospholipid and fatty acid content may vary according to nutrient limitation and temperature (Cozens and Brown 1981, Günter et al 1975, Minnikin et al 1971a,b). Specific nutrient limitation causes phenotypic changes in lipopolysaccharide (LPS) content (Cochrane 1987, Dean et al 1977, Tempest and Ellwood 1969) and cation content(Kenward et al 1979).

Alterations of cation content of magnesium depleted bacteria have been associated with increased resistance to ethylenediaminetetra-acetic acid (EDTA), polymyxin and cold shock (Boggis et al 1979, Kenward et al 1979). Specific nutrient limitation affects exopolysaccharide production: phosphate, nitrogen and iron depleted P.aeruginosa produced more exopolysaccharide than others grown under different nutrient depletions (Ombaka et al 1983). Carbon limited E.coli produced K1 polysaccharide and a 46Kd OM polypeptide which was associated with increased serum resistance, compared to magnesium limited cells which produced less of these polymers (Taylor 1984).

It is evident that specific nutrient deprivation causes many and varied changes in envelope composition. Such changes in vivo may well affect an organism's ability to survive in an infection and could potentially affect its susceptibility to antimicrobial agents and host defences (Anwar et al 1983, Brown and Melling 1969, Cozens and Brown 1983, Finch and Brown 1975, 1978, Taylor et al 1981).

1.4.2. Growth rate.

The growth rate of an invading microorganism will contribute to the outcome of an infection, especially in the early stages of an infection when the population of bacteria is small. Therefore at least in the initial stages of infection, the ability to replicate rapidly in vivo may be an important virulence factor. The growth rate will be affected by the site of infection, nutrient availability and by the strength of the host defences. Therefore it is hardly surprising to

find a large variation of *in vivo* growth rates. In some cases initial growth rates measured *in vivo* have been similar to those attained *in vitro*. For example, *P.aeruginosa* has been shown to grow with doubling times of 30 minutes in the lung and 20 minutes in the peritoneal cavity (Morris Hooke *et al* 1985, Sordelli *et al* 1985). In other cases slow growth rates have been reported: doubling times of 6 hours in the alimentary tract (Meynell and Subbaiah 1963) and 5-10 hours in the spleen (Maw and Meynell 1968) have been reported for *S.typhimurium* in mice. In many infections doubling times have been shown to increase as the infection becomes established. For example, the growth rate of *E.coli* in a rat meningitis model was 1.27 hours, 3 hours post infection, yet 24 hours post infection had increased to 29 hours (Cozens *et al* 1986a). Generation times of *P.mirabilis* and *E.coli* were found to be 2.3 hours and 0.9 hours respectively during the first 7 hours following experimental kidney infection. Later the doubling times increased to 20 hours (28-48h post infection) (Eudy and Burrous 1973). Finally both fast- and slow-growing bacteria may be present in an infection: the examination of several histologic sections of consolidated lungs, after a pneumococcal pneumonia infection in rats, demonstrated an apparent gradient of growth rates, from rapid bacterial multiplication at the expanding edge of the infection to no growth in the central core (Wood and Smith 1956).

Continuous culture of bacteria *in vitro* allows specific growth rate to be controlled over a range of growth rates, keeping the physical and chemical environment of the cells constant (Gilbert 1985). Using this technique, growth rate

has been shown to affect many properties of the bacterial cell including: expression of OMPs (Lodge et al 1986), amount of phospholipid (Gilbert and Brown 1978) and LPS (Tempest and Ellwood 1969) present in the envelope, the structure and pattern of cell wall synthesizing enzymes (Buchanan and Sowell 1982, De La Rosa et al 1983, Driehuis and Wouters 1987, Mengin-Lecreulx and van Heijenoort 1985, Pisabarro et al 1985), production of extracellular enzymes (Ombaka et al 1983) and permeability of the cell envelope (Broxton and Brown 1984). As a result of these changes to envelope structure, the susceptibility of bacteria to antimicrobial agents and host defences is altered by growth rate (Dean et al 1977, Finch and Brown 1975, 1978, Gilbert and Brown 1978, 1980, Harakeh et al 1985, Taylor 1984).

It has been known, even from the earliest studies on penicillin, that β-lactam antibiotics are bactericidal for growing bacteria (Hobby and Dawson 1944, Hobby et al 1942). Any factor which interferes with cell growth, whether it is the lack of an essential nutrient for auxotrophic mutants (Davis 1949) or concomitant use of a bacteriostatic agent, for example chloramphenicol (Jawetz et al 1951) will interfere with the killing action of β-lactam antibiotics. It is therefore a logical conclusion that slow growth rates in vivo may lead to β-lactam antibiotic tolerance (Tuomanen 1986). Recent studies by Cozens et al (1986b) have evaluated the bactericidal activity of a number of β-lactam antibiotics on slowly growing bacteria cultured in a chemostat. A number of the newer third generation cephalosporins; ceftazidime, ceftriaxone and ceftizoxime, considered to be among the most

rapidly bactericidal agents of this generation, were found not to be bactericidal against slow-growing cells. However a number of compounds were found to retain bactericidal activity. For these compounds the rate of kill per generation was constant at all growth rates. In other words, slowly growing bacteria were killed proportionally more slowly, thus in terms of absolute time, there is a marked decrease in the rate of kill as growth rate decreases. This relationship between growth rate and kill was also found for other β-lactam antibiotics and E.coli (Tuomanen et al 1986). Tuomanen (1986) found that imipenem was particularly effective in killing non-growing bacteria.

Sensitivity of Gram-negative bacteria to a number of other antimicrobial agents including: 3- and 4-chlorophenol (Gilbert and Brown 1978), EDTA (Finch and Brown 1975), cycloserine (Sterkenberg and Wouters 1981), chlorhexidine and 2-phenoxyethanol (Gilbert and Brown 1980) and chlorine dioxide (Harakeh et al 1985) have also been found to decrease with decreasing growth rate. Finally slow growth rate may also decrease the susceptibility of bacteria to host defence mechanisms, for example serum killing and phagocytosis (Finch and Brown 1978, Taylor et al 1981). It is therefore apparent that slow-growing bacteria in vivo in an established infection might be very difficult to kill, due to a general increase in resistance to antimicrobial agents and host defences.

1.5. The β-lactam Antibiotics.

The β-lactam antibiotics play a major role in treatment of bacterial infection especially in patients whose immune systems are severely compromised. These antibiotics have two distinct advantages for the treatment of infections in such patients; firstly they are potentially bactericidal antibiotics and secondly they are relatively non-toxic (Tipper 1985). In fact the ability of a patient to tolerate β-lactam antibiotics is limited mostly by allergy and effects on normal flora, since their direct toxicity is very low (Brown and Martin 1981). The β-lactam antibiotics include a diverse group of compounds, whose single common feature is the β-lactam ring.

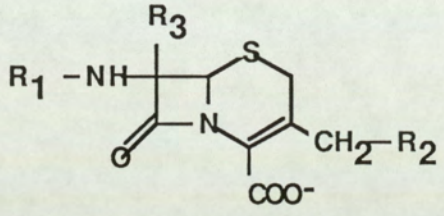
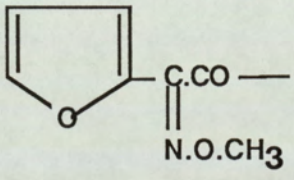
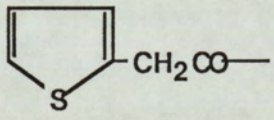
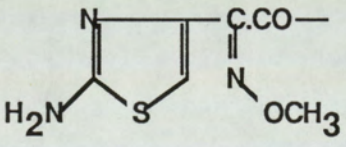
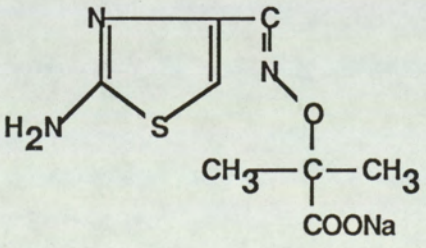
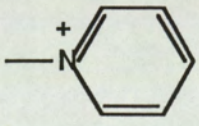
Major advances in the development and production of new β-lactam antibiotics have been influenced by the ability of bacteria to counteract the killing action of many agents. With the introduction of penicillin in the early 1940s, the first major obstacle proved to be the appearance in hospitals of β-lactamase-producing resistant strains of Staphylococcus aureus. This represented a serious clinical problem due to the failure of the penicillins then available, to cope with the infections caused by these organisms (Ridley et al 1970). This first challenge was met by the introduction of methicillin in 1960 (Rolinson et al 1960) and later by more effective semisynthetic penicillin derivatives which retained sufficient activity against S.aureus, while being resistant to the staphylococcal β-lactamase. Following this, the next major challenge was the appearance of hospital infections caused by Gram-negative bacteria, which possess a selectively

permeable OM along with the ability to produce β-lactamase (Nikaido 1985). Ampicillin was the first broad spectrum penicillin introduced which was active against a number of these Gram-negative organisms (Bear *et al* 1970). However β-lactamase determined resistance of *Enterobacteriaceae*, especially *E.coli*, *Salmonella*, *Klebsiella* and *Haemophilus* species, appeared very early due to the prevalence and ease of transmission of plasmids carrying transposons expressing class III β-lactamases. A number of non-fastidious Gram-negative organisms were also intrinsically resistant to ampicillin, for example *Pseudomonas*, *Proteus*, *Serratia* and *Enterobacter* species. In 1967, carbenicillin was introduced and was the first β-lactam to possess activity against these non-fastidious Gram-negative organisms (Hewitt and Winters 1973). Effort was then concentrated on the development of β-lactamase stable derivatives of these β-lactam antibiotics. In the 1970s there were many major developments including: the isolation of a new family of β-lactam antibiotics, the naturally occurring cephamycins (Nagarajan *et al* 1971). These antibiotics possess broad Gram-negative activity and high stability to inactivation by β-lactamases (Daoust *et al* 1973, Stapley *et al* 1972). A number of β-lactamase inhibitors were also isolated from naturally occurring sources including clavulanic acid (Howarth *et al* 1976) and olivanic acids (Brown *et al* 1977). These compounds are themselves poor antibiotics, but act synergistically when combined with β-lactamase susceptible β-lactam antibiotics such as ampicillin (see section 1.5.2). The development of many β-lactamase stable semisynthetic cephalosporins began with the second

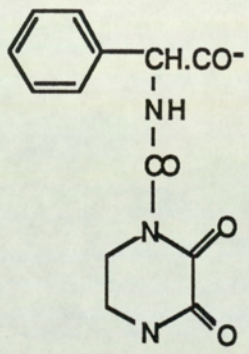
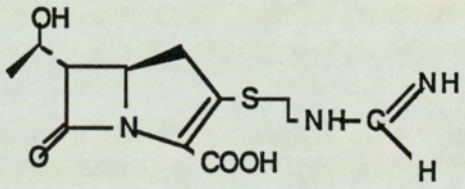
generation cephalosporins, for example cefuroxime (O'Callaghan et al 1976) and the semisynthetic cephamycins, for example cefoxitin (Onishi et al 1974). These were closely followed by the third generation cephalosporins, including the substituted aminothiazole oxime cephalosporins and oxacephems, which are potent broad spectrum antibiotics exhibiting a high stability to β-lactamases. These compounds include cefotaxime (Masuyoshi et al 1980), ceftazidime (O'Callaghan et al 1980) and moxalactam (Yoshida et al 1980). Table 1 shows the structures of some of the newer β-lactams.

The most recent mechanism of resistance to this generation of antibiotics is that afforded by the inducible chromosomal class I β-lactamase, as seen with E.cloacae and related species (Sanders and Sanders 1983). In this instance, clinical isolates become resistant to β-lactam antibiotics while the patient is receiving appropriate antibiotic therapy. A phenomenon that was rarely seen prior to the introduction of third generation cephalosporins (Sugarman and Pesanti 1980). This resistance is dependent on low rates of permeation of these antibiotics into the periplasmic space so that even slow rates of hydrolysis of the drug is sufficient to give protection to the organism (Nikaido 1985, see also section 1.6.1.). In addition mutations have occurred among the genes which encode the well established plasmid-determined β-lactamases, resulting in dissemination of novel transferable β-lactamases with an expanded spectrum of activity (Brun-Buisson et al 1987, Kliebe et al 1985, Sirot et al 1987). Concomitantly there has also been development of methicillin resistance in S.aureus, through PBP changes

TABLE 1. Structures of some third generation ß-lactam antibiotics.

Name (Class)	Structure		
Cephalosporins			
		R1	R2
Cefuroxime (2nd generation cephalosporin)			— O.CO.NH_2
Cefoxitin (Cephamycin)			— O.CO.NH_2
Cefotaxime (3rd generation cephalosporin)			— O.CO.CH_3
Ceftazidime (3rd generation cephalosporin)			

R_3 = -OCH_3 in cefoxitin and -H in other members. Table 1 is continued. overleaf

Name (Class)	structure
Piperacillin (ureidopenicillin)	
Imipenem (carbapenem)	

and so the continuous process of antibiotic development must go on to defeat the new mechanisms of resistance produced by bacteria. The mechanisms of resistance of Gram-negative bacteria to β-lactam antibiotics will be discussed in more detail in section 1.6.

1.5.1. Mode of action of β-lactam antibiotics.

Early work on the mode of action of penicillin culminated in the discovery that it inhibited the final cross-linking of the peptide sidechains of nascent peptidoglycan (PG). A single "transpeptidase" target was first postulated (Tipper and Strominger 1965, Wise and Park 1965), along with the suggestion that penicillin acted as an analogue of acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala) (Tipper and Strominger 1965). As a result of this structural resemblance, it was postulated that β-lactam antibiotics would interfere with PG metabolism, permitting biosynthesis of PG, but preventing the normal degree of transpeptide crosslinking (Martin 1964). The preexisting wall would become mechanically weakened with the incorporation of such poorly linked PG and eventually would rupture under the combined osmotic and mechanical pressure exherted by a normally expanding cytoplasmic mass. The result would be lysis and death of the bacterium.

It is now apparent from later investigations, that the mechanism of action of β-lactam antibiotics is much more complex. There are multiple targets for penicillin action: E.coli possesses three independent lethal targets, which are PBP 1A and PBP1B's, PBP2 and PBP3 and at least three non-essential targets, designated PBP4, PBP5 and PBP6. Selective

inhibition of these different targets results in different morphological effects; only inhibition of PBP1A and PBP1B's results in rapid lysis, whereas inhibition of PBP2 results in conversion of bacilli to osmotically stable ovoid cells with loss of viability, and inhibition of PBP3 results in filamentation. Thus implicating three mechanisms of bactericidal action of β-lactams. It is clear that other factors contribute to the mode of action, in particular the control of the autolytic enzymes (Tomasz 1979a,b). The action of β-lactam antibiotics can now regarded as a two stage process: (i) binding to primary receptors (PBPs) and (ii) the physiological effects on the sensitive cell initiated by this primary receptor-ligand interaction and which are related to the particular binding target (PBP1A + PBP1B's, PBP2 or PBP3) (Tipper 1985).

(i) Binding to the primary receptors.

The original hypothesis that β-lactam antibiotics act as structural analogues of acyl-D-Ala-D-Ala in order to acylate and inactivate these enzymes (Tipper and Strominger 1965) is now generally accepted as correct. However, since β-lactam structural types are diverse - including both bicyclic and activated monocyclic compounds, it is clearly not exact (Tipper 1985). In addition the highly selective inhibition of individual PBPs by certain β-lactam antibiotics (e.g. PBP2 by amdinocillin (mecillinam); PBP3 by aztreonam) must be due to recognition of substituents on sidechains or thiazolidine rings since the structural analogy is restricted to the β-lactam ring, which is common to all β-lactam antibiotics

(Tomasz 1986). Results from studies on Streptomyces R61 and R39 enzymes have suggested that once recognition of the substrate D-Ala-D-Ala peptide or of the analogous component of a β-lactam antibiotic results in reversible binding to the active site, interaction of other components of the substrate or inhibitor may be involved in an "induced fit" which, by modifying both substrate and enzyme conformation, enhances the acylation rate (Ghuysen et al 1980). In this way the binding related to substrate mimicry is relatively non-selective and it is secondary interactions which confer discrimination between PBP targets and are a major determinant of the acylation efficiency. It is apparent that a more detailed knowledge of the structure and chemistry of sufficient PBP active sites is a prerequisite for rational drug design.

The basic validity of the substrate-analogue theory (Tipper and Strominger 1965) has been proven by demonstrating that model acyl-D-Ala-D-Ala substrates and β-lactam antibiotics acylate the same active site serine residue in a number of PBPs from various sources (Georgopapadakou et al 1981, Waxman and Strominger 1980, Yocum et al 1982). Structure-activity analyses by Boyd (1982) have demonstrated that a potent β-lactam antibiotic must have sufficient chemical reactivity in its β-lactam bond in order to acylate PBP sites effectively. However, while necessary to ensure acylation, such reactivity is not sufficient to ensure potency. This will depend on the "fit" to the active site itself and on resistance to hydrolysis of the acyl enzyme.

The "fit" in turn will depend on the mimicry of the normal acyl-D-Ala-D-Ala substrate.

Studies with model transpeptide/carboxypeptidase enzymes by Ghuysen et al (1980) have established the effectiveness of β-lactam antibiotics as enzyme inhibitors.

$$E + \text{penicillin} \underset{K2}{\overset{K1}{\rightleftharpoons}} \text{penicillin.E} \xrightarrow{K3} \text{penicilloyl}\sim E \xrightarrow{K4}$$

E active + penicillin inactive

Good inhibitors were found to be the ones that trapped the enzyme (E) in an antibiotic complex both rapidly (high K3 value) and for a long time (low K4 value). Thus it is acylating efficiency (K3) rather than binding affinity (K1) which is paramount in PBP inhibition. It must be realised, however, that kinetic parameters alone cannot fully predict the nature or extent of the eventual antibacterial effect (e.g. bactericidal potency). One major complicating factor may be the continued production of new PBPs by penicillin treated cells (Tomasz 1986). In addition bound PBPs may be released into the growth medium through normal turnover (Gutmann et al 1981) or related to the "secretion" phenomenon observed in several bacteria during treatment with cell wall inhibitors (Horne et al 1977). Finally bacteria may be deficient in autolysin activity, resulting in a tolerant response to β-lactam antibiotics (Tomasz 1979a).

(ii) Physiological effects on sensitive cells.

Studies of the physiological consequences of inactivation of E.coli PBPs 1,2 and 3, demonstrate that β-lactam antibiotics can kill the bacterium by inactivation of a single PBP species. However many β-lactam antibiotics have similar affinities for several targets and at concentrations above their MIC may kill by inactivating more than one type of PBP. To determine the mechanism of lethality for inhibition of individual species of PBP it is necessary to look at concentrations of β-lactam antibiotics that saturate only one target or to use β-lactam antibiotics that have a high affinity for a particular target. The selective inhibition of PBPs has been most extensively studied in E.coli and many of the conclusions have been comprehensively reviewed (Spratt 1975, 1977a, 1979). Cephaloridine and cefsulodin saturate PBP1A and PBP1B's before PBP2 or PBP3. These antibiotics cause rapid and extensive killing of E.coli with degradation of cell wall material and rapid lysis. Amdinocillin (formerly called mecillinam), clavulanic acid and thienamycin derivatives all have a high affinity for PBP2, which is saturated at their minimum inhibitory concentration (MIC). These β-lactam antibiotics convert bacilli to osmotically stable ovoid cells, followed by loss of viability, but not accompanied by extensive cell wall degradation. Cephalexin and aztreonam are highly selective for PBP3 and cause selective inhibition of septation. This leads to the formation of long filaments, but to only limited, if any, bactericidal activity and lysis (Spratt 1977a). Low concentrations of many β-lactam antibiotics including

penicillin, ampicillin and cephalothin saturate PBP3 before any other PBPs. At these concentrations filamentation is also seen, however at higher concentrations above their MICs, swelling and eventual lysis occurs, corresponding to saturation of PBP1B's at these concentrations. There is considerable doubt that PBP3 is a killing target in the strict sense. When E.coli is treated with cephalosporins at concentrations only inhibitory for PBP3 (Chase et al 1981) the cells stop dividing, but continue to grow as non-septate filaments. Removal of the inhibitor results in resumption of cell division by break up of the filament into normal sized viable cells. Lysis is only observed when the concentration of cephalosporin is increased so that it combines with the PBP1 complex (Chase et al 1981). It is conceivable that the reported high in vivo efficacy of aztreonam (Bonner et al 1981) may be related to some aspect of the filamentous morphology that is disadvantageous for the bacterium in vivo, for example, increased susceptibility to phagocytosis (Andreana et al 1984). In conclusion, inhibition of either PBP1A and PBP1B's, PBP2 or PBP3 is lethal to growing cells via different mechanisms, perhaps in the case of PBP3 via some in vivo growth disadvantage. Thus the inactivation of the major transpeptidase (likely to be PBP1B's in E.coli) is not the sole route by which β-lactam antibiotics kill bacteria.

Autolysin activation in bacteria has been implicated in death due to exposure to β-lactam antibiotics where lysis occurs (e.g. in death caused by PBP1A and PBP1B's inactivation in E.coli). The association of the bactericidal

action of penicillin and activation of autolytic enzymes has been demonstrated most convincingly by Tomasz and colleagues with Streptococcus pneumoniae (Horne and Tomasz 1980, Tomasz 1979a,1980, Williamson and Tomasz 1980). Autolysin activation may involve a number of sequential steps, whose control is tied to the cell growth cycle (Tomasz 1986, Williamson and Tomasz 1980). Blockage of protein synthesis, for example as caused by bacteriostatic antibiotics, may block the cell cycle at some quiescent stage, removing some precondition for autolysin activation and resulting in protection from killing by β-lactam antibiotics. This tolerance to β-lactam antibiotic action may also be found in mutants with defective autolysin activity. Such mutants contain the normal complement of PBPs and these are normally susceptible to acylation by penicillin in vivo (Tomasz 1980). Thus the exogenous addition of lysine can restore the lethality of penicillin causing lysis (Tomasz 1980). It has been suggested by Tomasz (1980) that the autolysin type responsible for the massive penicillin induced lysis in species such as S.pneumoniae is normally responsible for extensive PG hydrolysis during normal cell separation. A second type of highly controlled PG hydrolase activity may be required for subtle remodelling of presynthesized PG, to allow for cell expansion - possibly to allow insertion of a new PG polymer (Weidel and Pelzer 1964). It is possible that abnormal inactivation of this system is responsible for the death of E.coli through PBP2 or PBP3 inhibition.

1.5.2. Combination therapy with β-lactam antibiotics.

Double β-lactam antibiotic combinations.

A combination of two β-lactam antibiotics may be advantageous in certain clinical situations, providing a synergistic activity against specific organisms or a broad spectrum of antibiotic coverage (Gutmann *et al* 1986). Synergism may occur with two β-lactam antibiotics that have high affinites for different PBPs in the same organism, or if one β-lactam antibiotic acts as a β-lactamase inhibitor leaving the β-lactamase susceptible antibiotic free to reach its target (Gutmann *et al* 1986). Most studies on the former mechanism have involved amdinocillin, which binds specifically to PBP2 (see previous section 1.5.1.). Combinations of this antibiotic with a number of β-lactam antibiotics which bind preferentially to PBP1A or PBP1B's resulted in increased lysis of *E.coli* (Kramer *et al* 1983). It has also been shown that combinations of amdinocillin with β-lactam antibiotics which have a preferential affinity for PBP3, (moxalactam, ceftazidime, cefotaxime and aztreonam) at least at low concentrations, cause rapid lysis of *E.coli* or *E.cloacae* with a very effective bactericidal effect (Gutmann *et al* 1986). In some cases organisms that are resistant to amdinocillin or to other β-lactam antibiotics, may be sensitive to the synergistic effects when these antibiotics are combined (Chattopadhyay and Hall 1979). It is important to note, however, that synergism is both strain- and concentration-dependent. Therefore the synergism between amdinocillin and other β-lactam antibiotics, or indeed any double β-lactam

antibiotic combination, cannot be considered a general assumption. With the second mechanism of synergy, the best examples of β-lactamase inhibitors are clavulanic acid and sulbactam. Although both of these compounds have high affinities for β-lactamases and act as suicide inhibitors, they have poor intrinsic antibiotic activity due to their low affinity for PBPs (Bush and Sykes 1983, Labia 1985). Carbenicillin plus these inhibitors has been shown to be effective against Enterobacter, Citrobacter, Serratia and indole positive Proteus with an acquired plasmid-mediated β-lactamase (Labia 1985, Wise et al 1980). However, for these same organisms, results of such combinations are not always predictable, due to other mechanisms of resistance besides plasmid-mediated β-lactamases (i.e. derepression of cephalosporinases, decrease in permeability).

Certain combinations of the newer β-lactam antibiotics have been reported to produce synergistic effects. With Enterobacter and Serratia, different combinations of ureidopenicillins with ceftizoxime, moxalactam, cefotaxime or cefoperazone showed synergism (Buesing and Jorgensen 1984, Moody et al 1984, Stutman et al 1984). In other cases indifference was reported for such combinations (Kurtz et al 1981). The synergism between the newer β-lactam antibiotics are also species and concentration dependent, with much variation in different reports of results (Gutmann et al 1986).

When double β-lactam antibiotic combinations are used in treatment, there is always the risk of antagonism. One major mechanism of antagonism is when one antibiotic is a good

inducer of the chromosomal class I β-lactamases and the other antibiotic is a good substrate. Many observations of this type have been made, especially after the introduction of cefoxitin (Fu and Neu 1980,1981, Goering et al 1982, Kuck et al 1981, Miller et al 1983, Moellering et al 1982, Moody et al 1984, Neu and Labthavikul 1982b, Sanders et al 1982b, Stutman et al 1984). This antagonism has been shown to occur mostly with species such as Enterobacter, Serratia, Citrobacter, indole positive Proteus, Aeromonas and Pseudomonas (Collatz et al 1984, Moellering et al 1982, Sanders and Sanders 1985). In species lacking the inducible class I enzyme this antagonism is not observed, for example E.coli, K.pneumoniae and P.mirabilis (Sanders et al 1982b). Imipenem is another new β-lactam antibiotic which is a good inducer of class I β-lactamases (Gootz and Sanders 1983) and has been shown to be antagonistic in combinations with other β-lactam antibiotics (Bertram and Young 1984, Miller et al 1983). However, imipenem has good antibacterial activity due to its high penetration rate (Nikaido and Vaara 1985), stability towards cephalosporinases and preferential binding to PBP2 (Spratt 1983). Since there are only a small number of PBP2 molecules present in E.coli cells, only a small amount of imipenem would be needed to inhibit this PBP. Thus antagonism with this antibiotic was only obtained at sub MIC levels.

There is no evidence that combined β-lactam antibiotic therapy prevents the emergence of resistant mutants, which has occurred after the introduction of many of the newer β-lactam antibiotics (Collatz et al 1984, Sanders et al 1982a,

Sanders and Sanders 1985). Combinations of cefotaxime with cefoxitin, cefamandole, cefoperazone or moxalactam, were found to provide no protection against the selection of derepressed cephalosporinase mutants of E.cloacae in vitro (Gutmann et al 1986).

β-Lactam and aminoglycoside antibiotic combinations.

Enhanced antibacterial activity has been noticed when aminoglycosides are combined with β-lactam antibiotics. The explanation of this effect is thought to be the alteration of the bacterial cell wall by β-lactam antibiotics, resulting in increased penetration of the aminoglycosides to their target, the ribosomes (Holm 1986). Synergism between these two types of antibiotics has been known for a long time and such combinations are used in the treatment of infections caused by Enterococcus faecalis, Staphylococcus, Pseudomonas, Klebsiella, Enterobacter, Proteus, Escherichia and Acinetobacter strains (Bryant and Kimbrough 1978, Klastersky et al 1977, White et al 1979, Winston et al 1984). Although synergism is a common finding, some exceptions are observed; however, true antagonism is rarely seen with this type of combination (Winston et al 1984). The major disadvantage of the aminoglycoside antibiotics is their nephro- and oto-toxicity. However the advantages of these compounds on their own and in combination with β-lactam antibiotics outweigh their disadvantages, especially in the treatment of septicaemias of unknown origin in critically ill immunocompromised patients.

In many clinical situations it may be useful to have an enlarged spectrum of antibiotic activity that could be provided by the combination of two antibiotics. The double β-lactam antibiotic combination has an advantage over the β-lactam/aminoglycoside combination due to the low toxicity of β-lactam antibiotics as compared to the aminoglycosides. However, antagonism and the selection of resistant mutants may limit the use of the double β-lactam antibiotic combinations. In the absence of clinical and experimental data indicating definite advantages of certain combinations, double β-lactam antibiotic combinations should be avoided.

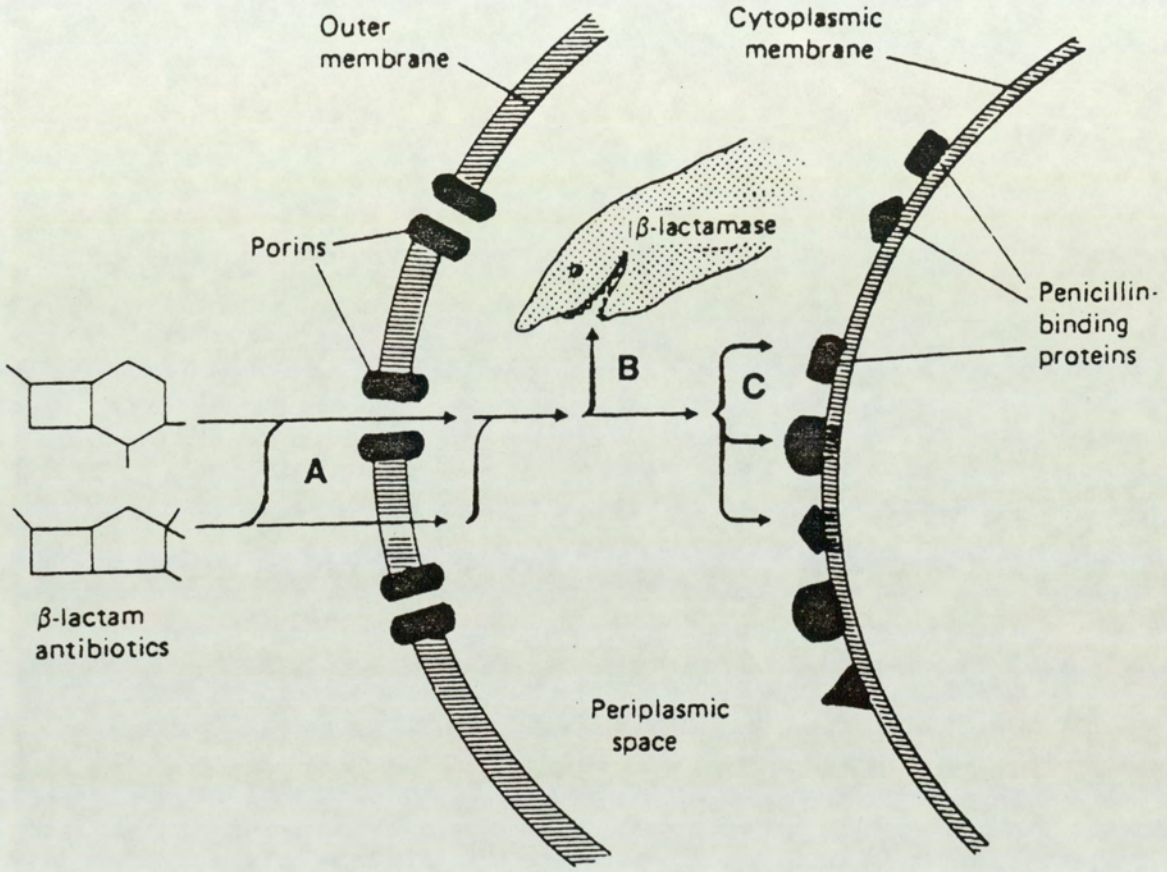
1.6. The mechanisms of resistance to β-lactam antibiotics.

As with other Gram-negative bacteria, resistance to β-lactam antibiotics in E.cloacae can evolve through at least three different mechanisms: (i) reduced penetration of the antibiotic to its target, (ii) enzymatic inactivation of the antibiotic and (iii) alteration of the target site (PBPs), (see Figure 2). These three mechanisms will be described in the following sections together with a detailed appraisal of current knowledge in these areas.

1.6.1. Resistance due to a change in permeability.

In order to be effective the β-lactam antibiotics must be able to reach their target sites in sufficient concentration. To reach these targets, the PBPs, they need to pass through potential cellular barriers. The first of these barriers may

FIGURE 2. Mechanisms of resistance to ß-lactam antibiotics.



A Reduced penetration of antibiotic to target

B enzymatic inactivation of antibiotic

C Alteration of target site

Data from (Normark and Lindberg 1985)

be capsular materials or exopolysaccharide. These materials may retard the passage of β-lactam molecules to the outer membrane by a general frictional resistance to diffusion, or by their charged nature reducing the permeation of a charged β-lactam molecule (Slack and Nichols 1982, Nichols *et al* 1988). *E.cloacae* is generally less often and less heavily encapsulated than related Gram-negative bacteria, for example *Klebsiella* spp. (John *et al* 1982) thus the permeability barrier afforded by capsular material will be of much less significance than that provided by the outer membrane. Studies on the properties of the OM have defined the roles of various OM components in the production of a permeability barrier (Nikaido 1979a,b, Nikaido and Nakae 1979, Nikaido and Vaara 1985).

The following sections will summarize the current knowledge in this area ending with examples of emergence of resistance through permeability changes in both *in vitro* studies and clinical reports of treatment failures.

The OM must function as a barrier to a number of hazardous agents in order to protect the inside of the bacterial cell. This is particularly important for the enteric Gram-negative bacteria, which may be exposed to bile salts, fatty acids and hydrolytic enzymes in the mammalian gut. At the same time the OM must allow an influx of nutrients and efflux of waste products to ensure the survival of the bacterium. There are three possible pathways across the OM: (i) non-specific protein channels (porins), (ii) specific diffusion channels and (iii) diffusion through the lipid bilayer (Nikaido 1979a,b).

Porin pathway

The porin proteins present in Gram-negative OMs are transmembrane proteins with the ability to form water-filled channels (Nakae 1976a,b). Porins usually exist as trimers in the OM (although dimers have also been found; Zalman and Nikaido 1985). Three channels are present at the outer surface of the OM and these coalesce to form one channel at the inner surface of the OM (Engel et al 1985). The presence of LPS is required to form these stable channels (Schindler and Rosenbusch 1981), however the role of LPS in the promotion of channel formation remains unknown. These channels are apparently non-specific and allow the diffusion of any hydrophilic solute as long as it is small (<600d in enterobacteria), (Nakae 1976a). Many nutrients and waste products of Gram-negative bacteria are small hydrophilic solutes and thus may pass through these porin channels. Porins in most Gram-negative bacteria are proteins with a molecular weight of 35-40Kd and are very often the most abundant proteins in the cell; for example, a single E.coli cell usually contains 10^5 molecules of porin (Rosenbusch 1974). Since porin is essential for the uptake of nutrients, mutants lacking the porin completely will not be able to survive. All "porinless" mutants isolated so far simply produce diminished amounts of this protein (Bavoil et al 1977). Using porin-deficient mutants it was found that the route of entry of β-lactam antibiotics is through these channels (Bavoil et al 1977, Nikaido et al 1977). Thus the study of OM permeability for β-lactam antibiotics essentially means the investigation of the properties of the porin

channel, at least in E.coli and other enteric Gram-negative bacteria.

Different porin species of Gram-negative bacteria. Enteric bacteria frequently produce multiple species of porin: E.coli K-12 produces OmpF and OmpC porins, S.typhimurium produces OmpF, OmpC and OmpD porins (Lugtenberg and van Alphen 1983, Osborn and Wu 1980) and E.cloacae has recently been shown to produce two porin species; 37Kd and 39-40Kd proteins (Kaneko et al 1984, Sawai et al 1982). Under certain conditions E.coli K-12 can produce further species of porins: PhoE is expressed under conditions of phosphate starvation or if the cell acquires a mutation in which the synthesis of OmpF and OmpC ceases (Foulds and Chai 1978, Nakae 1976a), Protein 2 is expressed at the expense of OmpF expression (Diedrich et al 1977, Pugsley and Schnaitman 1978), Protein K is expressed only in pathogenic E.coli producing capsular polysaccharide (Sutcliffe et al 1983, Whitfield et al 1983), NmpC may be expressed in conditions in which the cells are missing OmpF and OmpC proteins (Hindahl et al 1984) and LamB, a more specific porin, is produced in the presence of maltose or maltodextrins (Hazelbauer 1975, Szmelcman and Hofnung 1975). In total an E.coli cell can express seven species of porins depending upon the conditions to which the cells are exposed. No further porins have been identified in E.cloacae as yet, but it is reasonable to assume that more do exist. A summary of E.coli, S.typhimurium and E.cloacae porins is given in Table 2.

Regulation of porin expression. The regulation and expression of these porins in bacteria must be related to

TABLE 2. Porins of E.coli, S.typhimurium and E.cloacae

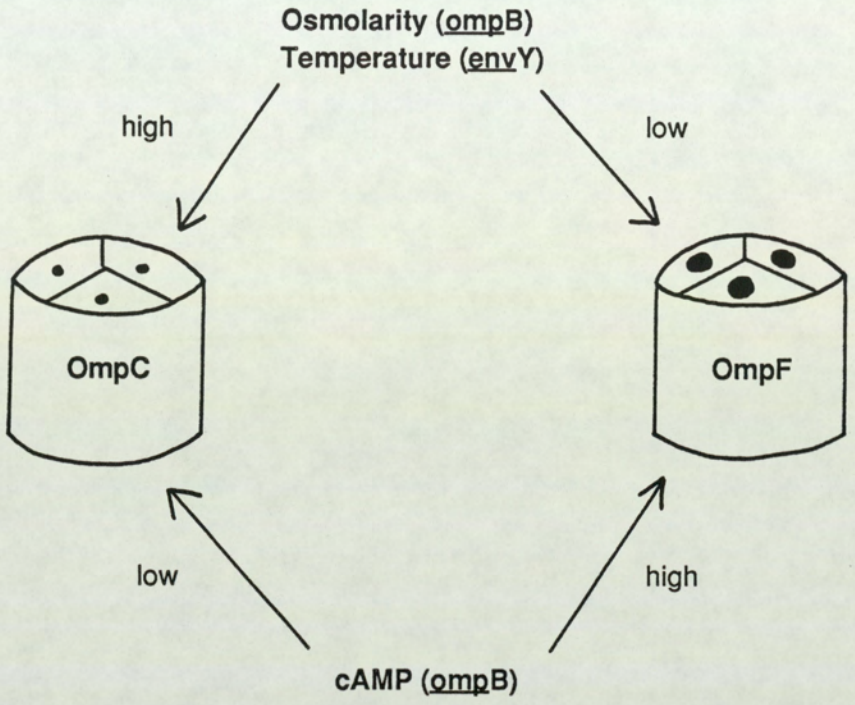
Source	Nomenclature	Molecular weight (kd)	Pore diameter (nm)
E.coli K12	OmpF	37	1.16
	OmpC	38	1.04
	LamB	47	?
	PhoE	36.8	0.8-1
	Protein 2	36.5	?
	Protein K	40	1.2
	NmpC	39.5	1.2
S.typhimurium	OmpF	40	1.4
LT2	OmpC	39	1.4
	OmpD	38	1.4
E.cloacae 206	'37'	37	1.2
	'39-40'	39-40	1.6

Data obtained from (Nakae 1986, Nikaido 1985, Kaneko et al 1984)

their physiological role. Of these porins the regulation of OmpF and OmpC in E.coli has been the most thoroughly investigated.

OmpF and OmpC are reciprocally regulated, this regulation is controlled by medium composition and temperature (Kawaji et al 1979, Lugtenberg et al 1976), see Figure 3. The ompB locus regulates OmpF and OmpC according to the osmolarity of the media and to the levels of cAMP (Lugtenberg et al 1976, von Mayenburg 1971). The envY locus regulates OmpF and OmpC according to the temperature of the growth medium (Lugtenberg et al 1976). These two genetic loci regulate the production of OmpF and OmpC independently of each other. The OmpC porin is a smaller porin which is much less efficient than OmpF (Jaffe et al 1982). A large amount of OmpC porin is produced in a medium of high osmolarity at 37°C at the expense of a lower level of OmpF production. It is thought that the biological significance of this regulation is related to the condition that bacteria are exposed to in the parasitic state (i.e. in the mammalian gut where ions and carbon sources are abundant). Under these conditions OmpC porins suffice to support nutrient entry across the OM, yet protecting the cells from antibiotics produced by other organisms or from fatty acids and bile salts in the mammalian gut. The situation would be reversed when bacteria are exposed to the non-parasitic state (e.g. in streams where ions and carbon sources are very limited). Under these conditions the cells would need the more efficient OmpF porin to scavenge the last trace nutrients (Nakae 1986).

FIGURE 3. Regulation of OmpF and OmpC porin expression



OmpC = smaller less efficient porin

OmpF = larger more efficient porin

Diffusion of β-lactam antibiotics through porins. The estimated diameters of the porin channels are very close to the size of the β-lactam molecules (Nikaido and Rosenberg 1983). The physicochemical properties of these molecules will therefore affect their rate of diffusion, even though porin channels exhibit no configurational specificity (Nikaido 1979b). Such properties include: size, charge and hydrophobicity.

i) Effect of size: the rate of diffusion through porin channels is strongly affected by the size of the solute molecules (Nikaido and Rosenberg 1983). This is especially significant for the OmpC porin whose permeability is at least several times lower than that through the OmpF porin, due to its narrower channels (Jaffe et al 1982, Nikaido and Rosenberg 1983). Below 500d small differences in size of the β-lactam molecules do not produce striking differences in penetration rates (Yoshimura and Nikaido 1985). However when compounds are greater than 500d or have bulky side chains, they have great difficulty passing through the OmpC channels (Yoshimura and Nikaido 1985). Compounds such as cefoperazone (Mr 664d), ceftriaxone (Mr 552d) and ceftazidime (Mr 545) which penetrate the OmpF channel at significant rates, cannot pass through the OmpC channels with measureable rates (Yoshimura and Nikaido 1985). This observation is important in view of the physiological regulation of OmpF and OmpC.

ii) Effect of charge: the presence of a positive charge aids penetration of a molecule through porins whereas the presence of a negative charge retards penetration. Zwitterionic compounds penetrate much more rapidly through

porins than compounds with two negative charges plus a positive charge, while compounds with two negative charges and no positive charge diffuse through porins rather poorly (Yoshimura and Nikaido 1985). The presence of multiple negative charges appears to retard the penetration of molecules through the OmpC channel slightly more than it does through the OmpF (Nikaido and Rosenberg 1983). The more severe retardation of molecules with a net negative charge may be due to the presence of a Donnan potential (inside negative) across the OM (Stock et al 1977). In addition to the electrical effect, charged groups tend to increase the hydrophilicity of the surface of the molecule through the presence of hydration shells. This latter effect could accelerate the permeation through the pore (Nikaido and Rosenberg 1983).

iii) Effect of hydrophobicity: Biagi et al (1970) determined the hydrophobicity of various β-lactam antibiotics and found with Gram-negative bacteria, the higher the hydrophobicity, the less effective was the agent. The negative effect of hydrophobicity in Gram-negative organisms was due to the slowing down of the diffusion through the OM (Nikaido et al 1983, Zimmerman and Rosselet 1977). Since the OmpC channel is narrower than the OmpF channel, then hydrophobicity affects the penetration through OmpC more severely than through OmpF (Nikaido and Rosenberg 1983). Among the zwitterionic compounds the calculated hydrophobicity of the uncharged form does not have much influence on the permeability. Thus in β-lactam antibiotics the nature of the nucleus seems to affect the penetration

rates much more than the extent of hydrophobicity determined by the peripheral substituents (Yoshimura and Nikaido 1985). Penicillins are more hydrophobic than cephalosporins and are therefore less permeable through porins (Yoshimura and Nikaido 1985). It has been claimed that penicillins tend to penetrate through non-porin pathways: the relatively high rate of penetration through a phospholipid bilayer has been correlated with this notion (Yamaguchi et al 1982). Since many penicillins have poor permeability through the porin channel due to their high degree of hydrophobicity, it is conceivable that even a slow leakage through non-porin pathways (e.g. diffusion through the lipid bilayer) could come to represent a major share in the overall penetration process. However, this does not necessarily indicate the presence of any specific penetration process for penicillins and does not take into account the presence of LPS in the Gram-negative OM (discussed later in this section). Thus much of the current data on the permeability of β-lactam antibiotics can be explained on the basis of gross physicochemical properties of the compounds and their influence on permeation through porin channels.

Specific pathways.

In the E.coli OM there are several specific pathways of diffusion in addition to the non-specific porin channel. These specific pathways appear to be divided into two classes.

The first group includes proteins which produce transmembrane channels similar to the porin channel. However,

these channels have additional discriminatory mechanisms that favour the diffusion of specific solutes: for example, maltose and maltodextrins (Luckey and Nikaido 1980). These proteins are often also receptors for phage lambda or phage T6 (Hantke 1976, Luckey and Nikaido 1980). Since the configurational discrimination of these channels becomes stronger for larger molecules (Luckey and Nikaido 1980), it is unlikely that β-lactam molecules would diffuse through these channels at significant rates.

The second group includes proteins which bind solute specifically and with a high affinity. These proteins transport vitamin B12 and iron-chelator complexes and require the wild-type allele of the tonB gene to function (Neilands 1982), see section 1.4.1. for more detailed discussion of these proteins. Since these pathways have a high degree of specificity it seems unlikely that they can transport β-lactam antibiotics. However, recently a new cephalosporin E-0702 was found to be incorporated into E.coli cells via this system (Watanabe et al 1987). E-0702 is the first β-lactam antibiotic which may be incorporated into cells by the active transport system that is dependent on the tonB function.

Lipid bilayer pathway.

Lipid bilayers are permeable to lipophilic (or hydrophobic) solutes, which traverse the OM by first dissolving into the hydrocarbon interior of the membrane (Cohen and Bangham 1972). Since some β-lactam compounds are rather lipophilic (e.g. penicillins) this pathway might be expected to play a

significant role in their penetration through the OM. However the Gram-negative OM is rather impermeable to lipophilic molecules (Nikaido 1976). The LPS was shown to be responsible for this impermeability, since removal by EDTA treatment (Leive 1974) or mutational changes in structure (Nikaido 1976, Roantree et al 1977) renders the OM permeable to lipophilic molecules. Thus when dealing with wild-type enteric Gram-negative bacteria, the possibility of diffusion through lipid bilayer regions may be discarded. However this pathway may become significant in deep rough mutants with incomplete LPS structure, or perhaps in very fast growing (low LPS) "open" cells (Gilbert and Brown 1978)

Factors affecting the rate of β-lactam diffusion across the OM.

From the evidence summarised above, it would seem that the pathway for the β-lactams, currently available for clinical use, across the OM is through the porin channnels. The factors affecting the rate of β-lactam diffusion across the OM therefore will be those affecting diffusion through porins and are summarised below:

1) The external concentration of the β-lactam antibiotic; since diffusion through the porin channel is a passive process, a diffusion gradient is required. To be effective the external concentration of the β-lactam antibiotic must be at least the MIC of the organism.

2) Properties of the β-lactam molecule; for example, size, charge and hydrophobicity as described earlier.

3) The number of functional porins present; pores are not "open" at all times, i.e. every pore is not always capable of admitting hydrophilic substances up to their individual exclusion limits. In situations where the transmembrane potential is high there are thought to be fewer "open" pores than when it is low (Schindler and Rosenbusch 1981).

4) The species of porin present; for example, under physiological conditions the OmpF porin is strongly repressed in favour of the OmpC porin, which has narrower channels. Permeability to β-lactams is thus reduced.

5) The rate of removal of β-lactam from the periplasm; Since diffusion through the porin is a passive process, the periplasmic content of any agent will eventually reach the external concentration, regardless of the magnitude of diffusion rates, if there is no process to inactivate or remove the agent from the periplasmic space. In Gram-negative bacteria, β-lactam inactivating enzymes (β-lactamases) are present in the periplasm. Even if the activity of the β-lactamase is very weak, when external concentrations of β-lactam antibiotics are in the range of their MIC, this low activity will be sufficient to destroy a significant fraction of the periplasmic β-lactams. Thus it is a balance between the rate of influx and the rate of removal, rather than the OM permeability alone that determines the periplasmic concentrations of the agent and thus its efficacy.

Clinical emergence of resistance due to permeability changes. A number of in vitro studies with E.cloacae and related organisms have linked reduced susceptibility to β-lactam antibiotics with a decrease in OM permeability. The basis for this decrease in permeability was in many cases shown to be a modification in the quantity of porins expressed by the resistant isolates (Bush et al 1985, Büscher et al 1987a, Godfrey and Bryan 1987, Harder et al 1981, Jaffe et al 1982, Mitsuyama et al 1987, Nikaido and Nakae 1979, Nikaido et al 1977, Sawai et al 1982). Stoorvogel et al (1987) cloned a restriction fragment of E.cloacae chromosomal DNA into E.coli, which caused β-lactam antibiotic resistance in both E.coli and the parental E.cloacae strain. The gene was found to encode a 20Kd polypeptide involved in the regulation of OMP synthesis. Alterations in OM permeability appear to be particularly important in cross-resistance between β-lactam and aminoglycoside antibiotics (Goldstein et al 1983, Preheim et al 1982). Several workers have described rapid emergence of broad spectrum resistance through diminished permeability, involving multiple classes of antibiotics including: β-lactam agents, trimethoprim-sulphamethoxazole, chloramphenicol and quinolones during β-lactam therapy for Enterobacter and Serratia infections (Gutmann et al 1985, Sanders and Watanakunakorn 1986). Clinical emergence of resistance to β-lactam antibiotics through permeability has been shown for a number of Gram-negative bacteria including: Serratia (Goldstein et al 1983, Sanders and Watanakunakorn 1986); E.coli (Bakken et al 1987); S.typhimurium (Medeiros et al 1987) and P.aeruginosa (Quinn et al 1986). However, clinical

emergence of resistance through permeability occurs relatively infrequently (c.f. resistance due to β-lactamase), one explanation for this is the inherent physiological disadvantage of porin deficient organisms in nutrient uptake. This may act against them in the long run, especially when organisms may possess other mechanisms of resistance, for example, β-lactamase production. Possibly this type of resistance mechanism will become more prevalent among clinical isolates when the β-lactamase resistance mechanism has been overcome.

1.6.2. Resistance due to a change in penicillin-binding protein affinity.

Penicillin-binding proteins.

Penicillin-binding proteins (PBPs) are defined as those bacterial proteins that covalently bind penicillins and other β-lactam antibiotics (Waxman and Strominger 1983). They can be visualised with the use of radioactive penicillin and sodium-dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) as radioactive bands on fluorographs (Spratt 1977a). Most bacteria contain between 10^3 and 10^4 total PBP molecules per cell (Waxman and Strominger 1983,, although the copy number of individual PBPs per cell may be as low as 20 (Spratt 1977a). Quantitatively PBPs are relatively minor proteins comprising approximately 1% of the total membrane protein (Blumberg and Strominger 1974). They are situated on the outer face of the cytoplasmic membrane (CM): for those

which have been studied in some detail (e.g. Bacillus subtilis), the proteins seem to be anchored to the membrane by a COOH-terminal hydrophobic region with their amino terminus and active site directed "outwards", towards the periplasm (Waxman and Strominger 1979, 1981). All bacteria contain multiple PBPs, which may include between three and eight distinct PBPs (Blumberg and Strominger 1974, Curtis et al 1979a). The PBPs of E.coli have been most extensively studied and will be described in some detail here. Taxonomically related bacteria tend to have similar PBP profiles. Curtis et al (1979a) have suggested that the PBPs of E.cloacae, K.pneumoniae, Proteus rettgeri and P.aeruginosa probably perform the same roles in elongation, shape and division as has been shown for E.coli K-12.

E.coli PBPs.

E.coli contains seven PBPs (see Table 3) which fall into two distinct classes: the first comprises the high molecular weight PBPs -1A, -1B's, -2 and -3 (60-90Kd). These proteins frequently lack demonstrable in vitro activity and are much less abundant than the second class, the relatively low molecular weight PBPs -4, -5 and -6 (40-50Kd). These proteins have been shown to possess in vitro D,D-carboxypeptidase activity. The role of the PBPs of E.coli in growth, morphogenesis and the killing action of β-lactam antibiotics has been elucidated using two principal approaches: (i) selective inhibition of PBPs by β-lactam antibiotics (Spratt 1975, see also section 1.5.1.) and (ii) the isolation of

TABLE 3. PBPs of E.coli

PBP	Molecular weight	Molecules per cell	Function	Consequences of inactivation
1A	92	200	Transglycosylases and primary transpeptidases. Essential for cylindrical wall synthesis	Rapid lysis
1B's	90(approx)	250		
2	66	20	Transpeptidase required for initiation of cylindrical wall growth at sites of septation	Non-viable spherical cells
3	60	50	A transglycosylase transpeptidase required for septum cross-wall synthesis	Filamentous non-septate cells
4	49	110	A secondary transpeptidase, D,D-carboxypeptidase or D,D-endopeptidase acting on maturing PG	Delayed transpeptidation absent
5	42	1800	A D,D-carboxypeptidase	none obvious
6	40	600	A D,D-carboxypeptidase	none obvious

Data from (Tipper 1985)

mutants that lack or produce thermolabile forms of these proteins (Spratt 1975, 1977b).

High molecular weight PBPs. PBP1 of E.coli consists of two components: PBP1A and PBP1B, which can be distinguished on a fluorograph. The latter component itself consists of a cluster of closely related species and so is referred to as PBP1B's (Spratt et al 1977, Suzuki et al 1978, Tamaki et al 1977). Specific inhibition of these PBPs by β-lactam antibiotics causes rapid lysis, suggesting roles for these PBPs in cell elongation (Spratt 1975). Mutants lacking detectable activity of either PBP1A or PBP1B's are viable, although a temperature sensitive mutant has been described lacking only PBP1B's (Tamaki et al 1977). In mutants lacking PBP1B's, PG biosynthetic activity is significantly retarded, suggesting that PBP1B's catalyses the majority of this activity in vitro (Suzuki et al 1978, Tamaki et al 1977). Double mutants lacking both PBP1A and PBP1B's have not been successfully isolated (Suzuki et al 1980). However a double mutant that lacks PBP1B's and produces a thermolabile PBP1A has been isolated and this mutant lyses when shifted to the restrictive temperature (Suzuki et al 1980). PBP1A and PBP1B's have been shown to possess combined transglycosylase and transpeptidase activities in vitro (Nakagawa et al 1979, Suzuki et al 1980). Since β-lactam antibiotics act only by inhibiting transpeptidation (see section 1.5.1.), it is apparent that the two active sites function independently (Tipper 1985). The data discussed in this text suggests that PBP1A and PBP1B's perform similar functions which are essential for cell viability. They are involved in the

extension of the peripheral cell wall in E.coli and may compensate reciprocally for each other when one is lost through mutation. The persistance of both of these PBPs is perhaps an indication of some discrete in vivo function which may improve survival of E.coli (Tipper 1985).

PBP2 is the least abundant E.coli PBP and has a restricted topographical distribution in the CM (Buchanan 1981). Specific inhibition of PBP2 by amdinocillin results in the formation of non-viable ovoid cells (see section 1.5.1.). Mutational loss of detectable levels of PBP2 also results in the formation of round shaped cells, however these cells are viable (Iwaya et al 1978). The formation of round shaped cells indicates that PBP2 functions in the maintenance of the rod shape of E.coli. The very small amounts of PBP2 present in normal cells, therefore, apparently control the initiation of new annular zones of cylindrical cell wall synthesis at sites of septation in E.coli (Tipper 1985). It is not clear why inactivation of PBP2, but not mutational loss is lethal. PBP2 is deduced to act as a transpeptidase, since cells carrying the cloned PBP2 gene and overproducing PBP2 give membrane preparations having amdinocillin sensitive transpeptidation (Ishino et al 1982). Transglycosylase activity of PBP2 has not been assessed.

Specific inhibition of PBP3 with a β-lactam antibiotic or growth at the restrictive temperature of a mutant producing a thermolabile form of PBP3, results in the inhibition of cell division and the growth of E.coli into long filamentous cells (Spratt 1975, 1977a). Similarly to PBP1, PBP3 has been demonstrated to possess combined

transglycosylase and transpeptidase activity *in vitro* (Ishino and Matsuhashi 1981). Thus PBP3 is required for cross wall synthesis at the septa during cell division.

The high molecular weight PBPs are essential for the growth of *E.coli*, controlling its elongation, shape and cell division. All of these PBPs appear to be transpeptidases *in vitro* and all but PBP2 have been shown to be transglycosylases *in vitro*. Due to their essential roles in the *E.coli* cell cycle, they are also lethal targets for β-lactam antibiotics.

Low molecular weight PBPs. Mutants lacking PBP4 activity are viable, showing no gross morphological defects. These mutants, however, do have a low level of cross-linking in their PG (Spratt 1983). De Pedro and Schwarz (1981) have suggested that these PBPs are responsible for secondary transpeptidation, after initial transpeptidation by the high molecular weight PBPs. Thus PBP4 is involved in maturation of the cell wall. This function is apparently not vital for the survival of the cell, at least *in vitro*, neither is it vital for the strength of the PG, since loss of this activity produces no obvious morphological defects (Spratt 1983).

Mutants lacking PBP5 (Spratt 1980) or PBP6 (Broome-Smith and Spratt 1982) indicate that both PBPs are individually dispensable. However, the double mutant has not been reported and so, like PBP1A and PBP1B's, it would seem that these two PBPs may have very similar functions, the loss of one being compensated by the presence of the other (Tipper 1985). These PBPs have been shown to catalyse the D-alanine carboxypeptidase reaction *in vitro* and probably do so *in vivo*

(Spratt 1983). This reaction has been suggested to control the extent of the PG cross-linking by removing the terminal D-alanine residues from the pentapeptide sidechains of nascent PG, thereby preventing them from acting as peptide donors in transpeptidation (Izaki *et al* 1966). However this suggestion has not yet been substantiated with experimental evidence and so the role of D-alanine carboxypeptidases in PG synthesis remains unclear (Spratt 1983). Interestingly overproduction of PBP5 produces round shaped cells possessing the same abnormalities as cells with inactivated PBP2 (Markiewicz *et al* 1982). Since D,D-carboxypeptidase activity increases *in vivo* at cell division (Tipper 1985) it is possible that PBP5 plays a role in cell division.

The low molecular weight PBPs appear to be dispensable in *E.coli*, performing non-essential roles in the maturation of PG. These PBPs all have demonstrable carboxypeptidase activity *in vitro* and probably function as carboxypeptidases *in vivo* also. They contain no identified lethal targets. Recently, however, a low molecular weight PBP (possibly PBP7 as described by Spratt 1977a), whose expression was variable, was suggested to be a lethal target of non-growing bacteria to the penem β-lactam antibiotics (Tuomanen and Schwartz 1987).

PBP mutations and resistance to β-lactam antibiotics.

Resistance through production of PBPs that have a decreased affinity for β-lactam antibiotics was first shown clearly in laboratory mutants of *E.coli*. These mutants had increased resistance to amdinocillin due to a decreased affinity of

PBP2 for this antibiotic (Spratt 1978). Mutants of this type are easy to obtain, since amdinocillin kills E.coli exclusively by inactivation of PBP2. Such mutants show cross-resistance to other PBP2 specific inhibitors, but not to those that kill by binding to PBP1 or PBP3 (Spratt 1983). Similarly PBP3 mutants can be selected by using β-lactam antibiotics that bind preferentially to this PBP (Spratt 1979). However, many β-lactam antibiotics are less specific, saturating several essential PBPs over a narrow concentration range. Spratt (1978) suggested that high level resistance to these antibiotics would be unlikely: high level resistance would require a decrease in affinity of each of these essential PBPs. He also pointed out that if β-lactam antibiotics act as structural analogues of acyl-D-Ala-D-Ala, then mutants that drastically reduce the affinity of a PBP for penicillin might, in many cases, also reduce its affinity for substrate. This would prevent it from performing its essential function in PG synthesis. Nevertheless, such mutants have been seen both in the laboratory and clinically. As yet resistance by this mechanism has occurred mainly with the Gram-positive bacteria. The most notable example being methicillin resistance in S.aureus (Brown and Reynolds 1980). The mechanism of resistance appears to be the acquisition of a "new" PBP, PBP2', which takes over the function of the other essential PBPs when they are inactivated (Georgopapadakou and Lui 1980b, Hartman and Tomasz 1984, Rossi et al 1985, Ubukata et al 1985). Resistance due to PBP modification has also been demonstrated in S.pneumoniae, a species in which β-lactamase production has yet to appear

(Hakenbeck et al 1980, Williamson et al 1981, Ziegelboim and Tomasz 1980). Up to an intermediate level of resistance, resistant isolates exhibit gradually decreasing affinities in four out of five PBPs. Above this level of resistance an abrupt additional change is observable in the PBPs: PBP1a and PBP1b are no longer detectable, while a new PBP1c, with an extremely low affinity for penicillin appears (Zigelboim and Tomasz 1980). Other Gram-positive bacteria resistant through PBP alterations include; Streptococcus faecium and E.faecalis (Fontana et al 1983).

Gram-negative bacteria have also demonstrated increased resistance through PBP changes. For example, Haemophilus influenzae (Mendelman et al 1984) and Neisseria gonorrhoeae (Barbour 1981, Dougherty et al 1980). Since the introduction of penicillin, when all isolates of N.gonorrhoeae were extremely sensitive to penicillin (MIC $< 0.01\mu g.ml^{-1}$), there has been a documented gradual increase in low level resistance to benzylpenicillin (MIC $0.5\text{-}8\mu g.ml^{-1}$) (Spratt 1983). This resistance has been demonstrated to result from a reduction in affinity of two out of three of the gonococci PBPs: reduction in affinity is seen first for PBP2 and is then followed by second step alterations in PBP1 (Barbour 1981, Dougherty et al 1980). At present these organisms can still be treated successfully with β-lactam antibiotics, but if the level of resistance continues to increase, this may no longer be possible. Clinical emergence of resistance through PBP changes in Gram-negative bacteria has also been reported for P.aeruginosa (Godfrey et al 1981). In this instance a cystic fibrosis patient was treated with a high dosage

regimen of tobramycin and piperacillin. Two serotypes of P.aeruginosa (M and K) were present and persisted during treatment. Both serotypes displayed increased resistance to β-lactam antibiotics, to differing degrees, through PBP alterations: in the K serotype, low level resistance was observed concomitantly with loss of detectable PBP3; with the M serotype a more graduated response to treatment was observed up to intermediate-level resistance. There was a reduced affinity of all the PBPs in this case, and an increase in the quantity of PBP6.

It is apparent from the data described in this section, that reduced β-lactam antibiotic susceptibility caused by PBP alterations, can arise in a number of different ways: by a reduction in affinity of normally occurring PBPs, loss in quantity of a particular PBP, by the addition of low affinity PBPs or in some cases through a combination of these events.

As pointed out earlier, since penicillin is a structural analogue of the natural substrates of PBPs, it is surprising that PBP alterations may occur, since they may also cause decreased affinity for substrate. Defective growth might be expected or difficulties in the synthesis of normal cell wall material in such instances. Indeed the production of altered PG structure has been demonstrated in a strain of N.gonorrhoeae with altered PBPs (Dougherty 1985). However, it is clear that alterations in PBPs and consequently in cell wall structure are not incompatible with normal growth: all clinical isolates with altered PBPs have normal growth rates (Tomasz 1986). A recent study by Garcia-Bustos et al (1988) investigated the effects of altered PBPs, in genetically

transformed mutants of S.pneumoniae, on PG structure. These PBPs had a decreased affinity for penicillin or a change in size. Their results indicated that the structure of the PG portion of the cell wall in resistant strains underwent a major change compared with the pattern typical of a susceptible pneumococcus. This structure appeared to be fully compatible with the normal physiological function of the cells. It is possible that the reason why the low affinity PBPs can support apparently normal rates of cell wall synthesis in the resistant bacteria is because the mutational change is not in the active site itself, but on a neighbouring site. As described in section 1.5.1.(i), secondary interactions of the β-lactam antibiotic with the PBP causing the "induced fit" are important in the acylating efficiency of the antibiotic. A change in this site may provide an organism with increased resistance to this antibiotic, while not affecting its normal substrate reactions.

Spratt (1983) pointed out that resistance through PBP alterations has so far been seen only in circumstances where β-lactamase production plays no part. Therefore this mechanism of resistance may be expected to occur in any pathogen, once β-lactamase defences have been overcome. Although the Gram-positive organisms have provided the most fertile field for the selection of resistance due to PBP alterations so far, if and when the β-lactamase barrier in Gram-negative bacteria is overcome these organisms may provide the next mechanism of resistance through PBP alterations. Tipper (1985) has suggested therefore, that it

may be necessary to design or screen β-lactam antibiotics, or mixtures of such antibiotics, which strike at multiple lethal target PBPs and to tailor such mixtures to specific pathogens of groups of pathogens.

1.6.3. Resistance due to an increase in β-lactamase production.

β-Lactamases.

β-Lactamases have been known since the discovery of penicillin: in 1929 Fleming observed the first case of resistance to β-lactam antibiotics when "coli-typhoid" bacteria were not inhibited by penicillin (Fleming 1929). Later Abraham and Chain (1940) prepared an extract of *E.coli* and determined that the active penicillin destroying component was an enzyme, which they termed "penicillinase". The introduction of cephaloridine and subsequent cephalosporins have shown that some enzymes hydrolyse these compounds more rapidly than penicillins, while others hydrolyse both penicillins and cephalosporins at equal rates (Hamilton-Miller *et al* 1965). Therefore, these enzymes are better referred to as β-lactamases.

β-Lactamases are ubiquitous in nature, their activity has been demonstrated in most bacteria where its presence has been sought (Matthew and Harris 1976, Richmond and Sykes 1973, Sykes and Matthew 1976), including blue-green algae (Kushner and Breuil 1971). β-Lactamase activity has also been demonstrated in eukaryotic microbial cells such as *Candida*

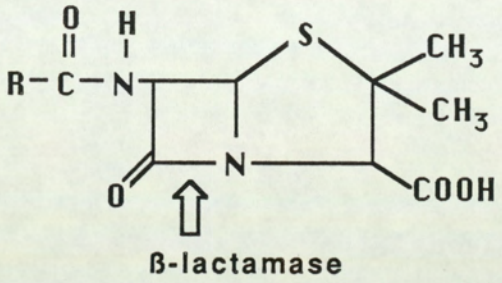
spp (Mehta and Nash 1978) and in the mammalian liver (Snow 1962), hence it is not confined to microbial sources.

In bacteria β-lactamases constitute the major single cause of resistance to β-lactam antibiotics (Livermore 1988, Medeiros 1984, Piddock and Wise 1985). β-Lactamases hydrolyse the amide bond that is present in the β-lactam ring of both penicillins and cephalosporins (see Figure 4). The hydrolysis of penicillin results in the formation of penicilloic acid in stoichiometric proportions (Figure 4). Penicilloates have one more acidic group than their parent compounds; they are stable and can be readily detected and assayed (Sykes and Smith 1979). With the cephalosporins the reaction is more complicated owing to the presence of a conjugated ring system and a substituent at the 3-position of the dihydrothiazine ring (Newton et al 1967). The first product of a β-lactamase attack on a cephalosporin is, hypothetically, a cephalosporoate. However, the majority of cephalosporoates are unstable, undergoing decomposition to smaller fragments (Hamilton-Miller et al 1970, see also Figure 4). The hydrolytic products of penicillins and cephalosporins are microbiologically inactive.

Over the last few years, antibiotically active molecules (other than penicillins and cephalosporins) containing a β-lactam ring structure have been reported and these molecules should be considered as substrates for certain β-lactamases. Examples of such compounds include: cephamycins, nocardicins, thienamycins, clavulins, olivanic acids, penems, carbapenems and monobactams (Rolinson 1988).

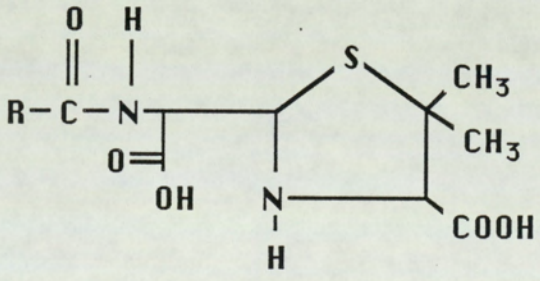
FIGURE 4. Hydrolysis of ß-lactam antibiotics

a) penicillins



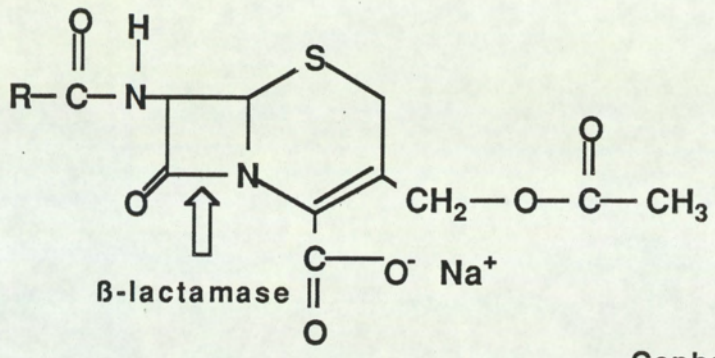
ß-lactamase

penicillin

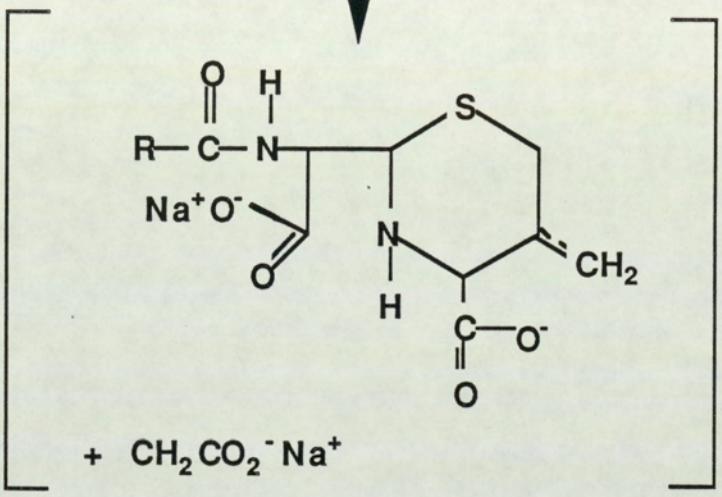


stable penicilloic acid

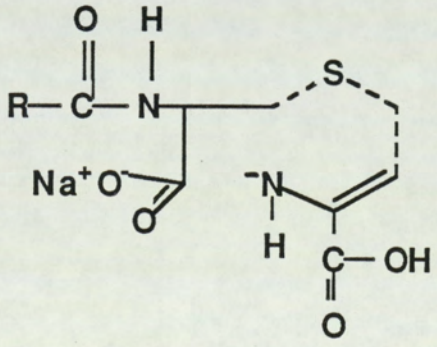
b) cephalosporins



Cephalosporin



Hypothetical intermediate: unstable cephalosporoic acid



Fragments

Data from (Neu 1980)

The new β-lactam antibiotics may interact with β-lactamases through a variety of mechanisms. Therefore to determine resistance or susceptibility of these compounds to hydrolysis by β-lactamases, detailed kinetic parameters are needed (Bush and Sykes 1986). The general reaction scheme for the action of β-lactamases is:

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

In this sequence, enzyme (E; i.e. β-lactamase) and substrate (S; i.e. β-lactam) combine first to form a reversible non-covalent complex (E.S). In the case of most clinically important β-lactamases at this stage 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). Hydrolysis is completed by deacylation of this covalent complex to yield free (active) enzyme and the ring-opened (inactive) β-lactam (P), which may fragment as mentioned previously for cephalosporins (Bush and Sykes 1984, 1986)

Affinity of β-lactamase for β-lactam antibiotics. The binding of a β-lactam to β-lactamase is evaluated by the expression K_{app}, based on the above reaction sequence.

$$K_{app} = \frac{k_{-1}}{k_1}$$

Binding of a β-lactam to a β-lactamase does not always result in facile hydrolysis. A number of β-lactam molecules may act as potent inhibitors for a variety of β-lactamases. This subject has been well reviewed (Bush and Sykes 1983, Wise

1982). Thus K_{app} may take the form of an inhibition constant K_i for β-lactamase inhibitors or of a Michaelis constant K_m for β-lactamase substrates. Values of K_m vary enormously according to the antibiotic and enzyme considered. A low K_m value represents a high affinity; the inducible chromosomally mediated β-lactamases (e.g. P99 enzyme from E.cloacae) possess low K_m values for many of the newer β-lactam compounds (Bush and Sykes 1984). Therefore such compounds are bound rapidly in the presence of this β-lactamase. However essential PBPs may also be extremely sensitive to β-lactam molecules (Curtis et al 1979b, Georgopapadakou and Lui 1980a). Therefore factors other than binding must be considered. Not only is the enzyme-β-lactam affinity important, but the destiny of the E.S complex must be examined.

Hydrolytic efficiency. The efficiency of an enzyme is dependent on its turnover number or K_{cat} value ($=k_3$ in above equation) and this value is directly proportional to V_{max}, a value readily obtained experimentally. The rate at which a β-lactam antibiotic is hydrolysed may be represented by the following equation:

$$v = \frac{V_{max}(S)}{K_m + (S)}$$

in which v = measured velocity of hydrolysis, V_{max} = maximum velocity attainable for the reaction, (S) = substrate concentration and K_m the Michaelis constant (which represents the equilibrium constant equal to the substrate concentration that will be hydrolysed at a rate equal to $0.5V_{max}$). The V_{max} value also varies according to the antibiotic and enzyme

under consideration (Bush and Sykes 1986). The best presentation of hydrolysis data for comparison purposes has therefore been suggested to be the ratio V_{max}/K_m - the "physiological efficiency" (Pollock 1965) or "efficiency of hydrolysis" (Sykes et al 1981). Such a ratio takes into account both binding and hydrolysis capability and becomes especially useful at antibiotic concentrations less than the K_m value; a situation often encountered within the periplasm of Gram-negative bacteria (Bush et al 1985, Livermore 1985, Vu and Nikaido 1985).

The kinetic parameters K_m and V_{max}, described above, govern the qualitative aspects of β-lactamase production, however, quantitative factors must also be considered. Some bacteria have the ability to produce exceptionally high levels of β-lactamase. In many of these strains the β-lactamase produced exhibits very inefficient hydrolysis of the substrate; resistance appears to be achieved nevertheless by the sheer numbers of β-lactamase molecules involved. Hence, whilst the efficiency of the β-lactamase remains an important factor, the amount of β-lactamase present may be the major resistance determinant (Simpson et al 1986).

Trapping versus hydrolysis. The biochemical mechanism by which large quantities of β-lactamase protect bacterial cells has been hotly debated. The fact that bacteria were resistant to many β-lactams which appeared not to be hydrolysed by β-lactamase led some workers to believe that this resistance was a result of non-hydrolytic action of β-lactamase. The theory of "trapping" was proposed (Then and Angehern 1982) whereby β-lactamase acted by non-covalently binding β-lactam

molecules, thereby inactivating them without hydrolysis. The presence of large amounts of β-lactamase in the periplasmic space binding in this fashion to β-lactam antibiotics was proposed to form a "sponge-type" barrier to incoming β-lactam molecules (Sanders 1983, Then and Angehern 1982). This hypothesis gained support from a number of workers (Olson et al 1983, Seeberg et al 1983). Others favoured a hydrolytic mechanism of resistance (Livermore 1985, Vu and Nikaido 1985) since quantitative consideration of the influx rates of β-lactams through the OM barrier (Nikaido 1985) showed that the high level resistance observed could not be explained by trapping alone (Vu and Nikaido 1985). This observation in turn stimulated a more careful reappraisal of the kinetics of hydrolysis of these compounds. Livermore (1985) and Vu and Nikaido (1985) pointed out that conventional β-lactamase assays were conducted under conditions of substrate excess and these could detect little or no hydrolysis of many of the newer expanded spectrum β-lactams. In contrast, when large amounts of β-lactamase are present in the periplasmic space, conditions of enzyme excess exist. Therefore, in the presence of lower, physiologically relevant, concentrations of β-lactams even low V_{max} values produced significant hydrolysis rates of these β-lactams because of the extremely low K_m values for these compounds. Thus hydrolysis that may be efficient under physiological conditions may go undetected in conventional assays. Livermore et al (1985, 1986) also constructed a novel model to represent β-lactamase inside a permeability barrier facing an influx of β-lactam molecules. Using this model they demonstrated that such β-lactamase,

when functioning jointly with a permeability barrier could afford a hydrolytic defence, even against very slowly hydrolysed substrates such as cefotaxime and ceftriaxone. This model afforded no support for the trapping hypothesis with these antibiotics.

It is now generally accepted that most β-lactamase-mediated resistance occurs through a hydrolytic mechanism, in combination with a permeability barrier. However, in a few cases, notably moxalactam and aztreonam, which readily form rather stable covalent adducts with class I β-lactamases, the mechanism may be a non-hydrolytic one (Bush et al 1982, Livermore 1987b). The original non-covalent binding, non-hydrolytic mechanism of trapping is not supported by direct evidence (Livermore 1985, 1987b).

Detection and Assay.

There are four principal methods available for the detection of β-lactamase hydrolysis of the β-lactam bonds (Sykes and Matthew 1979):

(i) acidimetric method; the hydrolysis of a penicillin or cephalosporin molecule by β-lactamase leads to the formation of at least one extra carboxyl group. The generation of this extra carboxyl group can be detected with pH indicators.

(ii) iodometric method; the products of β-lactamase hydrolysis act as reducing agents and remove iodine from its starch complex, resulting in a loss of colour intensity.

(iii) microbiological method; on hydrolysis by β-lactamases, β-lactam antibiotics lose all antibacterial

activity and so enzymes can be detected by microbiological assay techniques.

(iv) chromogenic substrates; hydrolysis of certain β-lactam-containing molecules by β-lactamase leads to the formation of products having an absorption spectrum in the visible range, different to that of the parent compound. Thus the presence of β-lactamase can be detected by colour changes in solution.

The most recent advance in the detection of β-lactamases has been the development of DNA probes for colony hybridisation (Cooksey et al 1985, Huovinen et al 1988, Jouvenot et al 1987). The advantage of this method being not only the detection of β-lactamase, but also the identification of specific β-lactamases in one simple test. However so far this method has been restricted to the TEM-type β-lactamases.

Methods of assaying β-lactamase activity are based on following the rate of the changes described in the four principal methods of detection described above. In addition, the absorption of penicillins and cephalosporins have a different wavelength from the absorption of their products on hydrolysis. Thus β-lactamase activity may be determined by spectrophotometric measurement of the substrate disappearance or product appearance according to the wavelength chosen. These methods are described in some detail by Neu (1980) and Sykes and Matthew (1979). The method used in this project is the spectrophotometric measurement of the appearance of the hydrolysis product of nitrocefin, a chromogenic

cephalosporin. This method is described in greater detail in the relevant methods and results sections.

Classification of β-lactamases from Gram-negative bacteria.

Most, if not all, Gram-negative bacteria produce β-lactamases (Sykes and Matthew 1976). Well over 50 different β-lactamases have now been identified, some of which are encoded by genes that reside on plasmids or transposons and others by chromosomally encoded genes (Datta and Kontomichalou 1965, Hedges and Jacob 1974, Medeiros 1984). Matthew and Harris (1976) found that virtually all Gram-negative bacteria produce some chromosomally-mediated β-lactamase. The types of β-lactamase produced are often specific for species and sometimes for subspecies. The wide variety of β-lactamases produced by Gram-negative bacteria has therefore led to a number of classification schemes. These schemes have been based on: substrate profiles, sensitivity to inhibitors, isoelectric point, molecular weight, inducibility, genetic origin and more recently on amino acid sequences (Ambler 1980, Jaurin and Grundstrom 1981, Matthew and Harris 1976, Richmond and Sykes 1973, Sykes and Matthew 1976). The earliest of the classification schemes (Jack and Richmond 1970, Sawai et al 1968) were based primarily on substrate profiles, with additional distinction provided by parameters such as enzyme inhibition and reaction to antisera. Richmond and Sykes (1973) elaborated on these criteria dividing the β-lactamases into five main classes and thus provided the first broadly useful frame of reference. This classification scheme

has recently been modified (Neu 1986) to include a sixth class (see Table 4).

Class I: These enzymes are characteristically produced by strains of E.coli, Shigella, Enterobacter, Citrobacter, indole positive Proteus, Morganella, Pseudomonas and Serratia species. The expression of class I β-lactamases varies between these species and this in turn determines their ability to cause resistance. Class I expression by E.coli, P.mirabilis and Shigella species is constitutive. These organisms produce only tiny basal amounts of enzyme (Lindberg and Normark 1986, Richmond and Sykes 1973). These small amounts of β-lactamase are insufficient to protect the bacteria even against readily hydrolysed β-lactam antibiotics such as cephaloridine and ampicillin (Lindberg and Normark 1986). Much greater potential for resistance arises in species such as P.aeruginosa, Enterobacter, Citrobacter, Serratia, Morganella and indole positive Proteus species. In these species class I expression is commonly (>80% of isolates) inducible (Lindberg and Normark 1986, Livermore 1987a, Medeiros 1984, Sanders 1983). This means that β-lactamase synthesis by these organisms is minimal in the absence of antibiotic, but can increase several hundredfold upon exposure to β-lactam antibiotics. The amount of β-lactamase induced will depend upon the structure of the β-lactam compound, its concentration and on the length of time for which the bacteria are exposed (Livermore and Yang 1987, Minami et al 1980, Nordstrom and Sykes 1974). Induction of class I β-lactamase expression is discussed in more detail later in this section. The class I β-lactamases hydrolyse

TABLE 4. Richmond and Sykes classification scheme

Group	Preferred substrate	DNA locus	Gene expression	Mr (kd)	pI	Further characteristics
I	cephalosporins	chromosome	constitutive inducible	20-42	3.9-8.7	inhibited by cloxacillin and carbenicillin, but not by P-CMB. Produced by E.coli, Enterobacter, Citrobacter indole +ve Proteus, Pseudomonas and Serratia spp.
II	penicillins	chromosome	constitutive inducible	32	5.3-6.4	inhibited by cloxacillin, but not by carbenicillin or P-CMB. Produced by Pseudomonas and Proteus spp.
III	cephalosporins and penicillins	plasmid	constitutive	17-44	5.2-8.1	inhibited by cloxacillin, but not by carbenicillin or P-CMB. Produced by Enterobacteriaceae, H.influenzae N.gonorrhoeae and P.aeruginosa
IV	cephalosporins and penicillins	chromosome	constitutive	19-24	6.5-7.5	inhibited by P-CMB, but not by cloxacillin or carbenicillin. Produced by Klebsiella, Bacteroides and N.catarrhalis
V	penicillins	plasmid	constitutive	12-43	5.3-7.7	not inhibited by cloxacillin, carbenicillin or P-CMB. Produced by Enterobacteriaceae and P.aeruginosa.
VI	cephalosporins	chromosome	constitutive	26-32	4.3-8.8	inhibited by carbenicillin, clavulanic acid and some strains also by cloxacillin. Not inhibited by cefoxitin Produced by Bacteroides.

Data from: (Neu 1986, Richmond and Sykes 1973, Sykes and Matthews 1976)

cephalosporins at a much higher rate than penicillins. With few exceptions they are inhibited by cloxacillin and carbenicillin, but are resistant to inhibition by para-chloromercuribenzoate (P-CMB) and clavulanic acid.

Class II: In contrast to the β-lactamases produced by Gram-positive organisms, only a relatively small number of Gram-negative bacteria have been reported to produce chromosomally mediated class II β-lactamases. These enzymes are distinguished by their predominant activity against penicillins and lack of activity against cephalosporins. The β-lactamases of certain Pseudomonas and Proteus species fall into this category. These enzymes are constitutive and are inhibited by cloxacillin, but not by carbenicillin or P-CMB.

Class III: These are the resistance plasmid TEM-type β-lactamases, which hydrolyse both penicillins and cephalosporins at equal rates. The enzymes are produced constitutively and have been found in Enterobacteriaceae, H.influenzae, N.gonorrhoeae and P.aeruginosa. Regulation of expression of these β-lactamases is dependent upon: the species of bacteria harbouring them, the gene dosage effect and cell copy number (Sykes and Smith 1979). The TEM-1 and TEM-2 β-lactamases are widely disseminated among clinical isolates. These two enzymes alone account for nearly 80% of the plasmid β-lactamase population (Matthew 1979, Simpson et al 1980). The only other enzyme found with any regularity is the SHV-1 enzyme. Typically, these enzymes have appreciable activity against the first generation cephalosporins, but almost no activity against the later generations. Recently,

however, a number of broad spectrum class III β-lactamases have been described. Kliebe et al (1985) described a probable mutant of SHV-1, designated SHV-2. This enzyme had an increased affinity for the expanded spectrum cephalosporins and was able to hydrolyse these compounds. In addition, a number of broad spectrum TEM-type β-lactamases have been described (TEM-3, -4, -5, -6, -7 and -8) which are active against the newer expanded spectrum β-lactam antibiotics (Bauernfiend and Horl 1987, Sirot et al 1987, Sougakoff et al in press). It is thought that these "new" TEM-type β-lactamases may have evolved by mutation from the original TEM-1 and TEM-2 β-lactamases (Sougakoff et al in press).

Class IV: The most important β-lactamases of this type are produced by strains of Klebsiella species and the enzymes exhibit a broad range of substrate specificity against penicillins and cephalosporins. The enzymes in this class are chromosomally mediated, invariably constitutive and are relatively resistant to inhibition by cloxacillin. Most are also resistant to inhibition by P-CMB. Apart from the Klebsiella enzymes, broad spectrum β-lactamases have also been found in strains of Bacteroides species and Branhamella catarrhalis.

Class V: This class is a rather heterogeneous grouping, which contains the plasmid mediated oxacillin- and carbenicillin-hydrolysing β-lactamases, which are constitutively produced. These enzymes are not inhibited by cloxacillin, carbenicillin or P-CMB. Organisms producing these enzymes include some Enterobacteriaceae and P.aeruginosa.

i) Oxacillin-hydrolysing enzymes; these β-lactamases, as their name suggests, have the ability to hydrolyse oxacillins rapidly. Of these OXA enzymes, OXA-2 is the most prevalent, although when compared to TEM it is relatively rare (Amyes 1988). Four new OXA enzymes were reported recently (Medeiros et al 1985). These enzymes, OXA-4, -5, -6 and -7 hydrolyse cefotaxime at a greater rate than do OXA-1, -2 and -3. As with the broad spectrum class III β-lactamases (SHV-2, TEM-3, -4, -5, -6, -7 and -8) this may be a direct result of selection pressure caused by the increased use of the newer cephalosporins over the last few years.

ii) Carbenicillin-hydrolysing enzymes; these enzymes have been classified by their high relative rates of hydrolysis of carbenicillin and their generally low rates against cephaloridine. The first members of this group were found in P.aeruginosa (Hedges and Matthew 1979) and later in further species of Pseudomonas, hence they were termed Pseudomonas-specific enzymes (PSE), of which PSE-4 is the most common (Williams et al 1984b). However, recent reports have shown that PSE-1, -2 and -4 may also be found in members of the Enterobacteriaceae (Livermore et al 1984, Medeiros et al 1982, Reid et al 1985).

Class VI: These enzymes are produced by Bacteroides spp. The enzymes are chromosomally mediated and constitutively produced. They preferentially hydrolyse cephalosporins and are inhibited by carbenicillin and clavulanic acid. Some but not all strains of Bacteroides produce β-lactamase that is inhibited by cloxacillin.

A major advance in β-lactamase classification occurred when Matthew et al (1975) showed that specific β-lactamases could be identified by flat-bed isoelectric focusing in polyacrylamide gel. Isoelectric points have proven to be a valuable marker, particularly for grouping plasmid mediated β-lactamases. The high sensitivity and reproducibility of the procedure has allowed the detection of chromosomal β-lactamases in virtually all bacterial species studied (Matthew and Harris 1976). The fingerprinting of these enzymes to the point of separation of β-lactamases different in only one amino acid (e.g. TEM-1 and TEM-2: Sutcliffe 1978) was made possible by isoelectric focusing.

In a review on the β-lactamases of Gram-negative bacteria and their role in resistance to β-lactam antibiotics, Sykes and Matthew (1976) moved away from the formally divided classification approach. Instead they grouped the enzymes on a genetic basis, that is; chromosomally mediated β-lactamases and R-factor mediated β-lactamases. Such a scheme relies on the knowledge of the genetic location of the enzyme, which is not always easy to determine.

The most up to date reclassification of the β-lactamases has been carried out using data obtained from sequence homology studies. In this scheme, three evolutionary distinct classes have been defined; A, B and C (Ambler 1980, Jaurin and Grundstrom 1981).

Class A: the penicillinases of S.aureus PC1, Bacillus licheniformis 749/C, Bacillus cereus 569/H β-lactamase 1 and E.coli TEM-1 and TEM-2 belong to this class of β-lactamases.

These enzymes share extensive sequence homology and preferentially hydrolyse penicillins. The molecular weight of the mature β-lactamase is approximately 29kd. Of interest there is homology between the amino acid sequence around the penicillin-binding sites of the D-alanine carboxypeptidases of Bacillus strearothermophilus and Bacillus subtilis and the sequence around the active site serine of these class A β-lactamases. This homology supports the hypothesis that these β-lactamases may be derived from PBPs (Waxman et al 1982, Yocum et al 1979). In spite of extensive sequence homologies, the β-lactamases in class A differ widely in their isoelectric points (pH 5.7 - pH >9) and in their substrate profiles, despite being penicillinases (Bauernfeind 1986).

Class B: the only enzyme of this class was detected in B.cereus (β-lactamase 2). This β-lactamase has a molecular weight of approximately 23kd and so far is the only β-lactamase requiring a metal cofactor - normally zinc II (Davies and Abraham 1974). Fragments of the enzyme protein have been sequenced and compared with the sequences of class A enzymes. No homologies with class A enzymes above background levels were detectable for any of the fragments (Ambler 1980).

Class C: the chromosomally-mediated AmpC β-lactamase of E.coli K-12 belongs to the class C β-lactamases. This enzyme shows no significant sequence homology with class A β-lactamases or D-alanine carboxypeptidases, despite having a serine residue at its active site also. This enzyme does, however, share extensive sequence homology with an active site fragment of P.aeruginosa cephalosporinase (Jaurin and

Grundstrom 1981). Sequence homology has also been shown with fragments of the same size of Shigella flexneri, Shigella sonnei, K.pneumoniae and Serratia marcescens (Bergstrom et al 1982). The amino-terminal sequence of the E.cloacae P99 enzyme is also very homologous to that of the E.coli enzyme (Joris et al 1985). The enzymes of this class are large proteins, having a molecular weight of approximately 39kd and hydrolyse cephalosporins at a much higher rate than do the class A enzymes.

No classification scheme is yet ideal. Complications have arisen with: the demonstration of cephalosporinases having most characteristics of chromosomal enzymes, but being coded for by plasmids (Levesque et al 1982, Sirot et al 1987); the discovery of chromosomally located transposons that encode β-lactamases (Sinclair and Holloway 1982) and the finding of a supposedly Pseudomonas-specific enzyme, PSE-2, in several species of Enterobacteriaceae (Livermore et al 1984). New enzymes are constantly being found. The advent of second and third generation cephalosporins have produced new classification schemes and terminology (e.g. TEM-3 to -8 including cefuroximases and cefotaximases) which has added to confusion. Although the formally proposed scheme by Richmond and Sykes (1973) is less than ideal, it at least provides a system of reference which has been cited extensively in current and past literature. In this project, therefore, the Richmond and Sykes terminology will be used.

Evolution and function.

It is thought that the β-lactamases may have evolved from penicillin-sensitive enzymes (PBPs) involved in PG synthesis (Pollock 1967, Tipper and Strominger 1965). As already explained in the previous section, this theory is supported by the sequence homology between the class A enzymes and the D-alanine carboxypeptidases of Bacillus species. Sequence homology has also been demonstrated between these enzymes and PBP5 and PBP6 of E.coli (Waxman and Strominger 1983). In addition Kelly et al (1985) have shown some similarity between the sequences immediately adjacent to the active site serines of the penicillin-sensitive D-alanine carboxypeptidase-transpeptidase from Streptomyces R61 and class C β-lactamases. No sequence similarity was detected between the class A and class C β-lactamases and so it has been concluded that they evolved independently (Kelly et al 1985).

β-Lactamases and PBPs also share functional characteristics. It is known that many β-lactamases are induced upon exposure to β-lactam antibiotics and recently it has been shown that the low affinity PBP of methicillin resistant S.aureus is also inducible (Chambers et al 1985, Ubukata et al 1985). Some PBPs have been found to act as weak β-lactamases, hydrolysing β-lactam antibiotics at a slow rate (Amaral et al 1986). However the reverse is not true, although as early as 1940 Abraham and Chain proposed a function of β-lactamase in cell metabolism, no direct function has yet been identified. Saz and Lowery (1979) have

suggested a role for β-lactamase in cell viability and maintenance, particularly in the sporulation of B.cereus.

β-Lactamases are produced in bacterial strains that were isolated before β-lactam antibiotics were used in antibacterial chemotherapy (Pollock 1967). β-Lactamases are also present in β-lactam susceptible as well as β-lactam resistant bacteria (Onishi et al 1974). Therefore it seems likely that β-lactamases must fulfil some, as yet unknown, function in bacterial cell metabolism. Whatever the function or origin of β-lactamases, it is clear that the clinical use of β-lactam antibiotics has played a major role in their widespread distribution and further evolution.

Regulation of expression of class I chromosomally-mediated β-lactamases.

As mentioned previously in this section expression of class I β-lactamases varies between different species. In species such as E.coli, β-lactamase is expressed constitutively at low basal levels. When these species are exposed to β-lactam antibiotics the small amounts of β-lactamase expressed are insufficient to protect them, even against readily hydrolysed β-lactam antibiotics (Lindberg and Normark 1986). However, β-lactamase expression in organisms such as Pseudomonas, Enterobacter, Serratia, Citrobacter, Morganella, indole positive Proteus and Providencia species may be induced several hundredfold upon exposure to β-lactam antibiotics. As a result of inducible expression of β-lactamase, these species possess a much greater potential for resistance. It is for this reason that much attention has been focused on

the kinetics of induction and on the genetic control of induction.

β-Lactamase induction.

β-Lactamase induction kinetics have been studied for a wide variety of bacteria and β-lactam antibiotics (Gootz and Sanders 1983, Minami et al 1980, 1983, Nordstrom and Sykes 1974, Okonogi et al 1985, 1986, Sanders and Sanders 1986, Then 1987). Generally β-lactamase synthesis commences 1-20 minutes after exposure of the bacteria to the antibiotic and ceases once all the antibiotic has been hydrolysed or removed physically. Thus induction is defined as a transient increase in β-lactamase synthesis following exposure of a bacterium to a β-lactam antibiotic. Once the inducer is removed, the quantity of enzyme returns to a basal level (Livermore 1988). Livermore (1988) defined four classes of inducers: (i) strong inducer, labile to β-lactamase, (ii) weak inducer, labile to β-lactamase, (iii) strong inducer, stable to β-lactamase and (iv) weak inducer, stable to β-lactamase.

(i) Strong inducer, β-lactamase labile. Most of the first generation cephalosporins are β-lactamase labile strong inducers along with benzylpenicillin, ampicillin and amoxycillin. In this case the drug induces β-lactamase to high levels and is hydrolysed by it. Consequently the inducible enzyme protects the bacteria. Gram-negative bacilli are uniformly resistant to these antibiotics (Livermore 1987a, Sykes and Matthew 1976).

(ii) Weak inducer, β-lactamase labile. In general all of the second and third generation cephalosporins (except

cefoxitin) fall into this category along with the ureidopenicillins, moxalactam and aztreonam. Such compounds can be inactivated by the class I enzymes, but fail to induce significantly below their MIC. Consequently β-lactamase-inducible strains are as susceptible as their enzyme basal mutants. This situation would be advantageous were it not for the presence of stably derepressed mutants in populations of bacteria with inducible β-lactamase. These stably derepressed mutants can be at least four times less susceptible than the inducible strains. The degree of lability varies between compounds and between the enzymes of different species. These factors will determine the level of resistance observed in these stably derepressed organisms. A number of problems arise with this group of antibiotics. Firstly, if induction is more efficient *in vivo* than *in vitro*, then susceptibility tests may underestimate resistance in inducible organisms (Livermore 1987a, Livermore and Yang 1987). Secondly, resistance to weak, labile inducers may arise transiently when exposed simultaneously to strong inducers (see section 1.5.2.). However, this antagonism is only temporary and only clinically significant in combination therapy. Finally, as mentioned above, inducibly expressed β-lactamase fails to protect against the labile, weak inducers and therefore these antibiotics place preexisting populations of stably derepressed variants at a competitive advantage. These variants tend, therefore, to overgrow the population and this is a serious and well established clinical problem, which will be discussed in the following section.

(iii) Strong inducer, β-lactamase stable. These β-lactam antibiotics induce β-lactamase synthesis strongly, however, they resist hydrolysis (or non-hydrolytic binding). Thus remaining equally active against β-lactamase inducible strains and stably derepressed mutants. β-Lactamase basal organisms are only marginally more susceptible, indicating that the enzyme affords only minimal protection even when produced in large amounts. The derepressed mutants therefore have no competitive advantage over the inducible strains and so do not overgrow the population as they do with labile, weak inducers. Unlike the β-lactamase labile weak inducers, induction occurs during the MIC tests and so these tests are unlikely to underestimate β-lactamase mediated resistance. Cefoxitin is a stable, strong inducer for Proteus and Serratia species, although it is a labile, strong inducer for P.aeruginosa, E.cloacae and Citrobacter freundii (Curtis et al 1986, Livermore 1987a). Other cephamycins, for example cefmetazole, also fall into this class (Sanders and Sanders 1986). Although the stable, strong inducers should not select stably derepressed mutants, cefoxitin has been shown to do so at sub-inhibitory concentrations (Kirkpatrick et al 1986). This implies that cefoxitin does not cause maximal induction at sub-inhibitory concentrations, thereby providing the stably derepressed mutants with a small competitive advantage over the inducible cells (Livermore 1987a). In vivo selection of stably derepressed mutants by cefoxitin has not been reported. Imipenem also approximates to a stable, strong inducer (Livermore and Yang 1987). Despite being very slowly hydrolysed by the class I β-lactamases of E.cloacae and

P.aeruginosa, this compound remains equally active against β-lactamase inducible and stably derepressed mutants of these species. A number of workers have tried, without success, to isolate stably derepressed mutants with imipenem (Büscher et al 1987b, Eng et al 1986, Kirkpatrick et al 1986).

(iv) Weak inducer, β-lactamase stable. These are the ideal drugs as they fail to induce β-lactamase and the enzyme is unable to protect the bacteria even when produced in large amounts in stably derepressed mutants. The MICs are virtually identical for inducible and mutationally basal or derepressed strains. Therefore there is no selection pressure for the stably derepressed strains and also there is no antagonism between these and other β-lactam antibiotics. Unfortunately, there are very few useful β-lactam antibiotics in this category. Carbenicillin is a stable, weak inducer for P.aeruginosa, but a labile, weak inducer for E.cloacae and P.vulgaris (Livermore 1988). Temocillin is a stable, weak inducer for E.cloacae and Proteus species, but is a rather narrow spectrum compound (Livermore 1988).

Differences in induction efficiency of β-lactam agents have been shown to be not only a function of the antibiotic itself, as they vary from species to species and strain to strain (Aronoff and Schlaes 1987, Bush and Sykes 1984, Gootz and Sanders 1983, Minami et al 1980, 1983, Okonogi et al 1985, Sanders and Sanders 1986). This variability may be due in part to the methods used in these studies, in particular the concentration of antibiotic used to induce β-lactamase. However, some of the differences may reflect a true

variability among species and drugs (Sanders and Sanders 1986).

In certain strains of bacteria, including E.cloacae, the growth media employed has been shown to markedly enhance β-lactamase production. For example, Schaedlers' broth was reported to have much greater induction potency than many β-lactams (Cullmann et al 1984, Dalhoff and Cullmann 1984). This so-called "non-specific" induction has been attributed to the bicyclic nature of the media molecules; tryptophan, thiamine, folic acid and haemin (Cullmann et al 1984, Dalhoff and Cullmann 1984) which is similar to that of β-lactams. Gatus et al (1986) also reported non-specific induction of β-lactamase in E.cloacae, but in this case by glycine in conjunction with acid-hydrolysed casein. However, the clinical relevance of glycine enhancement of β-lactamase is questionable. Much more relevant observations to the in vivo situation are those of Cullmann et al (1983). These workers found the addition of inactivated serum, urine, cerebrospinal fluid (CSF) or pleural fluid to overnight cultures of bacteria in Iso-sensitest broth led to β-lactamase induction comparable to that of cefoxitin. From these observations, they concluded that in a previous report, non-specific induction was responsible for the production of large amounts of β-lactamase by Morganella morganii in a group of animals not receiving treatment with β-lactam compounds (Dalhoff 1982). Such observations of non-specific induction of β-lactamase in vivo might have serious implications in the treatment of infections caused by organisms with inducible β-lactamases.

In order to investigate the genetic control of β-lactamase induction the chromosomal β-lactamase genes from both non-inducible species (E.coli) and inducible species (E.cloacae and C.freundii) have been cloned, (Bergstrom et al 1983, Grundstrom et al 1980, Honore et al 1986, Nicolas et al 1987) and the expression of their β-lactamases have been studied (Honore et al 1986, Jaurin et al 1981, Lindberg et al 1985, Nicolas et al 1987).

(i) E.coli: the gene encoding E.coli chromosomal β-lactamase is denoted ampC. This gene is poorly expressed due to an inefficient promoter and to an attenuator structure located between the promoter and the structural gene (ampC) (Jaurin et al 1981). In vitro approximately 90% of the transcripts initiated at the β-lactamase promoter terminate at this attenuator and hence do not contribute to the expression of β-lactamase. However the transcriptional readthrough beyond this attenuator is greater when bacteria are grown fast in rich media than at low growth rates with a poor substrate (Grundstrom and Normark 1985, Jaurin et al 1981, Jaurin and Normark 1979).

The ampC operon of E.coli partially overlaps the frd operon which codes for the fumarate reductase complex and consists of four genes frdA, B, C and D (Cole et al 1982, Grundstrom and Jaurin 1982). This enzyme complex is essential for anaerobic bacterial growth with fumarate as the electron acceptor (Ingledew and Poole 1984). Of particular significance for the evolution of β-lactamase overproduction is the fact that the same DNA segment that constitutes the ampC promoter, also encodes part of the frd structural genes

(Grundstrom and Jaurin 1982, Normark *et al* 1983). Thus mutations that would strengthen the *ampC* promoter may have a deleterious effect on the fumarate reductase enzyme. It is not only *E.coli* K-12 laboratory strains that have these overlapping genes, clinical isolates of *E.coli* also have them and so do *Shigella sonnei* (Olsson *et al* 1983).

(ii) *C.freundii* and *E.cloacae*: *C.freundii* and *E.cloacae* represent enterobacteria expressing inducible β-lactamases. In these species *frd* and *ampC* do not overlap, instead they are separated by a 1100-base-pair large DNA segment (Bergstrom *et al* 1983). Even though these species and *E.coli* carry highly related *ampC* β-lactamase genes, the regulation of these genes is markedly different. The structural basis for this difference is a regulatory gene, *ampR*, which is present on the 1100 base-pair segment (Lindberg *et al* 1985). This *ampR* gene codes for a 31kd polypeptide which, in the absence of β-lactam antibiotics, represses *ampC* expression 2.5-fold for *C.freundii* (Lindberg *et al* 1985) and 1.9-fold for *E.cloacae* (Lindberg *et al* 1987), (see Figure 5). Curtis *et al* (1987) isolated temperature conditional mutants of *E.cloacae* which underwent thermal transition from repressor to activator states at restrictive temperatures. In the absence of inducer at the restrictive temperature β-lactamase production increased 2-3-fold, a close agreement with the observations of Lindberg *et al* (1985, 1987). In the presence of inducing β-lactam antibiotics, the *ampR* protein acts as an activator leading to more than 100-fold induction of β-lactamase (Honore *et al* 1986, Lindberg *et al* 1985, 1987; see also Figure 5). β-Lactamase activity is not required for such

induction, as a non-functional β-lactamase has been induced in C.freundii (Lindberg et al 1985). However, induction is only possible with an intact ampR gene (Lindberg and Normark 1986). Synthesis of AmpR itself has not been found to be appreciably influenced by the addition of inducer, demonstrating that the effect is mediated via the AmpR product itself and not by a change in the ampR expression (Lindberg et al 1985). At present it is not known at which level AmpR regulates ampC β-lactamase expression. The simplest role envisaged for AmpR is that of a DNA binding protein which can directly modulate transcription of ampC (Honore et al 1986). An intriguing feature of the ampR gene is why it is not present in the Escherichia/Shigella branch of the Enterobacteria. Honore et al (1986) suggest that it may have been deleted from the ampC region of the chromosome following the divergence of these bacterial species and those such as E.cloacae from their common ancestor.

In organisms producing inducible class I β-lactamase, mutations may occur such that the bacteria then produce β-lactamase at high levels constitutively. These organisms referred to as stably derepressed mutants or variants, are resistant to virtually all β-lactams except imipenem and pose an interesting clinical problem (Sanders 1983, Sanders and Sanders 1985). Induction and stable derepression are often confused, but the two phenomena are distinct (Livermore 1986, Phillips 1986).

Stable derepression of β-lactamase.

Stable derepression results from a mutation in a β-lactamase regulatory gene (Lindberg and Normark 1986). Stable derepression may be partial, such that the organism produces an unusually high uninduced level of enzyme, but still retains inducibility. On the other hand stable derepression may be total, such that β-lactamase production is entirely unregulated by antibiotic presence (Williams et al 1984b). In the latter case, these mutants can produce an extraordinary amount of β-lactamase, in the range of 2×10^5 molecules per cell (Bush et al 1985, Vu and Nikaido 1985). The frequency of these mutations may be as high as 10^{-5} to 10^{-8} in inducible populations (Findell and Sherris 1976, Gootz et al 1982, Gwynn and Rolinson 1983, Lampe et al 1982, Olson et al 1983). In non-inducible populations, for example E.coli, the frequency is much lower (10^{-15}) (Lindberg and Normark 1986), therefore selection of such mutants is less likely. Nevertheless, they do occur clinically: six such isolates have been genetically studied (Bergstrom and Normark 1979, Olsson et al 1983). The data obtained favoured evolution of resistance through horizontal gene transfer from Shigella to E.coli, Shigella having a much higher rate of mutation (10^{-9}) than E.coli (Normark and Lindberg 1985).

Selection of stably derepressed mutants presents the greater proven problem of resistance rather than induction per se. There are many reports of the selection of stably derepressed mutants in vivo and these have been reviewed recently by Sanders and Sanders (1985). In such cases, typically a labile, weak inducer has been administered to a

patient who is infected with a β-lactamase-inducible organism. Subsequently stably derepressed mutants are isolated and ultimately these constitute the entire bacterial population in the infected site. This appears to be a particular problem with P.aeruginosa and E.cloacae, but also occurs commonly with other β-lactamase-inducible species (Sanders and Sanders 1985). The probability of derepressed mutants overrunning a population in vivo is likely to vary with the status of the patient, drug administered and the site of infection. Selection will be most likely in chronic infections or where the host is immunocompromised and thus wholly dependent on the antibiotic for the elimination of bacteria. If a strong inducer β-lactam is administered selection of derepressed mutants will be less likely than if a weak inducer is used (Livermore 1988, Sanders and Sanders 1986). In addition to selection of stably derepressed mutants in the individual, a further problem is posed by the accumulation of stably derepressed organisms in the hospital microflora. In the absence of strict infection control procedures, it is possible that these multiply resistant bacteria will spread. Instances where this has occurred in individual hospitals have already been described (Sanders and Sanders 1985).

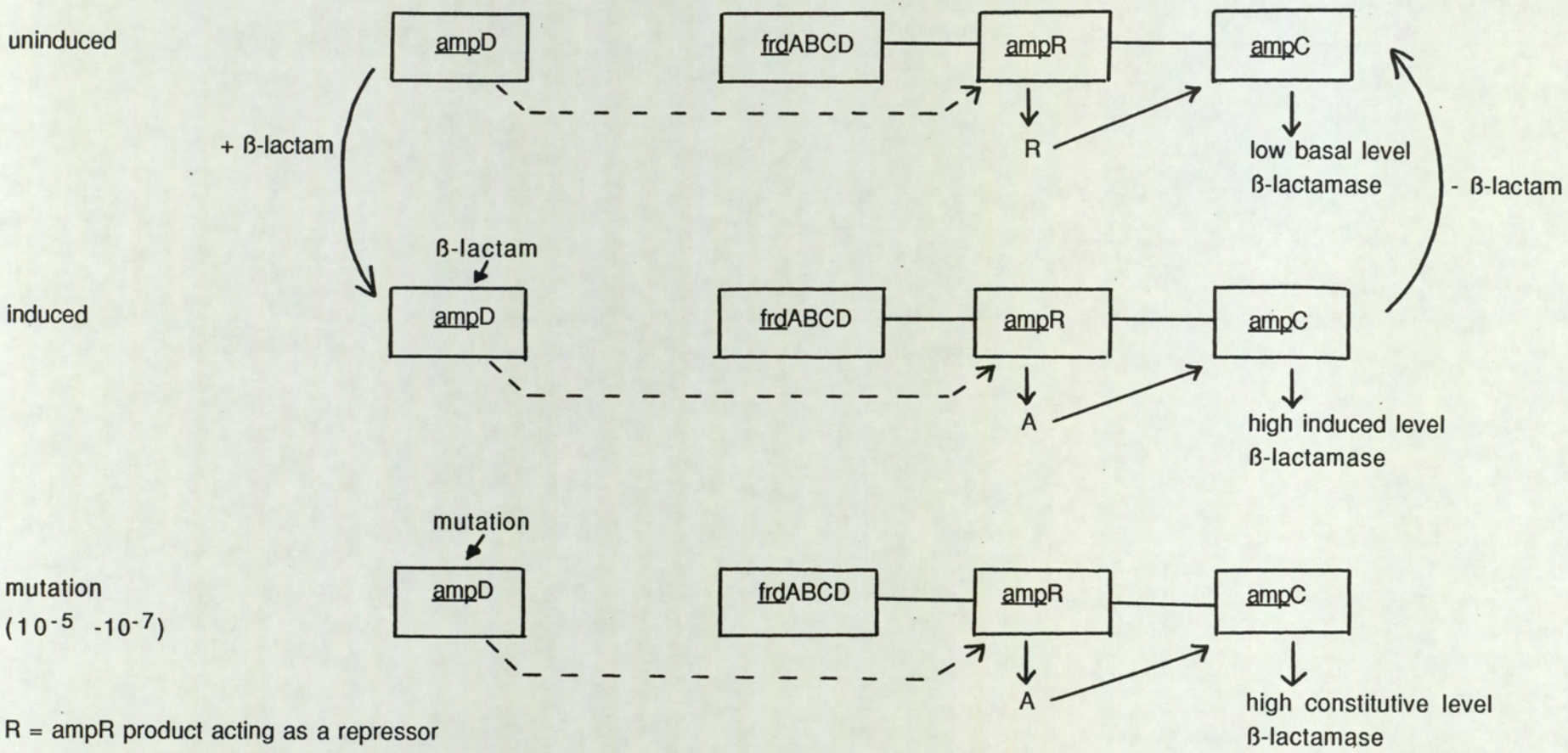
The resistance of the derepressed strains is due to the large amounts of β-lactamase produced (Sanders 1983, Seeberg et al 1983, Then and Angehern 1982). Recently Sanders et al (1983) described a possible method of overcoming such resistance. They found that in some Gram-negative bacterial strains clindamycin was capable of diminishing production of

β-lactamase, without inhibiting growth of the bacterial cells. Although at present this data has very limited clinical applicability, they suggest that this might provide a new approach for enhancement of antibacterial activity of certain β-lactam antibiotics.

The study of expression of β-lactamase from the cloned β-lactamase genes of E.cloacae and C.freundii has provided an insight into the control of constitutive overproduction of β-lactamase in stably derepressed bacteria (Lindberg et al 1985, 1987, Nicolas et al 1987). AmpR expression is essential for constitutive overproduction. However the mutation leading to stable derepression maps outside the ampR-ampC region of both C.freundii (Lindberg et al 1985, 1987) and E.cloacae (Nicolas et al 1987). This gene outside the ampR-ampC region has been denoted ampD (Lindberg et al 1987; see also Figure 5). It is present in non-inducible as well as β-lactamase inducible strains and is fully complementable between all species. This would suggest that its interaction with the rest of the system is very well conserved (Lindberg et al 1987). It is possible therefore that this gene plays a part in some process in cell wall metabolism. Mutations, or in the case of induction the action of β-lactam antibiotics, could lead to the accumulation or disappearance of a substrate, intermediate or end product. In E.coli β-lactamase expression would not be affected, but in C.freundii or E.cloacae, the change would be sensed by the ampR gene product. This would lead directly or indirectly to the induction or stable derepression of the C.freundii or E.cloacae β-lactamase (Lindberg et al 1987).

Summary of class I β-lactamase regulation: the genetic control of β-lactamase production in species possessing inducible class I β-lactamase is summarised in Figure 5. This figure illustrates how, in the uninduced state (absence of β-lactam), the ampR product represses production of the ampC β-lactamase, whereas in the induced state (presence of β-lactam) the ampR product activates expression of the ampC β-lactamase causing high levels of β-lactamase production. This process of induction is reversible, the removal of β-lactam leading to the uninduced state where low levels of ampC β-lactamase are expressed. A mutation in the ampD region causes the ampR product to activate production of the ampC β-lactamase, causing high levels of β-lactamase production. This process is irreversible and β-lactamase is produced constitutively.

FIGURE 5. Regulation of beta-lactamase production



R = ampR product acting as a repressor
A = ampR product acting as an activator

Contribution of β-lactamases to β-lactam antibiotic resistance.

Production of β-lactamase enzyme is the single most important mechanism of resistance to β-lactam antibiotics. A large number of treatment failures with the newer expanded-spectrum β-lactam antibiotics have been reported (Sanders and Sanders 1985). In most reports like these the resistant organism is found to be a stably derepressed mutant of the original infecting organism which is first reported as sensitive to many of the newer β-lactams (Beckwith and Jahre 1980, Follath et al 1987, Murray et al 1983, Olson et al 1983, Quinn et al 1987, Sanders et al 1982a, Weinstein 1986). Although in one such case the mechanism appeared to be through induction rather than stable derepression of β-lactamase (Sanders et al 1982a). An important contributing factor to the selection of stably derepressed mutants is the site of infection. Areas with larger numbers of bacteria and/or poorer antibiotic penetration appear to enhance the selection of these mutants (Weinstein 1986). In vivo studies by Xerri et al (1982) demonstrated the importance of the site of infection on the efficacy of β-lactamase-mediated resistance. These workers compared a rat paw model with a peritoneal infection of M.morganii. In the rat paw there were many bacteria within a restricted area, resulting in high concentrations of both intra- and extra-cellular β-lactamase and rapid inactivation of cefazolin. In contrast in the peritoneal cavity bacteria were spread throughout a large volume of the body cavity. Consequently the effects of β-lactamase were diluted and the concentrations of cefazolin were unaffected by the low

concentration of β-lactamase in the peritoneal cavity. Other *in vivo* studies have implicated β-lactamase as the main mechanism of β-lactam resistance, by demonstrating a reduction in resistance when a β-lactamase inhibitor is added (Bartlett *et al* 1983, Boon *et al* 1982) and still others by measuring the rate of drug breakdown with the use of radiolabelled antibiotic (O'Keefe *et al* 1978, 1979). A number of clinical studies have also demonstrated β-lactamase activity in body fluids taken from sites of infections (Bryant *et al* 1980, Masuda and Tomoika 1977).

As mentioned previously, new plasmid mediated β-lactamases, resistant to the expanded spectrum β-lactams are now emerging. There is therefore the potential widespread distribution of these β-lactamases to consider alongside the threat of spread of stably derepressed mutants throughout hospital flora. It is reasonable to assume that β-lactamases will continue to play the leading role in β-lactam resistance for some time to come. Of course β-lactamases are not the sole factors determining β-lactam resistance. As described previously in sections 1.6.1. and 1.6.2. they interact with permeability barriers and PBPs in effecting resistance. When the β-lactamase defences of the Gram-negative bacteria, especially the β-lactamase inducible species, have been overcome there is then the possibility that permeability and/or PBP mutations will take over their role as the major β-lactam antibiotic resistance determinant.

1.7. Aims of this study.

It is clear that resistance of *E.cloacae* and other Gram-negative bacteria is most profoundly influenced by the production of an inducible class I chromosomal β-lactamase (Livermore 1988, Medeiros 1984, Piddock and Wise 1985). Permeability through the porin channels (Nikaido 1985) and the affinity of the PBPs for the β-lactam antibiotic (Tipper 1985) also play a role in β-lactam resistance. However, resistance mediated through the latter mechanisms occurs relatively infrequently in the clinical situation, when compared to β-lactamase mediated resistance. All three mechanisms of resistance operate in the Gram-negative envelope, the composition and structure of which is profoundly influenced by the growth environment (Brown and Williams 1985a, b).

The purpose of this study was to investigate the influence of the growth environment upon the production of class I β-lactamase, porin or PBP expression in *E.cloacae*. Since β-lactamase-mediated resistance is the most common mechanism of resistance, the effect of the growth environment was studied in detail, including: comparison between *in vivo* and *in vitro* expression of β-lactamase, specific nutrient deprivation *in vitro*, growth rate *in vitro* and induction of β-lactamase by a third generation cephalosporin. In addition, the effects of specific nutrient deprivation on porin and PBP expression were examined.

2. MATERIALS AND METHODS

Addresses of suppliers and manufacturers are given in section 2.11.

2.1. Bacterial strains.

Five strains of *E.cloacae* and three strains of *Escherichia coli* were used in this study. Details of these strains are listed in Table 5. All strains were identified using the API-20E system (API Laboratories Ltd).

2.2. Clinical material.

Approximately 250ml of urine was obtained from each of three patients with *E.cloacae* urinary tract infections. Details of the patients clinical condition, antibiotic treatment and of the strain of *E.cloacae* isolated are given in Table 6. The urine was stored at +4°C for less than 12 hours before collection in each case. Viable counts were performed to determine the concentration of infecting organisms, and the bacteria harvested from the infected urine immediately. Samples of the pellet were plated onto Nutrient agar and MacConkey agar and also examined microscopically by Gram stain and wet film preparations to determine the purity of the infected urine. The identity of the infecting organism was determined using API-20E. The pellet was then used to prepare crude β-lactamase (see section 2.9.1.).

TABLE 5. Bacterial strains used in this study.

Species	strain	description	source
E.cloacae	E7	NCTC 11582 serotype O13	Colindale, UK
	E12	$P99^{+}$	N.A.C.Curtis, ICI Macclesfield, UK.
	E13	Clinical isolate[a]	Sandwell Hospital, Birmingham, UK.
	E14	Clinical isolate	Sandwell Hospital, Birmingham, UK.
	E16	Clinical isolate	Queen Elizabeth Hospital, Birmingham, UK.
E.coli	I113	NCTC 10418	Colindale, UK.
	I124	DCO, K-12, nal^{R}	N.A.C.Curtis, ICI, Macclesfield, UK.
	I150	$OmpF^{+}OmpC^{+}PhoE^{-}$ No. PC2911	Tomassen, Utrecht University, The Netherlands.

a Clinical data is given in Table 6.

TABLE 6. Clinical data of isolates of E.cloacae used in this study

Strain	Patient Age/sex	Clinical condition	Treatment[a]	Source
E13	83/F	total bed rest E.cloacae UTI[b]	none	Sandwell Hospital Birmingham
E14	79/F[c]	hip replacement E.cloacae UTI	none	Sandwell Hospital Birmingham
E16	85/F	Carcinoma of cervix Diabetes mellitus Congestive cardiac failure E.cloacae UTI	trimethoprim	Queen Elizabeth Hospital Birmingham

a treatment refers to treatment being given when the infected urine was collected for this study

b UTI = urinary tract infection

c this patient had previously been treated with cephradine, but was receiving no treatment at the time the infected urine was collected.

2.3. Antimicrobial agents.

Antibiotic powders were obtained as gifts from the following Pharmaceutical Companies: ampicillin and benzyl-penicillin (Beecham Pharmaceuticals); mezlocillin (Bayer); piperacillin and tetracycline (Lederle Laboratories); cephaloridine, cefuroxime and ceftazidime (Glaxo Group Research Ltd); cefoxitin (Merck, Sharp and Dohme Ltd); cefotaxime and gentamicin (Roussel Laboratories Ltd); chloramphenicol (Warner Lambert); nitrocefin was purchased from Oxoid Ltd; and benzyl [^{14}C] penicillin potassium was purchased from Amersham International plc.

2.4. Chemicals and media.

All chemicals were of analytical grade and supplied by BDH Chemicals Ltd. unless otherwise stated. Chemically defined medium (CDM) was prepared initially as described by Williams (1983) for Klebsiella aerogenes DL1 (see Table 7). The individual media constituents were prepared as sterile concentrated stock solutions, sterilised by autoclaving at 121°c for 15 minutes. The complete CDM was aseptically reconstituted immediately prior to inoculation. Nutrient agar (Lab 8) was obtained from Lab M, MacConkey agar (Oxoid CM7), Iso-sensitest agar (Oxoid CM471) and Iso-sensitest broth (Oxoid CM473) were obtained from Oxoid Ltd. They were prepared according to the manufacturers instructions and sterilised by autoclaving at 121°c for 15 minutes. Sterile, heat inactivated human serum used as a growth medium was obtained from Flow Laboratories. Urine used as a growth medium was obtained from equal numbers of male and female

TABLE 7. Summary of the medium composition used for each nutrient depletion

Nutrient	Depletion (mM)					
	C	N	Mg	K	PO_4	Fe
Glucose	*[a]	35.0	35.0	35.0	35.0	35.0
NH_4Cl	25.0	*	25.0	25.0	25.0	25.0
$MgSO_4$	0.4	0.4	*	0.4	0.4	0.4
KCl	1.5	1.5	1.5	*	1.5	1.5
Na_2HPO4/NaH_2PO_4 (pH 7.4)	66.7	66.7	66.7	66.7	*	66.7
$FeSO_4$	0.02	0.02	0.02	0.02	0.02	*
MOPS[b] (pH 7.4)	0.0	0.0	0.0	0.0	60.0	0.0
NaCl[c]	45.2	45.2	45.2	45.2	45.2	45.2
Trace elements [d]						

a nutrient under investigation, used at various concentrations

b MOPS = 3-(N-morpholino)propane sulphonic acid, adjusted to pH 7.4 with concentrated NaOH.

c non-essential for growth, included to achieve physiological osmolarity

d Trace elements also included in medium: $CaCl_2$ (5.0×10^{-7}), H_3BO_3 (5.0×10^{-7}), $CoCl_2$ (5.0×10^{-8}), $CuSO_4$ (5.0×10^{-8}), $ZnSO_4$ (5.0×10^{-8}), $MnSO_4$ (1.0×10^{-8}) and $(NH_4)_6Mo_7O_{24}$ (5.0×10^{-9}).

Based on CDM2+Fe (Williams 1983)

healthy volunteers. The urine was pooled and sterilised by filtration (glass fibre pre-filter and 0.2µm cellulose acetate membrane filter) before use as a culture medium.

2.5. Equipment.

Equipment not specified in text:

Automatic pipettes	Gilson pipetman P20, P200, P1000 and P5000, Anachem; Digital multichannel pipette, Flow Laboratories.
Balances	macro, Oertling HC22; micro, Sartorius 1702.
Centrifuges	Beckman J2-21 High speed, Beckman L8-M Ultracentrifuge.
French press	Amicon corp.
Filtration equipment	Clamped glass funnel, with 47mm, 0.2µm cellulose acetate filter pads, Millipore.
Gel drier	Model 224, Bio-Rad.
Gel electrophoresis	
apparatus	Large (250mm x 200mm x 1mm) made in house by Aston Services.
power pack	Model 500/200, Bio-Rad.
Incubator	Gallenkamp orbital shaking incubator
Laser Densitometer	Ultroscan Laser Densitometer LKB2202 run by an Apple II Europlus computer (Gelscan software).
Microscope	Wild model M20, Microscopic Instruments Ltd.
Osmometer	Knauer Regler.

pH Meter	PTI-15, Fisons Scientific Apparatus.
Photography	Nikon FG camera, Nippon, with Ilford Pan F film or Kodak technical pan film 2415.
Sonicator	Soniprep 150, MSE Scientific Instruments.
Spectrophotometers	Pye Unicam SP500 UV or SP6-400 UV.
Whirlimix	Fisons Scientific Apparatus.

2.6. Growth conditions.

2.6.1. Preparation of glassware.

All glassware was soaked overnight in EXTRAN 100 (BDH) rinsed six times in single distilled and three times in double distilled water. The glassware was air dried at 60°c, closed with aluminium foil and heat sterilised at 160°c for 3 hours. For iron-deprivation experiments, glassware was additionally immersed in a 0.01% solution of ethylenediaminetetra-acetic acid di-sodium salt (EDTA) for 18 hours. The glassware was then rinsed six times in double distilled water.

2.6.2. Batch culture.

Batch cultures were grown in Ehrlenmeyer flasks containing not more than one quarter of their volume of media. Flasks were incubated at 37°c on an orbital shaker (180rpm).

2.6.3. Continuous culture.

Continuous culture in a chemostat enables the effects of growth rate to be studied on bacteria grown under defined

conditions of nutrient availability. In the chemostat fresh media is constantly supplied and spent media and bacterial cells are constantly removed. The culture is mixed thoroughly and the volume is kept constant. A set constant flow rate (F), together with a constant volume (V) defines the dilution rate (D);

$$D = F/V$$

which under steady state conditions is equal to the growth rate of the population (μ). Growth rate can be calculated from the equation;

$$\mu = D = \frac{\log_e 2}{t_d}$$

where t_d is the doubling time. The operator can therefore define the growth rate of the population by manually adjusting the flow rate of the fresh medium into the culture vessel. The population density of the culture is controlled by the concentration of limiting nutrient in the inflowing medium, and consequently may also be controlled by the operator.

Chemostat cultures were grown in 50ml all-glass air-lift chemostats (Gilbert and Stuart,1977) in iron-limited CDM at various growth rates. The chemostats were allowed to stabilize for ten doubling times, at which point steady state was reached and bacteria could be harvested. Samples were removed directly from the culture vessel in volumes of not more than 5ml so that the equilibrium was subject to minimal disturbance.

2.7. Standard microbiological techniques.

2.7.1. Measurement of bacterial numbers.

Bacterial numbers were measured by viable counts, total counts and spectrophotometrically.

Viable counts. Viable counts were made using two methods; the spread plate method of Crone (1948) and a modified Miles and Misra (1938) method. Tenfold serial dilutions were made in sterile physiological saline. For the spread plate method volumes of 100µl were plated, in triplicate, on well-dried nutrient agar to produce 50-200 colonies per plate. For the Miles and Misra (1938) method drops of 50µl were deposited, in triplicate, on well-dried nutrient agar to produce 10-20 colonies per drop. For both methods the plates were incubated for 18 hours at 37°c.

Total counts. A Coulter Counter (model ZM with channeliser C256) was used to determine total counts in chemostat experiments. The sample was diluted in Isoton II (Coulter Counter) and counted three times.

Optical density. Measurements based upon optical density values are the most convenient for following changes in bacterial concentration during growth. At low concentrations the light scattered by a bacterial suspension is directly proportional to the concentration of bacteria in the suspension.

This relationship is expressed by the Beer-Lambert law:

$$OD \propto \log \frac{I_0}{I}$$

where I_0 = intensity of the incident light

I = intensity of the emergent light

provided that the light path is constant.

The relationship between measured and calculated OD at 420nm is linear for E.cloacae suspensions between a measured OD of 0.03 and 0.25. Above an OD of 0.25, this relationship does not apply due to secondary scattering of light (Meynell and Meynell, 1970). All suspensions with an OD exceeding this value were therefore diluted to restore linearity. An absorption wavelength scan of cell-free supernatants of iron-replete and iron-depleted stationary phase cultures showed that absorption by pigments and metabolic products was negligible at 420nm. This wavelength was therefore used for spectrophotometric growth measurements. An optical density of 1.0 at 420nm indicates a concentration of approximately 10^9 E.cloacae per ml.

2.7.2. Antibiotic suceptibility determination.

The minimum inhibitory concentration (MIC) of each compound was determined by the agar dilution method and for gentamicin, chloramphenicol, tetracycline, piperacillin, cefotaxime, ceftazidime and cefuroxime a microtitre tray broth method was also used.

Agar dilution method. E.cloacae strains E7, E12, E13, E14, E16 and a fully sensitive control strain of E.coli (NCTC 10418) were grown overnight in Iso-sensitest broth. The overnight cultures were used to inoculate dried, freshly poured Iso-sensitest agar plates, which contained a range of concentrations of each antibiotic. Inocula of 10^4 and 10^6 CFU were used to test all strains; the inocula were obtained by transferring 1µl of both the neat and a 10^{-2} dilution of each overnight culture to the surface of each plate using a multipoint inoculating device (Denly-Tech Ltd.). All plates were incubated at 37°c for 24 hours. The MIC of the antibiotic was defined as the concentration (in micrograms per milliliter) at which there was an estimated 99% reduction in the original inoculum.

Microtitre tray broth method. An inoculum of 10^5 CFU.ml^{-1} was used to test all strains in various growth media; urine, iron-supplemented urine, human serum, horse serum, iron-replete CDM, iron-deprived CDM, magnesium-deprived CDM, phosphate-deprived CDM or potassium-deprived CDM. The inoculum was obtained by adding 50µl of a suitable dilution of overnight culture in the appropriate growth medium to 50µl of an appropriate dilution of antibiotic in the same growth medium. The microtitre plates were incubated at 37°c for 24 hours. The MIC was taken as the concentration of antibiotic at which no visible growth occurred.

2.8. Nutrient limitation studies.

At a constant pH and temperature the rate of growth of an organism is dependant upon the concentrations of the nutrients in the growth medium. In the absence of toxic products, the onset of a slowing of growth rate at the end of exponential phase can therefore be assumed to correspond to the onset of depletion of a particular nutrient. When all other ingredients are in excess, the cell mass at this point is proportional to the initial concentration of that nutrient. By varying the initial nutrient concentrations a series of growth curves are obtained from which a growth medium can be constructed. The growth medium is defined in such a way that it may be varied to permit depletion of any individual ingredient, with all others in excess (Klemperer et al, 1979) .The nutritional requirements of E.cloacae E7 were determined in this way for carbon, nitrogen, phosphate, iron, magnesium and potassium. Strain E7 (NCTC 11582) was chosen for media development, to avoid the possibility of determining the nutritional requirements of an atypical or uncommon strain of E.cloacae.

2.8.1. Method of inoculation.

A series of growth curves of E7 were prepared for each nutrient under investigation, using OD_{420} as an indicator of cell mass. Growth depletion experiment inocula were prepared from an overnight culture of E7 grown in a chemically defined medium (see Table 7) in which all nutrients were present in excess, except for the nutrient under investigation. For each nutrient under investigation, the E7 inoculum was resuspended

in flasks containing fresh prewarmed media in a range of concentrations of that particular nutrient, to give an initial OD_{420} of 0.025 (approximately $1x10^7$ $CFU.ml^{-1}$). All limiting nutrient concentrations were present in duplicate. Growth of E7 was followed by measuring changes in OD of the culture with time until the bacteria were in stationary phase. Sampling volumes for OD measurements were kept as low as possible (<1ml) and constant between the flasks containing different nutrient concentrations. From the growth curves the OD_{420} at the onset of depletion was plotted graphically against initial nutrient concentration for each nutrient under investigation. From these graphs the concentration of each nutrient predicted to give an OD_{420} of 15 was calculated. Using this information it was possible to vary the composition of the medium to allow depletion of one particular nutrient, all other nutrients being in excess (i.e. sufficient to support growth to OD_{420} 15).

2.8.2. Determination of iron by atomic absorption spectrophotometry.

Atomic absorption spectrophotometry was used to determine the level of iron in iron-depleted CDM. A Perkin-Elmer manually loaded spectrophotometer (model 560) with a deuterium background corrector was used. A 20µl sample was loaded into the graphite furnace (model HCA 74 with argon gas flow), the analysis conditions were controlled using a model HGA 500 programmer. The levels of iron were determined from a calibration curve constructed using commercial standards

(Spectrosol grade, BDH Ltd.). The operational conditions of the spectrophotometer were as specified by the manufacturer.

2.8.3. Measurement of medium osmolality.

The osmolality of CDM was measured on a Knauer osmometer using commercial standards (Fiske Associates Incorporated) for the plotting of a linear calibration curve of osmolality versus machine reading (%). The osmolality of CDM was then adjusted to a physiological value (300mOsm.Kg^{-1}; Diem 1974) by adding NaCl.

2.9. Analysis of β-lactamase production by E.cloacae.

2.9.1. Preparation of crude β-lactamase.

In vivo bacteria. In vivo bacteria were obtained directly, without subculture, from approximately 250ml samples of infected urine. The urine was centrifuged (1400xg, 15min, 4°c) to remove non-bacterial cells and debris. Bacteria were harvested and washed in 50mM sodium phosphate buffer pH7 (15000xg, 15min, 4°c) then sonicated on ice (5x30secs, maximum power, MSE soniprep 1mm diameter probe, with 30secs intervening cooling). The sonicate was clarified by centrifugation (120000xg, 40min, 4°c) and the cell-free supernatant used as the crude β-lactamase preparation.

In vitro bacteria. In vitro, bacteria were grown in batch culture urine, iron-supplemented urine, human serum, horse serum, iron-replete CDM, iron-deprived CDM, magnesium-deprived CDM, phosphate-deprived CDM or potassium-deprived

CDM. The *in vitro* grown bacteria were prepared in a similar manner to the *in vivo*. However after washing in buffer, bacteria were resuspended to $OD_{420}10$ and were disrupted by French pressing twice (14000psi). The protein content of each crude β-lactamase preparation was determined using the method of Lowry *et al* (1951).

2.9.2. Typing of β-lactamase.

To determine the number of β-lactamases produced by each strain of *E.cloacae*, each crude β-lactamase preparation from CDM+Fe was analysed by isoelectric focusing. The isoelectric point of the β-lactamase also indicates whether the enzyme is plasmid or chromosomally encoded (Matthew *et al*, 1975). Antibiograms were performed to determine whether or not the β-lactamase produced was inducible.

Analytical isoelectric focusing. Isoelectric focusing (IEF) is an electrophoretic technique for separation of proteins according to their isoelectric points (pI) in a stable pH gradient. All electrophoretic separations of proteins make use of the fact that each protein has a net charge that varies with the pH of its environment. This net charge represents the sum of positive and negative charges on the surface of a protein. When passing from a very low pH to a very high pH, the net charge changes in a continuous manner from plus to minus. At a certain well defined pH, the net charge equals zero. This point is called the isoelectric point and this is the basis of protein separation by isoelectric focusing. Proteins differing by only a few

hundredths of a pH unit in their pIs may be resolved by this technique. To separate β-lactamases the method of Matthew et al (1975) was used. IEF was performed using an LKB 2117 Multiphor system. Thin layers (1mm thick) of polyacrylamide were mounted on thin glass plates with good heat-conducting properties. The composition of the gelling solution is given in Table 8. After mixing the acrylamide, BIS, glycerol and ampholine, the solution was made up to 60ml in distilled water and de-gassed for at least 10 minutes in a 250ml flask. The APS was then added and the complete gelling solution mixed by swirling the flask gently. TEMED was not required for polymerisation as the addition of ampholine provides enough amino groups for polymerisation. After polymerisation the mould was stored at 4°c for 30 minutes to facilitate dismantling. The covering thick glass plate was then carefully removed leaving the gel supported by one glass plate. A template (LKB) was placed on the cooling plate of the multiphor and wet thoroughly. The glass plate supporting the gel was then lowered onto the template, avoiding trapping air bubbles. Electrode strips soaked in the appropriate electrode solution (see Table 9a.) were placed on the ends of the long sides of the gel as indicated by the template. β-Lactamase samples (see above for preparation) were loaded onto the gel using small rectangular pieces of filter paper (Whatman 3mm Paratex, LKB) so that each loading contained approximately 100μg protein. Experimental time and electrical conditions are shown in Table 9b. All experiments were performed at 25 Watts constant power and at 10°c. After 1 hour the filter paper was removed. Marker proteins (LKB) of

TABLE 8. Composition of gels for isoelectric focusing.

Stock solutions		Composition of broad range gel (ml) (pH 3.5-9.5)
Acrylamide (29.1% w/v)		10.0
Bis (0.9% w/v)		10.0
Glycerol (87%)		7.0
1809 Ampholine	pH 4-6	0.2
	pH 5-7	0.2
	pH 9-11	0.4
	pH 3.5-10	2.8
Make up solutions to 60ml in distilled water, de-gas for 10 minutes on a vacuum pump (pressure: 20-25" mercury)		
Ammonium persulphate (1% w/v) freshly made		1.5
	mix solutions then fill mould	

TABLE 9. Conditions for isoelectric focusing:

a) electrode solutions.

pH range	anode	cathode
3.5 - 9.5	1M H_3PO_4	1M NaOH

b) electrical conditions.

pH range	voltage and current[a]				time (h)
	initial		final		
	V	mA	V	mA	
3.5 - 9.5	310	78	970	25	1.5

a constant power (25W)

known pI values were used to plot a linear calibration curve of pI versus distance from the anode (mm). β-Lactamase was detected using nitrocefin ($800\mu g.ml^{-1}$), 25-30µl was spread over one sample area at a time using a glass rod spreader and the distance from the anode to the first pink-coloured band(s) to show were measured. The pink bands were formed by the hydrolysis of nitrocefin by the focused β-lactamases.

Antibiogram. Each E.cloacae test organism and a fully sensitive control strain of E.coli (NCTC 10418) were plated onto Iso-sensitest agar to give single colonies after overnight incubation at 37°C. From these plates 6-10 colonies were suspended in 10ml sterile water. Each bacterial suspension was swabbed over the surface of dried Iso-sensitest agar plates. Freshly prepared 30µg discs of cefuroxime, cefoxitin, cefotaxime, cefuroxime and mezlocillin were placed in a semicircle, equidistant to each other, on each plate. The plates were incubated for 18 hours at 37°c. Zone sizes were assigned sensitive or resistant by comparison with a fully sensitive control (E.coli I113, see Table 5 for details). A resistant zone was taken as a zone diameter that was 6mm (or more) smaller than the control for the same antibiotic. The areas between cefotaxime and cefoxitin, plus cefuroxime and cefoxitin were examined for "blunting" - a feature where the zone sizes in these areas are diminished, due to antagonism between the antibiotics (Sanders and Sanders 1979). Drops of nitrocefin were placed on the edge of the cefoxitin zone and on an antibiotic free area. The rate of colour development in the two areas was visually compared.

If the cefoxitin area went rapidly pink compared to the antibiotic free area, this was taken as an indication of β-lactamase induction by cefoxitin.

2.9.3. Induction of β-lactamase.

In batch cultures, cefotaxime was added at varying concentrations (0, 0.5, 1, 2, 4 and 8 $\mu g.ml^{-1}$) to exponential phase cultures of E13 growing in CDM-Fe or urine, (for explanation of choice of this agent see section 5.6.5). Bacteria were harvested after two generations further growth. In chemostat cultures of E13 cefotaxime was added ($4\mu g.ml^{-1}$) in as small a volume as possible; simultaneously the inflowing medium was changed to one containing cefotaxime ($4\mu g.ml^{-1}$). In this manner the cefotaxime concentration was brought to the required level instantly and maintained at that level for the duration of the experiment. Control samples were removed from the chemostats before the addition of cefotaxime and further samples removed at a number of generations after. The samples were harvested and prepared for β-lactamase assay.

2.9.4. Selection of mutants of E.cloacae E13 and E16 with stably derepressed β-lactamase.

In this study cefotaxime was used to select for spontaneous stably derepressed mutants in β-lactamase expression of E.cloacae strains E13 and E16. Overnight cultures of E13 and E16, grown in Iso-sensitest broth were harvested (15000xg, 10min, 20°c) and resuspended in 50mM sodium phosphate buffer pH7, to give three concentrations of bacteria; 10^{10}, 10^{9} and

10^8 CFU.ml^{-1}. 100µl aliquots of each concentration were plated in triplicate onto Iso-sensitest agar plates containing cefotaxime (1 or 2µg.ml^{-1}). The plates were incubated at 37°c for 18 hours. Colonies growing on these plates were tested for β-lactamase production by adding a drop of nitrocefin to the side of the colony. Those colonies showing rapid nitrocefin hydrolysis were subcultured onto antibiotic-free Iso-sensitest agar. The antibiotic susceptibility of each putative mutant was measured. The strain (or clone) with the highest MIC value was taken as the stably derepressed mutant and referred to as E13DR1 or E16DR1. The expression of β-lactamase in these strains was measured to confirm stable derepression of β-lactamase and to determine whether this derepression was total or partial.

2.9.5. Measurement of specific β-lactamase activity and kinetic parameters.

Specific enzyme activity was determined using the method of O'Callaghan et al (1972) and was performed on a Pye-Unicam SP6-400 UV spectrophotometer, with a thermostatically regulated cuvette cell holder (37°c) and Euroscribe strip chart recorder (Gallenkamp). Stock solutions of 50mM sodium phosphate buffer pH7 and nitrocefin (0.516mg.ml^{-1} dissolved in 10% (v/v) dimethyl sulphoxide (DMSO), and 50mM sodium phosphate buffer pH7) were required prewarmed to 37°c. 0.1ml stock nitrocefin and 0.8ml phosphate buffer were mixed in a 1cm cell with a total volume of 1ml, which was placed in the spectrophotometer. 0.1ml of the crude β-lactamase preparation (or an appropriate dilution in phosphate buffer) was added

into the cuvette *in situ* using a Micro Lang-Levy pattern glass pipette (Gallenkamp) and mixed thoroughly. The chart recorder (speed $1cm.min^{-1}$) recorded the initial rate of hydrolysis as the appearance of the red hydrolysed product of nitrocefin at 482nm. Each rate was determined six times to give a mean value.

To determine the kinetic parameters K_m and V_{max} the same experimental conditions were used. However, the nitrocefin hydrolysis was allowed to go to completion and the full progress curve was recorded. K_m and V_{max} were calculated from this progress curve using the method of half-time analysis described by Nichols and Hewinson (1987) and based on the theoretical work on half-time analysis of a single reaction curve by Wharton and Szawelski (1982). In this method a Michaelian kinetic mechanism is assumed and the equation:

$$t_{1/2} = \frac{S_0}{2V_{max}} + \frac{K_m \ln 2}{V_{max}}$$

may be obtained by integration of the Michaelis-Menten equation (Wharton and Szawelski, 1982). Where S_0 is the local substrate concentration at any time on the reaction progress curve and the half-time, $t_{1/2}$, is the time taken for any S_0 to fall to $S_0/2$. The procedure is therefore to measure $t_{1/2}$ for several different S_0 values and plot graphically $t_{1/2}$ versus S_0. The slope of the straight line thus obtained has a value $1/2V_{max}$ and the intercept, when the line is extrapolated to zero S_0, has the relation:

$$t_{1/2} = \frac{K_m \ln 2}{V_{max}}$$

It is assumed that complete hydrolysis of the substrate (nitrocefin) has occurred at the end of the reaction. This method was checked using classical Lineweaver-Burk plots (Lineweaver and Burk 1934).

2.10. Electrophoretic analysis of envelope components of E.cloacae.

2.10.1. Preparation of outer membranes.

Bacteria were grown in batch culture in 250ml volumes of various growth media; urine, iron-supplemented urine, human serum, horse serum, iron-replete CDM, iron-deprived CDM, magnesium-deprived CDM, phosphate-deprived CDM or potassium-deprived CDM, for 18 hours. The bacteria were harvested (15000xg, 10mins, 4°c) and resuspended to approximately $OD_{420}10$. Bacteria were disrupted by French pressing twice (14000psi) then sodium-lauryl sarcosinate (sarkosyl; Sigma Chemicals Co.) was added to a final concentration of 2% (w/v) and incubated at room temperature for 45 minutes. Sarkosyl selectively solubilises the inner membrane portion (Filip et al 1973). Any whole cell debris was removed by centrifugation (15000xg, 10mins). The outer membrane fraction was then collected by centrifugation (120000xg, 40mins) and the pellet resuspended in a small amount of water. The protein content of each sample was determined by the method of Lowry et al (1951) and stored at -20°C until required.

2.10.2. Isolation of non-covalent peptidoglycan-associated proteins.

In order to distinguish which of the major outer membrane proteins were porins, the proteins which were tightly, but not covalently bound to peptidoglycan were isolated using the method of Mizuno and Kageyama (1979). Membranes were prepared as above. The sample was then extracted in SDS (2% SDS, 10% glycerol, and 10mM Tris-HCl buffer pH7.8 in 0.1M NaCl) for 30 minutes at 60°c to solubilize non peptidoglycan-associated proteins (Mizuno and Kageyama, 1979). The insoluble fraction was collected by centrifugation (120000xg, 40mins, 25°c). The extraction was repeated twice and the peptidoglycan protein complex obtained was washed once in distilled water before being resuspended in distilled water and stored until required at -20°c.

2.10.3. Preparation of penicillin-binding proteins.

Preparation of bacterial cell envelopes. Bacteria were grown in batch culture in various growth media; Iso-sensitest broth, urine, iron-supplemented urine, human serum, iron-replete CDM, iron-deprived CDM, magnesium-deprived CDM, phosphate-deprived CDM or potassium-deprived CDM. 500ml of fresh prewarmed medium was inoculated aseptically using a 2% inoculum from an overnight culture in the same medium. Growth was monitored by OD at 420nm until OD_{420} 0.9-1.0, at which point the bacteria were harvested (15000xg, 10min, 4°c). The pellet was resuspended to OD_{420} 10 in 50mM sodium phosphate buffer pH7, containing 140mM 2-mercaptoethanol (BDH) and 10%

v/v glycerol. Bacteria were disrupted by French pressing twice (14000psi). The suspension was centrifuged (10000xg, 5mins, 4°c) to remove any unbroken cells, then the supernatant was ultracentrifuged (120000xg, 40mins, 4°c). The pellet was washed in 50mM sodium phosphate buffer pH7, containing 10% v/v glycerol at 4°c and reultracentrifuged (120000xg, 40mins, 4°c). The pellet was resuspended in 500µl of 10mM sodium phosphate buffer pH7 at 4°c and frozen rapidly in liquid nitrogen. A small amount (≤30µl) was retained for assay for protein by the method of Lowry et al (1951). Protein concentrations of preparations were diluted if necessary to 10mg.ml^{-1} prior to assay.

Assay of penicillin-binding proteins in cell envelope preparations. The assay of penicillin-binding proteins (PBPs) from bacterial cell envelope preparations was essentially as described by Spratt (1977a) with minor modifications. Initially the saturating concentration of [^{14}C] penicillin G required was determined for all strains of E.cloacae. Envelope preparations of strains E7, E12, E13, E14 and E16 grown in Iso-sensitest broth were thawed to room temperature. The preparations were then adjusted to a protein concentration between 5 and 10mg.ml^{-1} (maximum 10mg.ml^{-1}) with 10mM sodium phosphate buffer pH7 at 25°c. For each strain, 100µl volumes of envelope preparation were aliquoted into nine separate, labelled "Eppendorf" tubes and prewarmed to 30°c in a water bath for 10mins. A vial of [^{14}C] penicillin G (50µci.mMole^{-1}) was reconstituted and diluted in 10mM sodium phosphate buffer pH7, 25°c, to give nine different concentrations; 1.1, 11, 55, 110, 220, 330, 550,

825 and 1100µg.ml^{-1}. 10µl of each dilution was added to the prewarmed envelope preparations, so that for each strain there were nine envelope preparations labelled with graded concentrations of radiolabelled penicillin (final concentrations; 0.1, 1, 5, 10, 20, 30, 50, 75 and 100 µg.ml^{-1}). The preparations were mixed rapidly and incubated at 30°c for 10 minutes. The reaction was terminated by the addition of 5µl of unlabelled penicillin G (120mg.ml^{-1}, ie. a saturating concentration). After mixing well this was immediately followed by the addition of 10µl 20% w/v sarkosyl. After careful mixing the assay was incubated at room temperature for 20 minutes, then ultracentrifuged (100000xg, 30mins, 10°c, AP Pegasus with "Eppendorf" tube attachment, MSE Scientific Instruments). 100µl of the supernatant, containing the sarkosyl soluble inner membranes, was carefully removed and placed in a fresh tube. 37µl sample buffer (containing 2.5ml 0.5M Tris-HCl buffer pH6.8, 2.0ml glycerol, 2.0ml 10% w/v SDS, 0.1ml 0.5% w/v bromophenol blue) was added and mixed well, followed by 13µl of 2-mercaptoethanol. The preparations were boiled for three minutes at 100°c in a heating block and then stored at -20°c until required for electrophoresis. The saturating concentration of labelled penicillin was taken as the lowest concentration of penicillin used which enabled all PBPs of all strains of E.cloacae to be visualised by fluorography (section 2.10.5), and above which the addition of more penicillin did not enhance the visualisation. The value obtained for E.cloacae E7, E13 and E16 was 30µg.ml^{-1} and for E12 and E14 was 75µg.ml^{-1}. For all further assays the same

procedure described above was followed, except that only one concentration of labelled penicillin was used for each strain; [^{14}C] penicillin G was reconstituted to 330 or 825 $\mu g.ml^{-1}$ and 10µl added to each 100µl envelope preparation. E.coli I124 was included as a control strain, this was labelled with 30 $\mu g.ml^{-1}$ [^{14}C] penicillin G.

2.10.4. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs and PBPs.

All electrophoresis was performed on a vertical system (200mm x 250mm)using 1mm spacers and with a discontinuous buffer system using an SDS-Tris glycine buffer (57.6g glycine, 12g Tris, 2g SDS made up to 2 litres with distilled water). 10% polyacrylamide gels with the ratio of acrylamide to bis-acrylamide of 30:0.3 were used for electrophoresis of both OMPs and PBPs (see Table 10 for composition). The chemicals used were of biochemical or electrophoretic grade and were mixed thoroughly, adding the APS (BDH) and TEMED (BDH) last. TEMED initiated polymerisation. The separating gel was poured between the plates of the electrophoresis equipment and allowed to set, with a layer of 0.1% w/v SDS overlaid to ensure a horizontal surface. When the gel had set, the 0.1% SDS was poured off and the stacking gel poured carefully on top. A sample slot-forming comb was carefully inserted, without trapping air bubbles, and the gel allowed to set. After the gel had polymerised the sample wells were washed with 0.5M Tris-HCl buffer pH6.8, diluted 1:4 with distilled water containing 0.1% SDS, in order to remove any acrylic acid present.

TABLE 10. Composition of gels for SDS-PAGE.

Stock solutions	Volume of solution added (ml)	
	separating gel	stacking gel
Acrylamide	33.3	2.0
1.5M TRIS-HCl buffer pH8.8	25.0	0.0
0.5M TRIS-HCl buffer pH6.8	0.0	2.5
10% (w/v) SDS	1.0	0.1
Distilled water	39.6	5.3
10% (w/v) APS	1.0	0.1
TEMED	0.05	0.01

Acrylamide solution contains 30% (w/v) acrylamide: 0.3% (w/v) BIS

BIS is N,N-methylene bisacrylamide

SDS is sodium-dodecyl sulphate

APS is ammonium persulphate

TEMED is N,N,N',N'-tetramethylethylene diamine

(Laemmli 1970)

SDS-PAGE of OMPs. The OMP preparation was denatured by mixing with an equal volume of freshly made sample buffer (containing; 1ml 0.5M Tris-HCl buffer pH6.8, 2ml 10% w/v SDS, 1ml glycerol, 0.1ml 2-mercaptoethanol, 2ml distilled water and bromophenol blue as a tracking dye) and heating to 100°c for 5 minutes. Samples, containing 20-60$\mu g.ml^{-1}$ protein (final concentration), were loaded into each well and electrophoresis carried out at room temperature at a constant current of 30mA per gel, until the tracking dye had moved 20cm from the interface of the stacking and separating gels. The gel was fixed and stained in 0.1% Coomassie Brilliant Blue (BDH) in 50% v/v methanol/10% v/v acetic acid and destained in 5% v/v methanol/10% v/v acetic acid. Protein standards (lysozyme 14.3K, carbonic anhydrase 29K, egg albumin 45K, bovine albumin 66K, phosphorylase B 97.4K and β-galactosidase 116K, Sigma) were used to construct a linear calibration curve of $\log_{10}$ molecular weight of protein standards against R_f, where

$$R_f = \frac{\text{distance moved by standard protein (mm)}}{\text{distance moved by bromophenol marker (mm)}}$$

The molecular weights of the OMPs were therefore determined from their R_f values using this calibration curve. The percentage of major OMPs that were peptidoglycan linked, were determined by scanning the Coomassie stained gels with a laser densitometer. Outer membrane preparations from E.coli I150 grown in Iso-sensitest broth were run on each gel as a control.

SDS-PAGE of PBPs. 50µl of boiled PBP assay was added to each well. Electrophoresis was carried out at room temperature at a constant current of 40mA per gel for three to four hours, until the bromophenol blue marker (in the sample buffer) had moved 10cm from the interface of the stacking and separating gels. The gels were fixed and stained with PAGE Blue 83 in 50% v/v methanol/10% v/v acetic acid and destained in 5% v/v methanol/10% v/v acetic acid.

2.10.5. Fluorography of PBP gels.

The destained PBP gels were soaked in approximately 250ml Amplify (Amersham International), containing 10% v/v glycerol, for 30 minutes with shaking. Amplify is an aqueous based scintillant which when impregnated into the PBP gel increases the sensitivity of [^{14}C] fluorography 15-fold and reduces the exposure time of the film to the gel. The gels were dried on thick filter paper (13CHR Whatman) on a gel drier for 2½ hours under a vacuum. The dried gels were pre-exposed (1-5mins exposure at approximately 3m to Ilford 904 safelight) to pre-fogged Amersham Hyperfilm MP, sandwiched between 1/16" aluminium sheets, wrapped in two light proof plastic bags and stored at -70°c for 12 weeks. The films used for fluorography were pre-fogged to allow the reactivity of the silver particles in the film to become linear and hence shorten exposure time. These films were pre-exposed to the safelight to allow for "dark adaption" - i.e. if the fluor-impregnated substrate was exposed to sunlight or certain types of fluorescent light, the light induced fluorescence of

the fluor would have caused the film to blacken. The films were developed in an automatic kodak RP developer.

2.11. Addresses of suppliers and manufacturers:

Amersham International, Lincoln Place, Green End, Aylesbury, Bucks, UK.
Amicon, 57, Queens Road, High Wycombe, Bucks. UK.
Anachem, Charles Street, Luton, Beds. UK.
API Laboratories Ltd. Grafton Way, Basingstoke, Hamps. UK.
Bayer, Bayer House, Strawberry Hill, Newbury, Bucks.
BDH Chemicals Ltd, Broom Road, Poole, Dorset, UK.
Beckman RIIC Ltd. Turnpike Road, Cressex Industrial Estate, High Wycombe, Bucks. UK.
Beecham Pharmaceuticals, Great West Road, Brentford, Middx. UK.
BioRad Laboratories Ltd. Caxton Way, Watford, Herts. UK.
Coulter Electronics Ltd. Northwell Drive, Luton, Beds. UK.
Denly Instruments Ltd. Nalts lane, Billingshurst, West Sussex, UK.
Eppendorf, Gerätebau, Nethelera and Hinz Gamh, West Germany.
Fiske Associates Incorporated, Uxbridge, Massachusets, USA.
FSA. Bishop Meadow Road, Loughborough, Leics. UK.
Flow Laboratories, Woodcock Hill, Harefield Road, Rickmansworth, Herts. UK.
Gallenkamp, Belton Road, West Loughborough, Leics. UK.
Glaxo Group Research Ltd. Greenford Road, Greenford, Middx. UK.
Knauer Regler, Berlin, West Germany.
Lab M, Topley House, Bury, Lancs, UK.
Lederle Laboratories, Fareham Road, Gosport, UK.
LKB Instruments Ltd. 232, Addington Road, Selsdon, South Croydon, Surrey, UK.
Merck, Sharp and Dohme Ltd. Four Marks, Alton, Hants. UK.
Microscopic Instruments Ltd. 7, Little Clarendon Street, Oxford, UK.
Millipore (UK) Ltd. 11-15, Peterborough Road, Harrow, Middx. UK.
MSE Scientific Instruments, Sussex Manor Park, Crawley, Sussex, UK.
Nippon Kogaku KK, Chiyoda-ku, Tokyo 100, Japan.
Oertling Ltd. Orpington, Kent, UK.
Oxoid Ltd. Wade Road, Basingstoke, Hamps. UK.
Perkin-Elmer Ltd. Post Office Lane, Beaconsfield, Bucks, UK.
Pye Unicam Instruments, Cambridge, UK.
Roussel Laboratories Ltd. Broadwater Park, North Orbital Road, Uxbridge, Middx. UK.
Sartorius Instruments Ltd. 18 Arenue Road, Belmont, Surrey, UK.
Sigma Chemical Co. Ltd. Fancy Road, Poole, Dorset, UK.
Warner Lambert Health Care, Mitchell House, Southhampton Road, Easleigh, Hamps. UK.

3. NUTRITIONAL REQUIREMENTS OF E.CLOACAE.

3.1. Nutrient deprivation studies of E.cloacae E7.

E.cloacae E7 was grown in duplicate in eight different concentrations of each nutrient under investigation. These concentration ranges are listed in Table 11.

Growth curves were plotted (OD_{420} versus time, see section 2.8.1.) for each concentration. An example of the results obtained using this method is given for carbon (glucose) deprived E7 cultures (Figure 6). Mean generation times or doubling times were measured for the log phase bacteria. (Stanier et al 1977). From this value, the growth rate (μ) can be calculated using the equation:

$$\mu = \frac{\ln 2}{t_d}$$

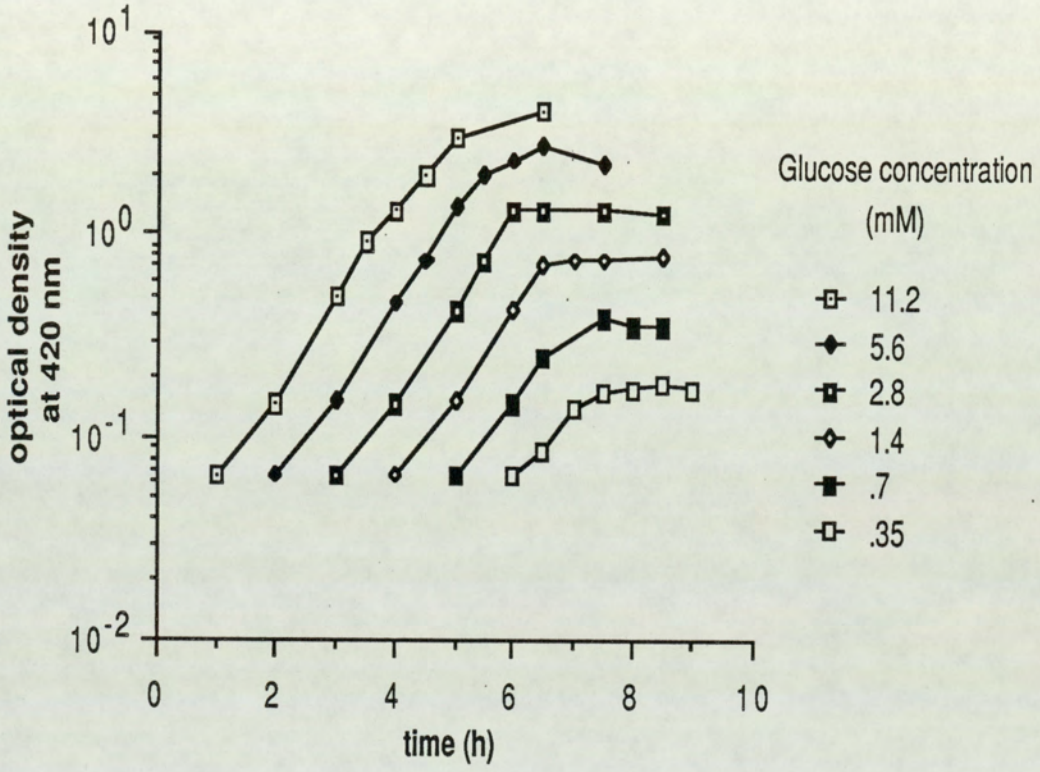
The growth rate of E7 during log phase was little changed (t_d +/- 1 min, μ +/- 0.02 h^{-1}) through the range of concentrations examined for each nutrient (e.g. see Figure 6). The growth rate was also very similar under different nutrient deprivations (t_d 40-46 min, μ 0.90-1.04 h^{-1}; see Table 11). However the onset of stationary phase, and hence the duration of log phase, varied with the initial concentration of nutrient. The OD_{420} at which the onset of stationary phase occurred was plotted against initial nutrient concentration (Figure 7). The relationship between OD_{420} and initial nutrient concentration was linear for all nutrients up to approximately OD_{420} 2.0.

TABLE 11. Growth rate of E7 under different nutritional deprivations

Limiting nutrient	Concentration range (M) of added nutrient	doubling time t_d (min)	growth rate μ (h^{-1})
Carbon (glucose)	0 - 1.12×10^{-2}	42 ± 1	0.99 ± 0.02
Nitrogen (NH_4Cl)	0 - 2.50×10^{-2}	42 ± 1	0.99 ± 0.02
Iron ($FeSO_4$)	0 - 2.00×10^{-5}	40 ± 1	1.04 ± 0.02
Magnesium ($MgSO_4$)	0 - 4.00×10^{-5}	46 ± 1	0.90 ± 0.02
Phosphate (Na_2HPO_4/NaH_2PO_4)	0 - 6.67×10^{-2}	45 ± 1	0.92 ± 0.02
Potassium (KCl)	0 - 1.50×10^{-3}	42 ± 1	0.99 ± 0.02

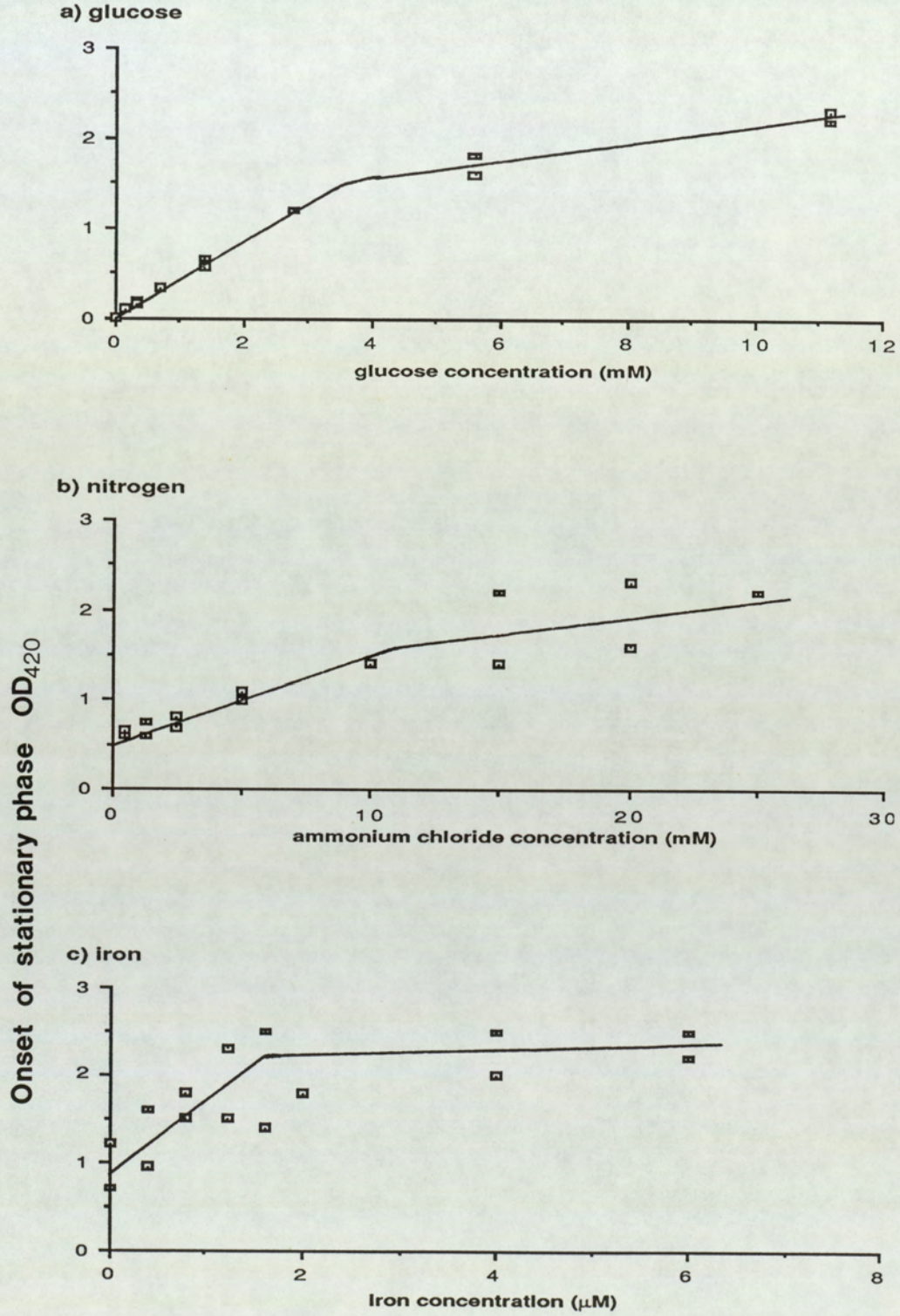
E.cloacae E7 was grown in chemically defined medium (CDM) under the different nutrient limitations listed above. In each case E7 was grown in duplicate in eight different concentrations of the limiting nutrient. The concentration ranges of each nutrient are listed above. Doubling times (t_d) and growth rate (μ) values represent the mean value of the cultures for each nutrient limitation.

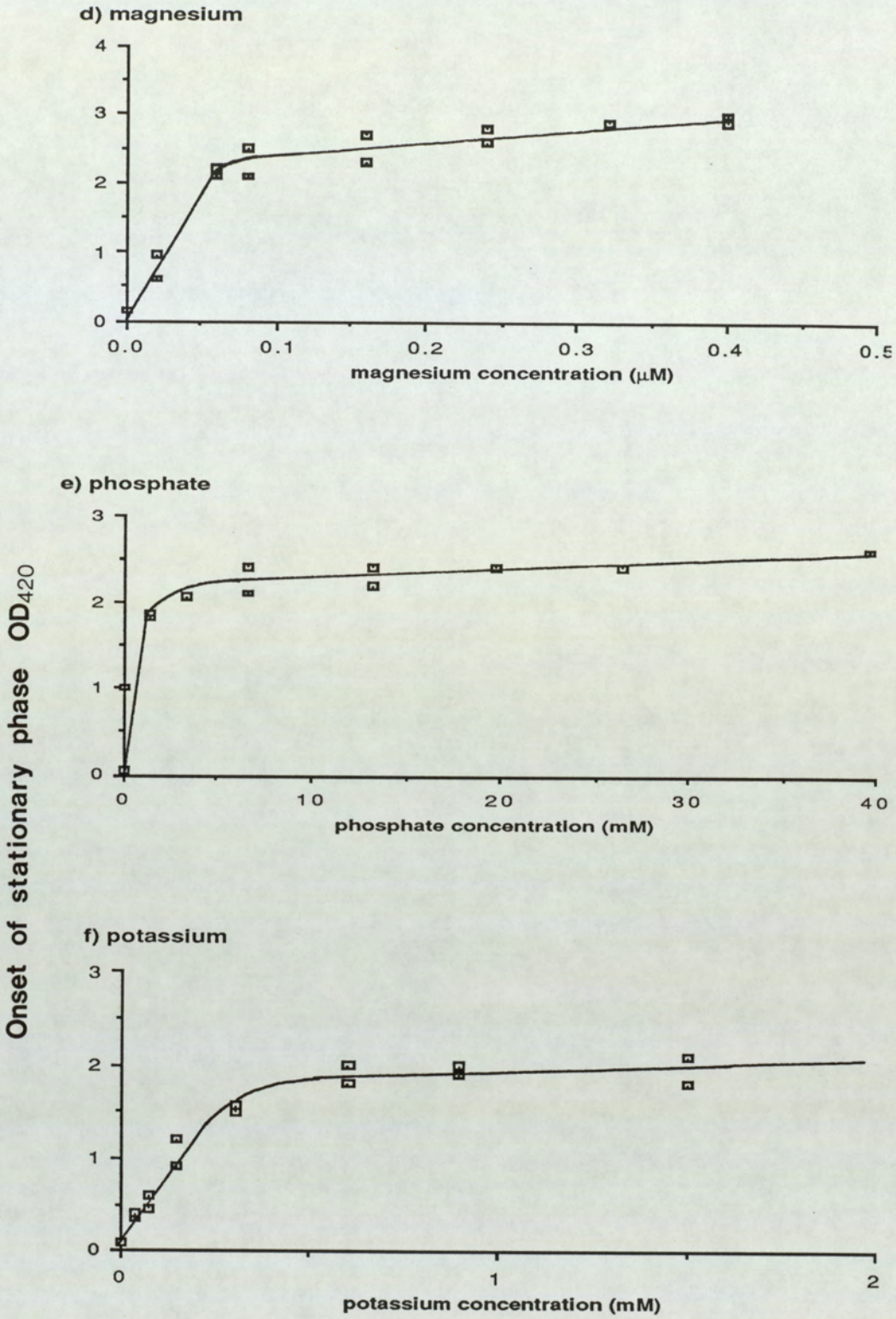
FIGURE 6. Growth of E7 under carbon deprivation.



Growth of E.cloacae E7 in chemically defined medium (CDM) containing six different concentrations of glucose. The growth curves have been offset for clarity and are in reality superimposable. No growth was observed in cultures containing 0.07 or 0.0 mM glucose. Note how the final OD_{420} is influenced by the initial nutrient concentration. Growth rate is identical in log phase of all cultures.

FIGURE 7. Relationship between onset of stationary phase and initial nutrient concentration for growth of E7





The onset of stationary phase of each culture varies with initial nutrient concentration of limiting nutrient. The results from two separate experiments are represented graphically above. The relationship, is linear for all nutrients up to approximately OD_{420} 2.0 and up to this point the nutrient is limiting. After this point, the nutrient is in excess of the requirements of E7. Using this data, a chemically defined medium (CDM) can be constructed in which each essential nutrient may be included at concentrations leading to cell depletion at a predetermined absorbance, or in known excess.

After this point the addition of greater nutrient concentrations than this did not alter the time (or OD_{420}) at which the onset of stationary phase began (i.e. bacteria did not grow exponentially above OD_{420} 2.0):the nutrient was now in excess of the requirements of E7 and this strain was probably deprived by another nutrient or prevented from further growth by inadequate oxygenation or a build up of toxic products. If the line representing the relationship between onset of stationary phase and initial nutrient concentration is extrapolated back through the x axis, the contaminating level of this nutrient in the growth medium can be determined. For example, this line passes through zero for glucose, indicating that no other carbon source was present other than the glucose added. In contrast in the case of iron, even if no $FeSO_4$ was added to the CDM, iron was still present in the growth medium as it was present as an impurity in the other nutrients added to the CDM. Contaminating levels of iron in CDM-Fe were determined more accurately by atomic absorption spectrophotometry (see section 3.2).

From the graphs in Figure 7, initial concentrations of each nutrient which would support the exponential growth of E7 to OD_{420} 1.0 (CDM 1) were determined (see Table 12). These values when multiplied by a factor of 15 provided a CDM in which all nutrients were present well in excess of the requirements of E7. The phosphate, due to its role as a buffer as well as an essential nutrient, was included at an even higher concentration (x 133). This CDM with all nutrients in excess is referred to as CDM+Fe (see Table 12).

TABLE 12. Final composition of CDM used for growth of E.cloacae under different nutrient deprivations.

Nutrient	concentration of each nutrient added (mM) [a]					
	CDM 1[b]	CDM+Fe	CDM-Fe	CDM-Mg	CDM-P	CDM-K
Glucose	2.4	36.0	36.0	36.0	36.0	36.0
NH_4Cl	5.0	75.0	75.0	75.0	75.0	75.0
$MgSO_4$	0.025	0.4	0.4	0.025	0.4	0.4
KCl	0.15	2.3	2.3	2.3	2.3	0.15
Na_2HPO_4/NaH_2PO_4	0.5	66.7	66.7	66.7	0.5	66.7
$FeSO_4$	0.0002	0.03	0.0	0.03	0.03	0.03
MOPs	-	0.0	0.0	0.0	60.0	0.0
NaCl[c]	-	38.0	38.0	50.0	15.0	43.0

a - each CDM composition also includes the trace elements listed in Table 7

b - CDM 1 = concentration of nutrients that will support exponential growth of E7 to OD_{420} 1.0. These values were obtained from the graphs in Figure 7 and are multiplied by a factor of 15 where excess nutrient is required. Phosphate was included at an even higher concentration (approximately 130-fold higher than CDM 1) as it was also acting as the buffer

c - non-essential for growth, included to bring osmolality up to physiological value (see section 3.3)

A chemically defined medium (CDM) was constructed in which all nutrients were present in known excesss (CDM+Fe) and in which iron, magnesium, phosphate and potassium could be included at limiting concentrations (CDM-Fe, CDM-Mg, CDM-P, CDM-K) leading to an onset of stationary phase at approximately OD_{420} 1.0. These CDMs were used in all nutrient deprivation experiments for E.cloacae

Magnesium-, phosphate- and potassium-deprived CDM were obtained by including the nutrient to be deprived at the initial concentration that is given for CDM 1 (see Table 12; CDM-Mg, CDM-P and CDM-K). Iron deprived CDM (CDM-Fe) was obtained by omitting $FeSO_4$ (Table 12).

3.2. Determination of iron by atomic absorption spectrophotometry.

Three constituents of CDM: phosphate buffer, glucose and ammonium chloride were found to contribute measurable contaminating levels of iron in CDM-Fe. Each of these nutrients were available in two grades of purity; Analar and Aristar (BDH). The contribution of iron contamination by these nutrients to CDM-Fe is given in Table 13. Since the Aristar quality chemicals provided approximately half the level of iron contamination compared to Analar, the Aristar chemicals were used for CDM-Fe.

3.3. Determination of medium osmolality.

The osmolality of CDM+Fe, CDM-Fe, CDM-Mg, CDM-P and CDM-K was measured by freezing point depression. The values obtained were; 230, 230, 209, 273 and 222 milliosmoles (mOs) respectively. The addition of sodium chloride; 38, 38, 50, 15 and 43mM to each respective CDM (Table 12) brought their osmolality up to a value of 300 $mOsm.Kg^{-1}$, which is the physiological osmolality of serum (Diem 1974).

TABLE 13. Contribution of iron contamination by various components of CDM

Nutrient	Grade (BDH)	Final concentration in CDM (mM)	Level of iron contamination in CDM (μM)
NH_4Cl	Analar	75.0	0.036
	Aristar	75.0	0.008
Glucose	Analar	36.0	0.041
	Aristar	36.0	0.016
Na_2HPO_4/	Analar	66.7	0.993
NaH_2PO_4	Aristar	66.7	0.571
Complete CDM	Analar	NA	1.07
	Aristar	NA	0.60

NA = not applicable

Aristar chemicals were used in iron-deprived chemically defined medium (CDM-Fe) as these contained the lowest level of iron contamination. Iron contamination was measured by atomic absorption spectrophotometry.

TABLE 14. Growth rates of E7, E12, E13, E14 and E16 in CDM+Fe

E.cloacae strain	doubling time t_d (min)	Growth rate μ (h^{-1})
E7	42	0.99
E12	48	0.87
E13	48	0.87
E14	42	0.99
E16	45	0.92

CDM+Fe was designed initially for E.cloacae E7. Strains E12, E13, E14 and E16 grew in CDM+Fe at comparable growth rates, indicating that they have similar nutritional requirements. No complex growth factors (e.g. vitamins) were necessary for growth.

3.4. Growth of E7, E12, E13, E14 and E16 in CDM+Fe.

The CDM+Fe was designed initially for E7. Once the media design was complete, the four other E.cloacae strains used in this study (E12, E13, E14 and E16) were grown in this medium. Their doubling times and growth rates are compared in Table 14. The growth rates obtained were very similar (t_d; 42-48 mins, μ; 0.87-0.99 h^{-1}) indicating that the CDM+Fe was providing excess nutrients for all five strains and that none of these E.cloacae strains required additional growth factors (e.g. vitamins). The CDM designed for E7 was therefore used for all five strains throughout this study.

3.5. Discussion.

Bacteria, like all living cells, require the essential nutrients carbon, nitrogen, phosphorus, sulphur, potassium, magnesium and iron in a metabolisable form in order to grow. In an organism's natural environment, availability of these essential nutrients will frequently be restricted (Brown 1977). When nutrient depleted, a dividing bacterium will manufacture an envelope characteristic of this depletion (Brown 1977) and this may result in changes of bacterial sensitivity to antibiotics (Brown 1977, Melling and Brown 1975). The aim of this study was to examine the properties of E.cloacae which determine its resistance to β-lactam antibiotics under such conditions of nutrient depletion. For this purpose the nutritional requirements were quantified for E.cloacae E7 by the method of Klemperer et al (1979), leading to the development of a chemically defined medium (CDM) for E.cloacae. The major advantage of such a CDM over other

simple salts media is that all essential nutrients have been defined quantitatively and each may be included at concentrations leading to cell depletion at a predetermined absorbance or in known excess.

The nutritional requirements of K.pneumoniae DL1 (Williams 1983), E.coli $RP1^+$ and $RP1^-$ (Klemperer et al 1979) and P.aeruginosa NCTC 6750 (Noy 1982) have previously been determined. The minimum requirements for these strains to grow exponentially to OD 1.0 are given in Table 15 for comparison with those of E.cloacae E7. This table also includes values of the normal ranges of these nutrients present in the urine of normal female and male adult humans on an average diet (Diem and Lentner 1975). The presence of all the nutrients of CDM in urine indicates that urine, like CDM, will act as a growth medium. Of course many other nutrients are present in urine and their content is highly variable. Other carbon sources than glucose are present, for example glucuronic acid, glycoproteins, mucopolysaccharides and glycopeptides (Diem and Lentner 1975). Glucose is only likely to be the major carbon source in cases of badly controlled diabetes mellitus, when high levels of glucose are excreted into the urine (Diem and Lentner 1975).

All strains of E.cloacae used in this study grew in the CDM at similar growth rates, indicating that they were not auxotrophic organisms. When all nutrients were present in excess, all strains grew to a final OD_{420} of approximately 10. Under nutrient deprivations, in most cases bacteria grew to a final OD_{420} of approximately 1.5-2, except when iron deprived, when they grew to OD_{420} 4-5. This higher OD_{420} for

TABLE 15. Nutritional requirements of K.pneumoniae, E.coli, P.aeruginosa and E.cloacae.

Nutrient added	Concentration of nutrient required for exponential growth to OD 1.0[a]					Nutrient concentration in urine from an average adult[b] (extreme range 95%)
	K.pneumoniae	E.coli		P.aeruginosa	E.cloacae	
	DL1	RP1⁻	RP1⁺	NCTC 6750	E7	
Glucose ($\times 10^{-3}$M)	3.5	2.5	2.8	4.0	2.4	0.09-0.73
NH_4^+ ($\times 10^{-3}$M)	2.5	1.9	1.8	4.0	5.0	20-70
PO_4^{3-} ($\times 10^{-4}$M)	2.2	1.7	5.6	3.2	5.0	258-645
Mg^{2+} ($\times 10^{-5}$M)	2.4	1.3	2.5	4.0	2.5	490-1650
K^+ ($\times 10^{-5}$M)	7.5	2.6	4.2	6.2	15.0	3500-8000
Fe^{2+} ($\times 10^{-7}$M)	2.4	-	1.0	6.2	2.0	<10

a) The concentration of each nutrient required to enable the organism to reach an OD of 1.0 was obtained from plots of onset of stationary phase against initial added nutrient concentation. Data shown in this table were obtained from Klemperer et al (1979), Noy (1982) and Williams (1983).

b) The composition of urine is highly variable. The data shown in this table was obtained from Documenta Geigy (Diem and Lentner1975) and is representative of the urine of an average adult on an average diet.

iron deprived bacteria was due to the difficulty in removing contaminating levels of iron from the media. In urine bacteria grew to a final OD_{420} of approximately 2, indicating that the bacteria were deprived of some essential nutrient. The iron requirement of E7 was similar to that of the other enterobacteria and was lower than that of Pseudomonas, hence it is more important to remove contaminating levels of iron from the iron-deprived growth media of enterobacteria than Pseudomonas species. The nutritional requirements of E7 for other nutrients were also similar to those of other enterobacteria.

4. ANTIBIOTIC SUSCEPTIBILITY OF E.CLOACAE AND THE EFFECT OF GROWTH MEDIUM.

4.1. Antibiotic susceptibilities of E.cloacae: agar dilution MIC method.

The susceptibilities of E.cloacae E7, E12, E13, E14, E16 and E.coli I113 were initially determined using the agar dilution method as described in section 2.7.2. The results are given in Table 16. E.coli I113 was included as a sensitive control and was sensitive to all of the antibiotics tested. Strains E7, E13 and E16 had similar susceptibility patterns. These strains were resistant to ampicillin, to the first and second generation cephalosporins; cephaloridine and cefuroxime and also to the cephamycin, cefoxitin. However they were sensitive to the expanded spectrum third generation cephalosporins; cefotaxime and ceftazidime, and to the ureidopenicillin, piperacillin. Differences in size of inocula affected the MIC values to cefuroxime, cefotaxime and piperacillin, being approximately fourfold greater for the larger inoculum. All three strains were sensitive to gentamicin and resistant to tetracycline and chloramphenicol. Strains E12 and E14 had similar susceptibilities to each other; they were both resistant to all the β-lactam antibiotics used in the test. The size of the inoculum did not affect the MIC value. They were both sensitive to gentamicin and resistant to tetracycline and chloramphenicol, however the MIC value of E12 to these antibiotics was much higher than the MIC values of the other four E.cloacae strains. Strain E12 probably harboured a resistance plasmid.

TABLE 16. Antibiotic susceptibilities of E.cloacae determined by the agar dilution method.

Antibiotic	E.coli I113[a]		E.cloacae									
			E7		E12		E13		E14		E16	
	10^4[b]	10^6	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
ampicillin	4	8	128	128	>1024	>1024	1024	>1024	>1024	>1024	256	1024
piperacillin	2	2	2	8	128	256	2	4	128	256	2	8
cephaloridine	<16	<16	512	>1024	512	>1024	>1024	>1024	>1024	>1024	1024	>1024
cefoxitin	<16	<16	64	128	128	256	512	>512	256	512	256	512
cefuroxime	4	4	8	32	>256	>256	32	128	>256	>256	16	64
cefotaxime	0.06	0.06	0.12	0.5	>32	>32	0.25	1	>32	>32	0.5	1
ceftazidime	0.12	0.25	0.25	0.5	>8	>8	0.25	0.5	>8	>8	0.5	1
gentamicin	0.12	0.25	0.25	0.5	0.12	0.25	0.25	0.5	0.25	0.5	0.25	0.5
chloramphenicol	2	2	8	16	>256	>256	8	16	8	16	8	16
tetracycline	1	1	4	4	>256	>256	2	4	4	8	4	8

a E.coli was included as a sensitive control

b MIC values were determined for two inocula 10^4 and 10^6 cfu.ml^{-1}

4.2. Antibiotic susceptibilities of E.cloacae in different growth media.

The susceptibilities of E.cloacae E7, E12, E13, E14 and E16 were determined in duplicate in ten different growth media; Iso-sensitest broth, normal pooled urine, iron-supplemented urine, human serum, horse serum, CDM+Fe, CDM-Fe, CDM-Mg, CDM-P and CDM-K. The results are presented in Table 17. The susceptibilities of each strain determined by the agar dilution method using a 10^4 cfu.ml^{-1} inoculum are included for comparison, since the microtitre tray broth method uses an inoculum of 10^5 cfu.ml^{-1}. The MICs for all five strains of E.cloacae in Iso-sensitest broth were identical to those from the Iso-sensitest agar. The sensitivity of all five strains to gentamicin did not change appreciably whatever medium it was measured in. For the other antibiotics tested, however, there seemed to be a general trend for the MICs in CDM-grown bacteria to be lower by a factor of approximately 2-8. These differences were not caused by the influence of medium osmolality or pH as these values were very similar for all media tested (Table 18).

E7: this strain was resistant to tetracycline and cefuroxime in Iso-sensitest media, urine and human serum, but was sensitive to these antibiotics in horse serum and CDM. It was resistant to chloramphenicol in Iso-sensitest media and urine, but sensitive in both human and horse serum and CDM. It was sensitive to piperacillin, cefotaxime and ceftazidime in all media except human serum. The MIC values for piperacillin, cefotaxime and ceftazidime were higher in Iso-sensitest media and urine than horse serum and CDM, even

TABLE 17. Antibiotic susceptibility of E.cloacae grown in different media.

Strain	Antibiotic	MIC [a] values in different media										
		ISA	ISB	UR	UR +Fe	Hu	Hr	CDM +Fe	CDM -Fe	CDM -Mg	CDM - P	CDM - K
E7	gentamicin	0.25	0.125	0.063	0.25	0.25	0.125	0.125	0.063	0.125	0.25	0.25
	chloramphenicol	8	8	16	8	2	2	2	2	2	2	2
	tetracycline	4	4	4	8	>16	0.5	0.5	0.5	0.125	0.5	0.5
	piperacillin	2	1	4	4	>16	0.25	0.25	0.5	0.5	0.25	0.5
	cefotaxime	0.12	0.125	0.125	0.125	16	0.015	0.015	0.015	0.015	0.015	0.015
	ceftazidime	0.25	0.125	0.125	0.063	2	0.031	0.031	0.031	0.031	0.063	0.031
	cefuroxime	8	8	8	4	>16	1	1	0.5	1	1	1
E12	gentamicin	0.12	0.063	0.063	0.25	0.25	0.063	0.125	0.063	0.125	0.063	0.25
	chloramphenicol	>256	256	256	256	128	128	64	64	32	128	64
	tetracycline	>256	>256	256	256	256	>256	128	64	64	64	128
	piperacillin	128	128	256	128	>256	>256	64	128	64	128	64
	cefotaxime	>32	128	64	64	128	256	8	8	8	16	8
	ceftazidime	>8	128	16	16	64	256	4	4	4	8	4
	cefuroxime	>256	>256	256	256	>256	>256	64	64	32	64	64

continued on following page

E13	gentamicin	0.25	0.125	0.125	0.5	0.5	0.25	0.5	0.25	0.125	0.25	0.5
	chloramphenicol	8	8	256	16	64	2	2	2	2	2	2
	tetracycline	2	4	256	2	>256	>16	0.125	0.5	4	0.125	0.25
	piperacillin	2	2	64	0.25	2	>16	0.5	0.5	0.125	0.25	0.5
	cefotaxime	0.25	0.5	8	0.015	0.25	>16	0.015	0.031	0.25	0.015	0.063
	ceftazidime	0.25	<0.25	4	0.015	0.25	>16	0.031	0.031	0.125	0.031	0.063
	cefuroxime	32	32	128	0.5	8	>16	1	1	0.25	0.5	1
E14	gentamicin	0.25	0.125	0.125	0.25	0.25	0.125	0.5	0.125	0.25	0.25	0.25
	chloramphenicol	8	4	256	16	8	4	4	4	2	4	4
	tetracycline	4	4	256	32	32	256	0.5	0.5	0.5	0.5	0.5
	piperacillin	128	>256	>256	64	256	>256	64	64	64	128	64
	cefotaxime	>32	256	128	32	128	256	8	16	16	16	16
	ceftazidime	>8	>256	>256	32	64	>256	4	4	4	8	4
	cefuroxime	>256	>256	>256	256	>256	>256	32	64	32	128	64
E16	gentamicin	0.25	0.125	0.125	0.5	0.25	0.125	0.5	0.25	0.125	0.25	0.5
	chloramphenicol	8	8	16	16	8	8	2	4	2	8	2
	tetracycline	4	4	4	2	4	16	0.25	0.5	0.25	0.25	0.25
	piperacillin	2	2	2	0.25	2	16	8	2	8	0.5	0.5
	cefotaxime	0.5	0.5	0.5	0.063	0.5	>16	4	0.25	0.125	2	0.015
	ceftazidime	0.5	0.25	1	0.031	0.5	16	1	0.5	4	0.125	0.063
	cefuroxime	16	16	2	1	16	16	16	1	0.5	1	1

ISA = Iso-sensitest agar, ISB = Iso-sensitest broth, UR =urine, Hu = human serum, Hr = horse serum, CDM = chemically defined medium

a an inoculum of 10^5cfu.ml^{-1} was used for MIC determination by the microtitre tray broth method

TABLE 18. Osmolality and pH of media used for MIC determination

medium	osmolality (mOs)	pH
urine (1)[a]	480	6.8
urine (2)	316	6.8
human serum	295	7.7
horse serum	243	7.3
CDM+Fe	300	7.2
CDM-Fe	300	7.2
CDM-Mg	300	7.2
CDM-P	300	7.2
CDM-K	300	7.2
ISB[b]	316	7.1

a number in brackets indicates batch of urine, two batches were used

b ISB = Iso-sensitest broth

MIC values were determined for E.cloacae in a number of different growth media. Two factors which can affect the reproducibility of MIC values are osmolality and pH (Lorian 1980). However, in the growth media examined above, values of both osmolality and pH varied very little from physiological values (300mOsm.Kg^{-1}. pH 7).

though all values were still in the sensitive range. Interpretation of sensitivity or resistance through MIC values was achieved using the recommended breakpoint concentrations of these antibiotics (gentamicin 1mg.ml^{-1}, chloramphenicol 8mg.ml^{-1}, tetracycline 1mg.ml^{-1}, piperacillin 16mg.ml^{-1}, cefotaxime 1mg.ml^{-1}, ceftazidime 2mg.ml^{-1} and cefuroxime 4mg.ml^{-1}; Working Party for the BSAC 1988).

E12: this strain was resistant to all the antibiotics except for gentamicin. However, there was still a variability in MIC values for all antibiotics except gentamicin; MIC values were approximately 2-8 fold greater in Iso-sensitest media, urine and serum.

E13: this strain was resistant to piperacillin, cefotaxime and ceftazidime in urine and horse serum, but sensitive to these antibiotics in Iso-sensitest media, iron-supplemented urine, human serum and CDM. It was resistant to chloramphenicol in Iso-sensitest media, urine and human serum and sensitive in horse serum and CDM. With tetracycline it was resistant in Iso-sensitest media, urine, serum and CDM-Mg and sensitive in other nutrient-deprived CDM. With cefuroxime it was resistant in Iso-sensitest media, urine and serum, but sensitive in iron-supplemented urine and CDM.

E14: this strain was resistant to all of the β-lactam antibiotics tested. The MIC values were approximately 2-8 fold greater in Iso-sensitest medium, urine and serum than CDM. It was resistant to chloramphenicol in Iso-sensitest media, urine and human serum, but sensitive in horse serum and CDM. It was also resistant to tetracycline in Iso-sensitest media, urine and serum, but sensitive in CDM.

E16: this strain was resistant to chloramphenicol and tetracycline in Iso-sensitest media, urine and serum, but sensitive in CDM (except in CDM-P E16 was resistant to chloramphenicol). It was resistant to cefuroxime in Iso-sensitest media, serum and CDM+Fe, but sensitive in urine CDM-Fe, -Mg, -P and -K. It was sensitive to piperacillin, cefotaxime and ceftazidime in most media, but resistant to them all in horse serum. In CDM+Fe and CDM-P it was resistant to cefotaxime and in CDM-Mg it was resistant to ceftazidime.

4.3. Discussion.

4.3.1. Antibiotic susceptibilities of E.cloacae.

The agar dilution method was initially used to provide information on the antibiotic susceptibility patterns of the strains of E.cloacae used in this study. The β-lactam antibiotics were of main interest in this respect. Gentamicin, chloramphenicol and tetracycline were included for comparison with the broth microtitre dilution method which will be discussed in the following pages. As mentioned in section 1.1 E.cloacae was not included in the antibacterial spectra of the earlier β-lactam antibiotics. This point was reflected by the resistance of all five strains of E.cloacae used in this study to ampicillin, cephaloridine, cefuroxime and cefoxitin. The susceptibilities of these same strains to the newer β-lactam antibiotics (cefotaxime, ceftazidime and piperacillin) however, divided the five strains into two groups; those which were sensitive

(E7, E13 and E16) and those which were resistant (E12 and E14) to these drugs. The use of the two inocula in the test (10^4 and 10^6cfu.ml^{-1}) indicated the presence of inducible β-lactamase or mutationally derepressed β-lactamase. If an inducible β-lactamase was present, it had a much greater effect on the larger inoculum. Induction was therefore indicated when the MIC of the 10^6cfu.ml^{-1} inoculum was 4-8 fold greater than that of the 10^4cfu.ml^{-1} inoculum. A difference of twofold was not counted as significant as this lay within the error of the procedure. Such differences were seen with strains E7, E13 and E16 with cefuroxime, cefotaxime and piperacillin, indicating that these strains probably each possessed an inducible β-lactamase. This observation was supported by later work (see section 5.1). In the case of mutationally derepressed β-lactamase MIC values were high and were not affected by the size of the inoculum (i.e. they could not be induced to higher β-lactamase concentrations). Both strains E12 and E14 exhibited this characteristic indicating that they were probably stably derepressed mutants. For strain E12 this was merely confirmation of a known fact as it was a P99$^+$ strain (see Table 5) known to overproduce the P99 β-lactamase constitutively.

4.3.2. Effect of growth medium on antibiotic susceptibilities.

The broth dilution microtitre plate method was used to determine the effect of growth media on the β-lactam susceptibility of all five strains of E.cloacae. The β-lactam antibiotics used in this study were chosen as they represent

some of the newer β-lactam antibiotics in clinical use. Cefuroxime was the oldest of the four, representing the second generation cephalosporins. Cefotaxime and ceftazidime represented the third generation cephalosporins. Piperacillin represented the ureidopenicillins. Increases in β-lactam resistance caused by β-lactamase usually result in large changes of MIC (c.f. E7 and E12 MICs; Table 16). However when changes in porin or PBP expression are responsible the changes in MIC are usually smaller and incremental. As an indicator for changes in porin expression and permeability three non-β-lactam antibiotics were included in this study; gentamicin, chloramphenicol and tetracycline. Any changes in porin expression affecting the permeability of β-lactam antibiotics might also affect these antibiotics. Mutants cross-resistant to β-lactam and aminoglycoside antibiotics through permeability changes have been reported clinically for S.marcescens (Goldstein et al 1983). However, changes in PBP or β-lactamase expression will not affect these antibiotics.

In the strains producing basal levels of β-lactamase (i.e. E7, E13 and E16) there was no evidence of an increase of MIC to the β-lactam antibiotics large enough to suggest overproduction of β-lactamase (through induction or stable derepression) being selected by any growth medium used. However, there were changes in the region of 2-16 fold, not only for β-lactam antibiotics, but also for chloramphenicol and tetracycline, suggesting a possibility of permeability changes of E.cloacae in the various media.

Generally strains E7, E13 and E16 were sensitive to the newer β-lactams (cefotaxime, ceftazidime and piperacillin). However, resistance did occur in human serum (E7), horse serum (E13, E16) and urine (E13) grown bacteria. Even though these strains were sensitive when grown in the remaining media, differences in MIC were apparent. In most cases the MIC value for bacteria grown in Iso-sensitest media, urine or serum was greater (approximately 4-8 fold) than CDM grown bacteria. Similarly with strains E12 and E14 although resistant to the newer β-lactams in all growth media, there were differences of at least 4-8 fold in the MIC values, being lower in the CDM grown bacteria. In general all five strains were resistant to cefuroxime except for CDM grown E7, E13 and E16 which were sensitive. Overall differences between complex media and simple salts medium (i.e. CDM) were reflected by the MIC values for chloramphenicol and tetracycline. The factors which may cause variation in MIC measurements were therefore explored.

A number of factors may affect the determination and reproducibility of an MIC. The density of the inoculum is one of the most important variables. The inocula for broth MIC determinations were standardised to 10^5cfu.ml^{-1} +/- one half a log. Each time the MICs were determined, the inocula were checked by viable count. If any inoculum was too high or low, then the results of that particular MIC were discarded and repeated. Only one factor may have affected the standardisation of the size of the inocula for serum grown bacteria and this was the clumping of bacteria when they grew in this medium. The effects of clumping on the accuracy of

viable counting were reduced by vortexing these bacteria, however they were not completely eliminated.

The pH of the medium may affect the activity of certain antibiotics (Thrupp 1980), therefore the pH of each medium used was measured (Table 18). However, the pH of the media used did not vary greatly from physiological pH (7.2 +/- 0.3). In this study, therefore, pH should not be responsible for the changes of MIC observed.

The osmolality of growth media may also affect the activity of antibiotics (Thrupp 1980) and is also involved in porin regulation (see section 1.6.1). As with pH, the osmolality varied around the physiological value (Table 18; 315 +/- 61mOsm.Kg^{-1}). If osmolality were a major contributor to the changes in MIC observed, then bacteria grown in horse serum, with the lowest osmolality, should consistently have lower MICs than those grown in CDM and this did not occur. Only with strain E7 are the MICs between horse serum and CDM grown bacteria comparable.

Some antibiotics are highly bound to plasma proteins and only the free fraction is microbiologically active. However the clinical significance of this phenomenon is controversial (Craig and Suh 1980, Wise 1985, 1986, Working Party of the BSAC 1988). The protein binding of the antibiotics used in this study were reported by the Working Party of the BSAC (1988) to vary between 20 and 30%. In the same report this group stated that protein binding under 70-80% was unlikely to be significant.

Previous work from this and other laboratories have demonstrated effects of nutrient deprivation and excess on

the sensitivity of bacteria to antimicrobial agents (Brown and Melling 1969, Cozens and Brown 1983, Finch and Brown 1975, 1978). In particular the concentration of divalent cations, especially magnesium and calcium, have a major effect on certain antibiotics, most notably gentamicin (Thrupp 1980). Magnesium deprivation also modifies the response of certain bacteria to antimicrobial agents- notably the effects of EDTA and polymyxin on P.aeruginosa (Brown and Melling 1969, Finch and Brown 1975, Kenward et al 1979). In contrast with some species, for example K.pneumoniae, magnesium deprivation did not affect the organisms response to polymyxin (Williams 1983). The effect of nutrient deprivation on the antibiotic susceptibility of E.cloacae has not yet been studied in detail. The results of this study imply that at least iron-, magnesium-, phosphate- or potassium-deprivation did not greatly affect the sensitivity of E.cloacae to gentamicin, chloramphenicol, tetracycline, piperacillin, cefotaxime, ceftazidime or cefuroxime by the methods used here. The reason for the differences in MIC between the complex and simple growth media were investigated more thoroughly in the following three chapters; the effect of growth media upon β-lactamase, porin and PBP expression was investigated.

5. E.CLOACAE β-LACTAMASE PRODUCTION

5.1. Typing of β-lactamase.

The β-lactamases produced by E.cloacae strains E7, E12, E13, E14 and E16 were identified and classified by isoelectric focusing and by the use of antibiograms. The isoelectric points (pIs) of the β-lactamases from these strains are listed in Table 19. Each strain expressed one major β-lactamase which rapidly hydrolysed nitrocefin and showed as a strong pink band on the gel. In addition to the major bands, several satellite bands occurred, these hydrolysed nitrocefin more slowly and had a weak colour compared to the first band detected. The pI values for the latter β-lactamases are given in parentheses in Table 19. The pI values of β-lactamase preparations from strain E13 grown in different media (i.e. urine, serum and nutrient deprived CDM) were determined. The pI value remained the same, regardless of the growth medium, indicating that the growth media used did not induce production of a different β-lactamase - only one β-lactamase was produced.

The antibiogram results are given in Table 20. E.coli was used as a control strain and was sensitive to all antibiotics used in the test. Nitrocefin hydrolysis and zones of inhibition (blunting) were not detected with E.coli (see also Figure 8a). Strains E7, E13 and E16 had identical antibiogram patterns, and were only resistant to cefoxitin. The zones between cefuroxime (CXM) and cefoxitin (FOX) and also between cefotaxime (CTX) and cefoxitin were blunted (see Figure 8b) indicating antagonism between these antibiotics.

Nitrocefin hydrolysis was rapid when nitrocefin was placed onto bacteria surrounding the cefoxitin disc, but slow when placed on bacteria in antibiotic free areas. This implies that cefoxitin had induced β-lactamase production in those bacteria surrounding the cefoxitin disc. A few single colonies were present in cefotaxime and cefuroxime zones of inhibition (Figure 8b), these were stably derepressed mutants. Strains E12 and E14 also had identical antibiogram patterns. Both strains were resistant to all four antibiotics (Figure 8c) and hydrolysed nitrocefin rapidly in areas both near the cefoxitin disc and in antibiotic free areas. No blunting occurred. These data indicates that both strains were producing high quantities of β-lactamase even in the absence of antibiotic. It is highly probable from the antibiogram patterns observed that strains E7, E13 and E16 produced β-lactamases at low basal levels which were inducible by cefoxitin, whereas strains E12 and E14 were stably derepressed mutants producing high amounts of enzyme constitutively.

TABLE 19. Isoelectric points of ß-lactamases of *E.cloacae*

E.cloacae strain	Isoelectric point (pI)
E7	7.4 (7.8)[a]
E12	7.6
E13[b]	8.8 (8.4) (8.2)
E14	7.6
E16	7.6 (7.9)

a numbers in brackets indicate isoelectric points of satellite bands

b the isoelectric point of this strain was measured using ß-lactamase preparations from bacteria grown in urine, iron-supplemented urine, human serum, horse serum, iron-replete chemically defined medium and also iron-, magnesium-, phosphate- and potassium-depleted chemically defined medium.

TABLE 20. Antibiograms of E.cloacae

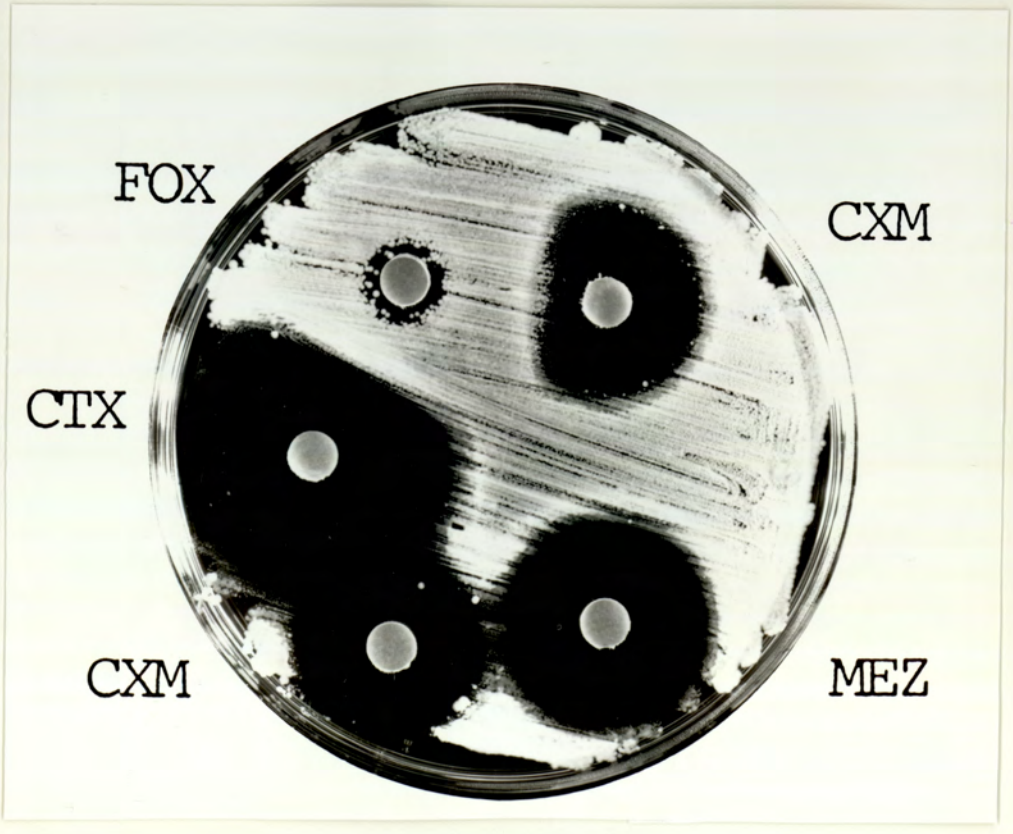
Strain	Sensitivity pattern				Blunting		Nitrocefin hydrolysis	
	CXM	FOX	CTX	MEZ	CXM/FOX	CTX/FOX	FOX	AB Free
E.coli I113	S	S	S	S	-	-	ND	ND
E.cloacae E7	S	R	S	S	+	+	rapid	slow
E.cloacae E12	R	R	R	R	-	-	rapid	rapid
E.cloacae E13	S	R	S	S	+	+	rapid	slow
E.cloacae E14	R	R	R	R	-	-	rapid	rapid
E.cloacae E16	S	R	S	S	+	+	rapid	slow

CXM = cefuroxime, FOX = cefoxitin, CTX = cefotaxime, MEZ = mezlocillin, AB = antibiotic, ND = none detected, S = sensitive, R = resistant.

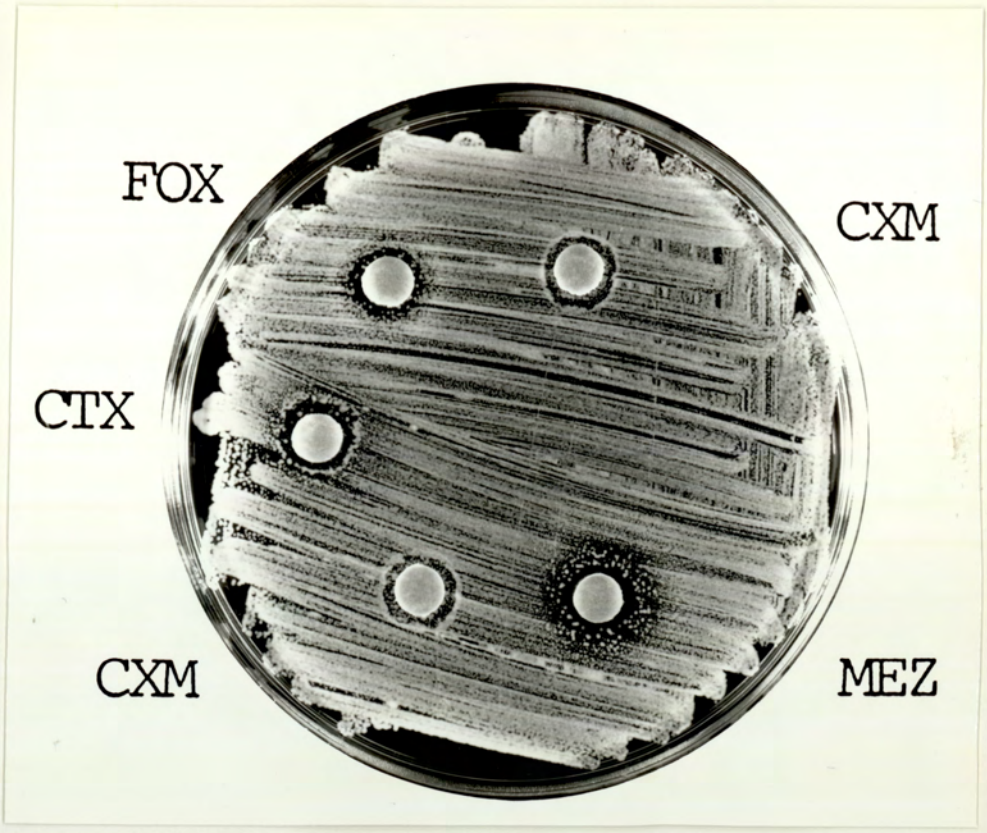
FIGURE 8. Antibiograms of E.coli and E.cloacae.

a)

b)



c)



CXM = cefuroxime

FOX = cefoxitin

CTX = cefotaxime

MEZ = mezlocillin

a) E.coli sensitive control

b) E.cloacae strains E7, E13 and E16

c) E.cloacae strains E12 and E14

5.2. Selection of stably derepressed mutants of E.cloacae.

Stably derepressed mutants of E13 and E16, referred to as E13DR1 and E16DR1 respectively were selected using cefotaxime. The mutants were found to occur at a frequency of 10^{-5} to 10^{-6} for both strains. The antibiotic susceptibility of these mutants was determined by the agar dilution method using piperacillin, cefotaxime and ceftazidime and was compared to the MIC values of the parent strains (Table 21). From these MIC values E13DR1 was shown to express a higher level of resistance than E16DR1 to the test antibiotics. This factor was confirmed with the measurement of specific enzyme activity; E13DR1 having much greater β-lactamase activity than E16DR1 (see section 5.3.2). These data indicate that E13DR1 may have been fully derepressed, but that E16DR1 was most probably only partially derepressed. That E16DR1 might still have been an inducible enzyme was illustrated by the inoculum effect with the MIC values for ceftazidime and piperacillin (i.e. the 10^6 inoculum had an MIC value twofold higher than the 10^4; fully derepressed strains have the same MIC for both inocula).

5.3. Effect of growth medium on kinetic parameters and specific enzyme activity of β-lactamases of E.cloacae.

5.3.1. Kinetic parameters.

K_m and V_{max} values were obtained using the method of half-time analysis (Nichols and Hewinson 1987). However, before this method was used extensively it was first compared with the classical Lineweaver-Burk method.

TABLE 21. Antibiotic susceptibility of stably derepressed mutants E13DR1 and E16DR1 compared to parent strains E13 and E16.

Antibiotic	MIC ($\mu g.ml^{-1}$) of *E.cloacae* strains							
	E13		E13DR1		E16		E16DR1	
	10^4 [a]	10^6	10^4	10^6	10^4	10^6	10^4	10^6
piperacillin	2	4	128	>128	2	8	64	128
cefotaxime	0.25	1	>32	>32	0.5	1	16	16
ceftazidime	0.25	0.5	8	8	0.5	1	1	2

a) MIC values were determined for two inocula: 10^4 and 10^6 $cfu.ml^{-1}$

Three enzyme samples were measured in duplicate by each technique (see Table 22). Both methods showed very close agreement, indicating that the half-time analysis method may be used to obtain accurate enzyme kinetic data.

K_m and V_{max} values were measured for strains E13 and E16 in all growth media (i.e. urine, serum and nutrient-deprived CDM). These values are shown in Figure 9. K_m values for E13 varied between 45 and 78µM, (mean value: 63 +/- 15). However in the case of E16 there was even less variability, 57-68µM, (mean value: 61 +/- 10). The conclusion drawn from this data is that the K_m probably does not vary with the growth medium, but is a fixed value.

The V_{max} values obtained from β-lactamases of strains E13 and E16 grown in the different media were found, as expected, to reflect the specific enzyme activity as measured by initial rates of nitrocefin hydrolysis. Since the specific enzyme activity is presented in detail here (section 5.3.2.), for all five strains grown in the different media, the V_{max} data is not given.

5.3.2. Specific enzyme activity.

in vivo: crude β-lactamase samples were obtained directly from in vivo infected urine samples from human patients. Details of the examination of each sample are given in Appendix 1. The specific enzyme activity from in vivo grown E13 (0.29 µmol nitrocefin. min^{-1}. mg $protein^{-1}$) was at least tenfold greater than that for the same strain grown in vitro in most of the media used

TABLE 22. Measurement of enzyme kinetics: half-time analysis method compared to Lineweaver-Burk.

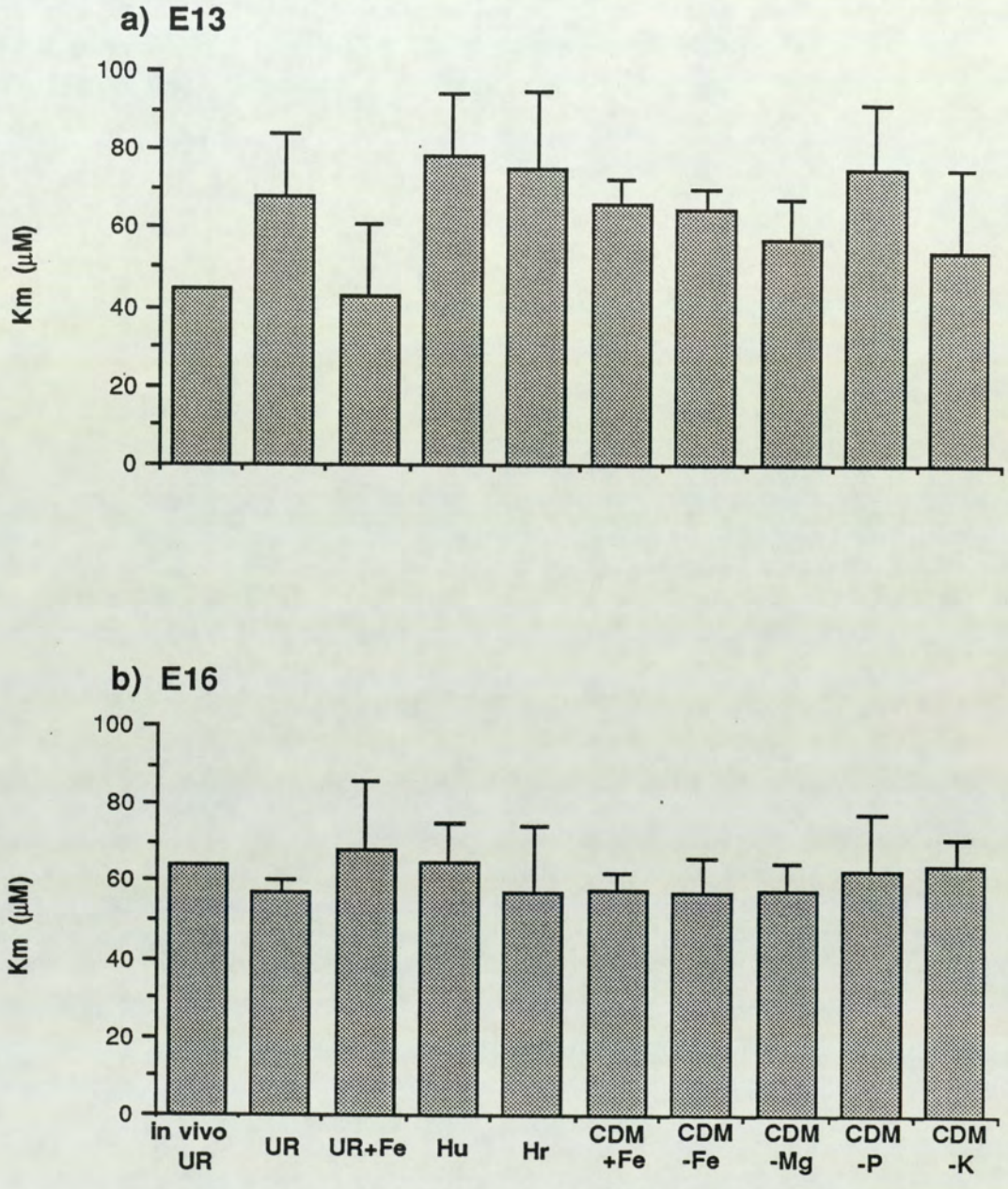
	Kinetic parameters of ß-lactamase			
E.cloacae E13 ß-lactamase samples	Lineweaver-Burk		Half-time analysis	
	K_m [a]	V_{max} [b]	K_m	V_{max}
in vivo urine	44.2	0.391	40.5	0.347
	47.4	0.454	49.5	0.523
in vitro urine	72.7	0.008	81.4	0.009
	70.2	0.027	75.6	0.030
CDM+Fe	42.1	0.018	48.5	0.029
	57.3	0.025	56.7	0.022

a K_m (µM)

b V_{max} (µmol nitrocefin. min^{-1}. mg $protein^{-1}$)

The method of half-time analysis (Nichols and Hewinson 1987) was compared to the classical Lineweaver-Burk method for the determination of kinetic parameters of ß-lactamase. Both methods showed close agreement. The half-time analysis method was adopted for further measurements of K_m.

FIGURE 9. Effect of growth medium on K_m of E.cloacae ß-lactamase



crude ß-lactamase extracts were prepared from E.cloacae E13 and E16 obtained directly from in vivo urinary tract infections (in vivo UR) and from in vitro cultures grown in duplicate in: urine (UR); iron-supplemented urine (UR+Fe); human serum (Hu); horse serum (Hr); iron-replete chemically defined medium (CDM+Fe) and also iron-, magnesium-, phosphate- and potassium-deprived chemically defined medium (CDM-Fe, CDM-Mg, CDM-P and CDM-K). The Km values from each ß-lactamase preparation were determined and are represented graphically above. Note: error bars represent the standard error of Km values obtained from the duplicate in vitro cultures.

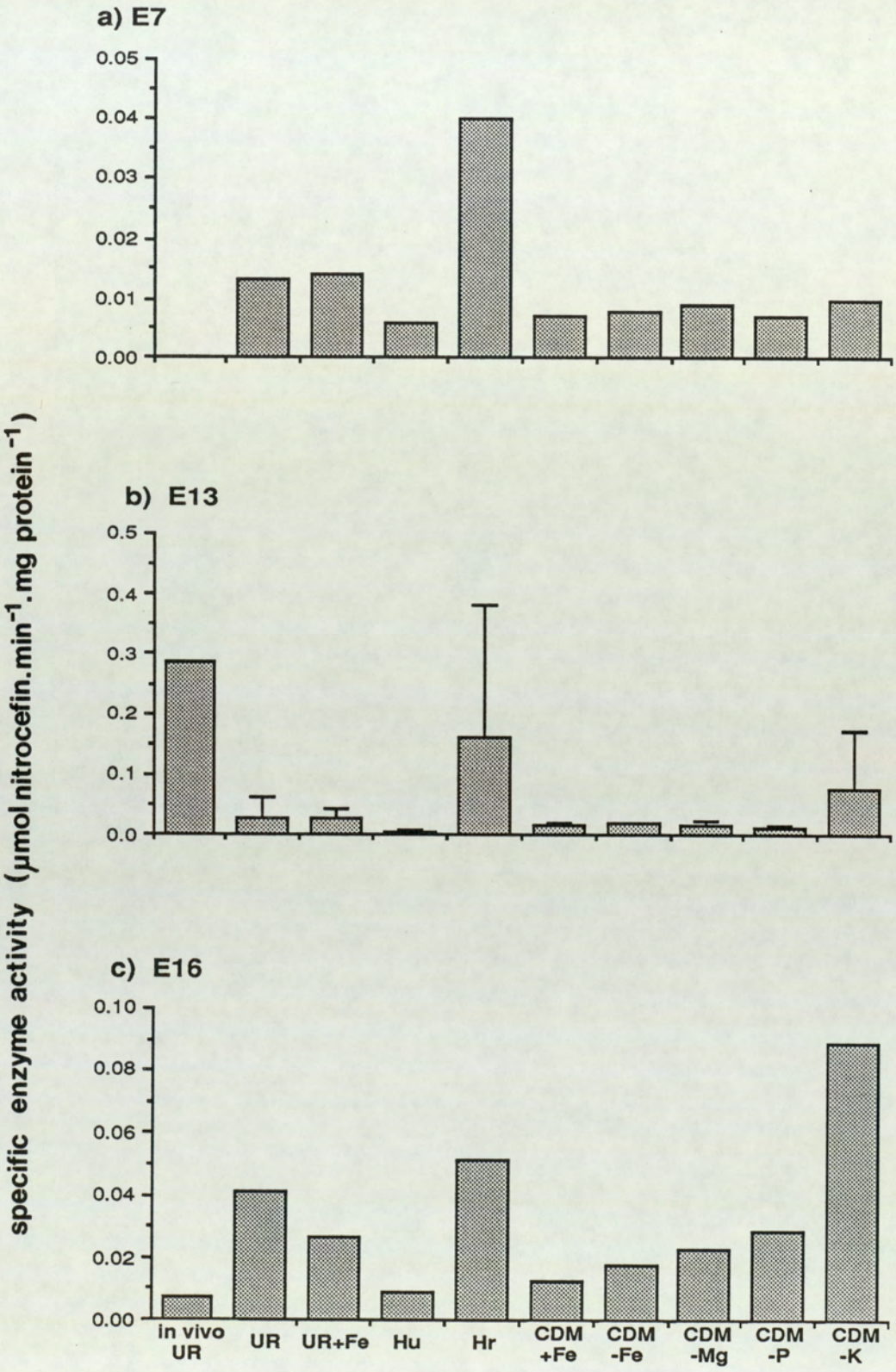
(0.005-0.03 μmol nitrocefin. min^{-1}. mg $protein^{-1}$) except for horse serum and CDM-K (0.161 and 0.08 μmol nitrocefin. min^{-1}. mg $protein^{-1}$ respectively; see Figure 10b). The *in vivo* β-lactamase activity for strains E14 and E16 was in most cases up to fivefold lower than their *in vitro* β-lactamase activities (see Figure 10c and e).

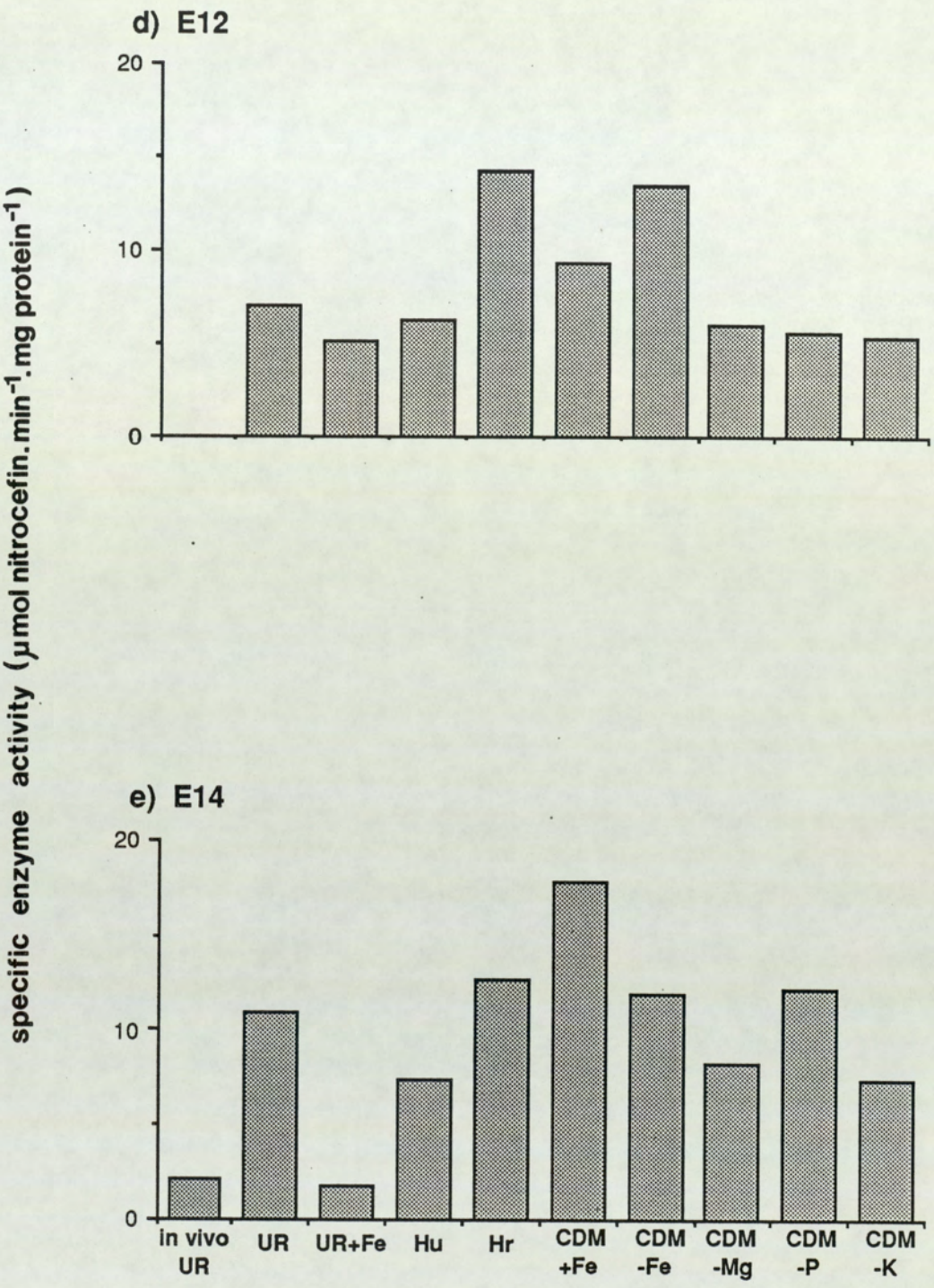
In vitro low level enzyme expression: this group includes E7, E13 and E16, which produced low basal levels of enzyme (Figure 10a, b and c). Specific enzyme activity in these strains was generally below 0.05μmol nitrocefin. min^{-1}. mg $protein^{-1}$. Only occasionally did the levels exceed this value, occurring in horse serum and CDM-K for both E13 and E16.

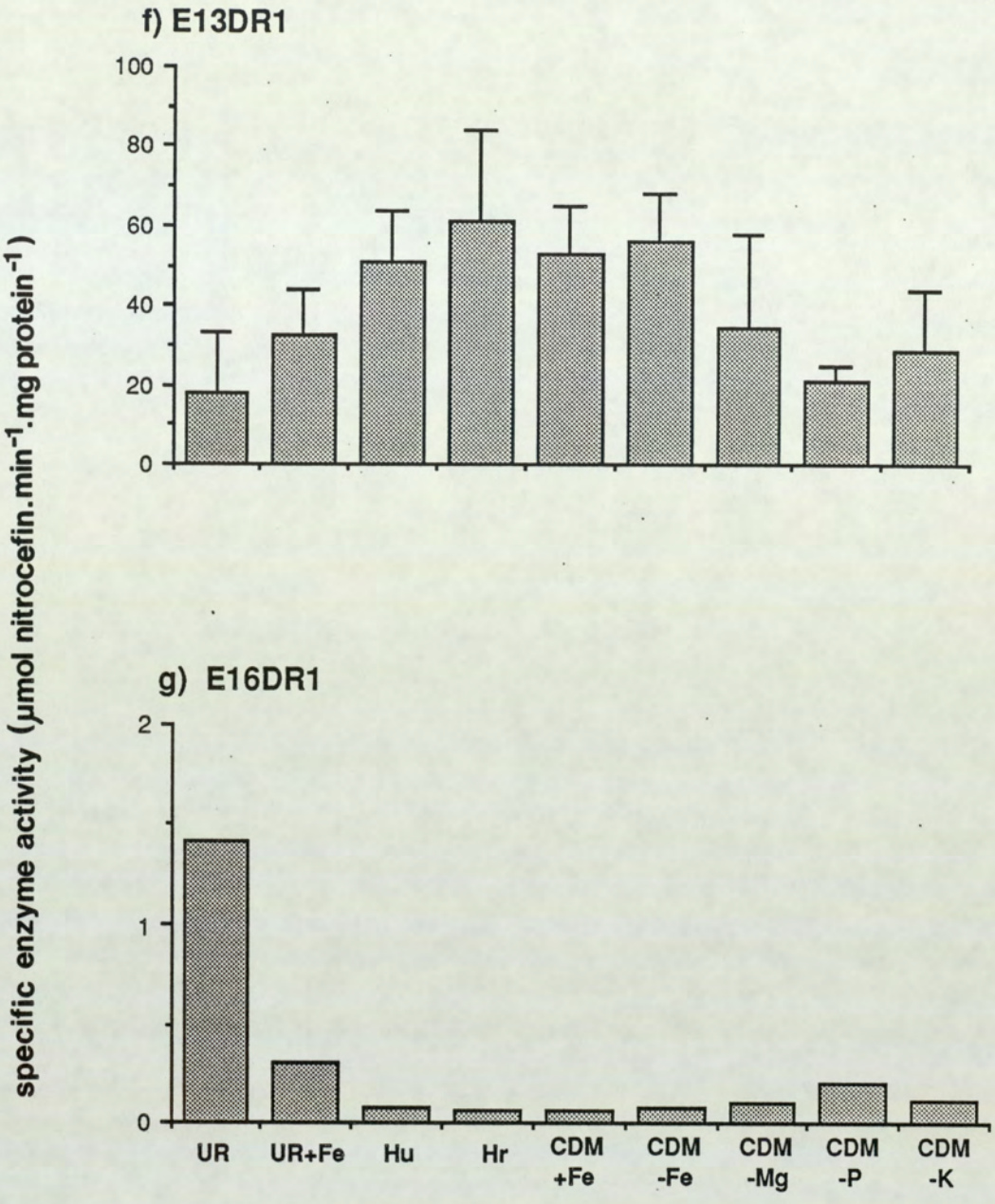
In vitro high level enzyme expression: this group includes E12, E14 and E13DR1 (Figure 10 d, e and f). The levels of enzyme activity in these strains were averages of 8, 10 and 40μmol nitrocefin. min^{-1}.mg $protein^{-1}$ respectively. In general, these strains possessed a greater variability in specific enzyme activity than the low level producers. Media consistently showing higher enzyme expression in all three strains included horse serum, CDM+Fe and CDM-Fe.

E16DR1, for enzyme expression, lies somewhere between these two groups of high and low β-lactamase activity. The activity of this enzyme was generally 0.1μmol nitrocefin. min^{-1}. mg $protein^{-1}$, only showing a much higher expression in urine.

FIGURE 10. Effect of growth medium on specific enzyme activity of E.cloacae.




d) E12
20
10
0
e) E14
20
10
0
specific enzyme activity (µmol nitrocefin.min-1.mg protein-1)
in vivo UR
UR
UR+Fe
Hu
Hr
CDM +Fe
CDM -Fe
CDM -Mg
CDM -P
CDM -K



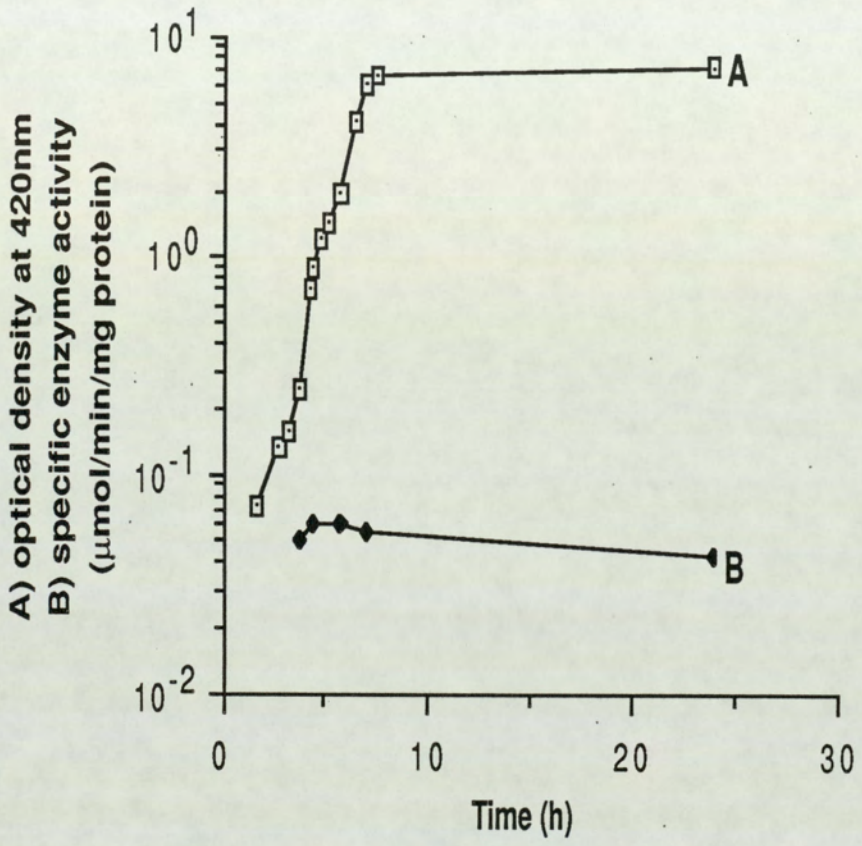
crude ß-lactamase extracts were prepared from E.cloacae E13, E14 and E16 obtained directly, without subculture, from in vivo urinary tract infections and also from E.cloacae E7, E12, E13, E13DR1, E14, E16 and E16DR1 grown in vitro in: urine (UR); iron-supplemented urine (UR+Fe); human serum (Hu); horse serum (Hr); iron-replete chemically defined medium (CDM+Fe); iron-, magnesium-, phosphate- and potassium-deprived chemically defined medium (CDM-Fe, CDM-Mg, CDM-P and CDM-K). The specific ß-lactamase activity of each preparation was measured using the method described by O'Callaghan et al (1972) and the results are represented graphically above. The error bars represent the standard error of specific ß-lactamase activity where this was measured from more than one culture of E.cloacae.

5.4. Effect of growth rate upon β-lactamase activity.

5.4.1. Batch culture.

E.cloacae E7 was grown in batch culture in CDM+Fe in five separate culture flasks. At various times throughout the growth curve (Figure 11) each flask was harvested and crude β-lactamase extracts prepared. The specific enzyme activity of each sample was measured and was found not to vary greatly between sample times (1 = 0.052, 2 = 0.061, 3 = 0.061, 4 = 0.055 and 5 = 0.043 μmol nitrocefin. min^{-1}. mg $protein^{-1}$; see also Figure 11). These data indicate that β-lactamase was produced consistently throughout the different growth phases in strain E7. β-lactamase production was also measured for strain E13 in both log and stationary phase bacteria in CDM+Fe, CDM-Fe, CDM-Mg, CDM-P and CDM-K. The specific enzyme activities of these β-lactamase preparations are given in Table 23. Once again, no difference was detectable between log and stationary phase bacteria. In this study, therefore, β-lactamase preparations from batch cultured bacteria were made from stationary phase bacteria due to the greater number of bacteria available for harvesting at this time.

FIGURE 11. ß-Lactamase production at different stages of growth of E.cloacae E7.



A Growth curve of E.cloacae E7 (OD_{420} versus time)

B ß-Lactamase activity of E.cloacae E7 (specific enzyme activity versus time)

E.cloacae E7 was grown in batch culture in CDM+Fe in five separate flasks. The growth of E7 was monitored spectrophotometrically by measurement of optical density at 420nm. The values plotted on the graph (**A**) represent mean values from the five culture flasks. At five different stages in the growth of E7 one flask was harvested in order to make ß-lactamase preparations from these bacteria. The specific enzyme activity of each ß-lactamase preparation was measured and is represented graphically (**B**). ß-lactamase activity did not change significantly throughout the different stages of batch culture.

TABLE 23. ß-Lactamase activity in log and stationary phase E.cloacae E13.

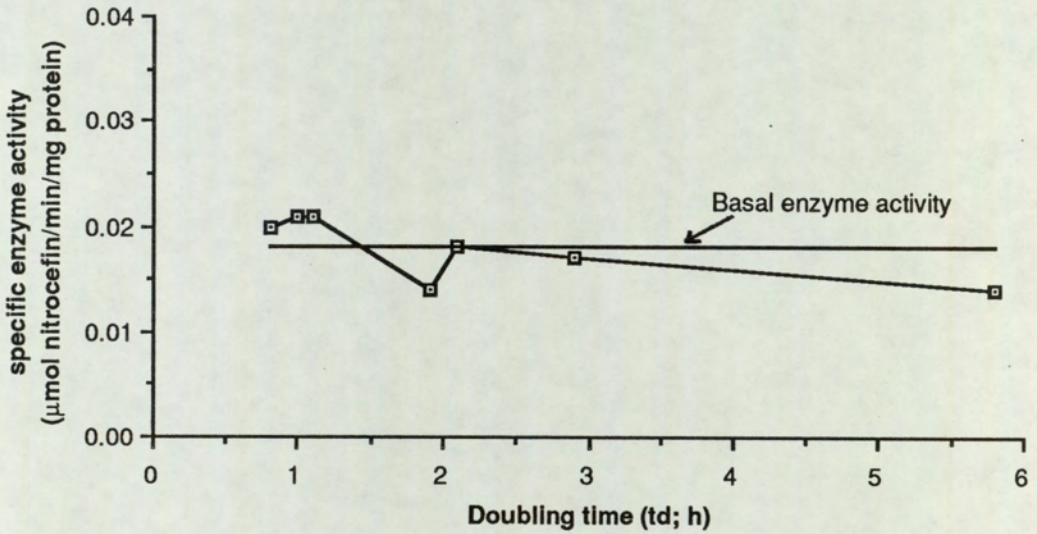
Medium	Specific enzyme activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	
	log phase	stationary phase
CDM+Fe	0.018	0.015
CDM-Fe	0.018	0.018
CDM-Mg	0.015	0.015
CDM-P	0.014	0.012
CDM-K	0.014	0.080

Batch cultures of E13 growing in iron-replete chemically defined medium (CDM+Fe), iron-deprived CDM (CDM-Fe), magnesium-deprived CDM (CDM-Mg), phosphate-deprived CDM (CDM-P) and potassium-deprived CDM (CDM-K) were harvested in both log phase and stationary phase. Crude ß-lactamase preparations were made from these cells and the specific enzyme activity measured using the method described by O'Callaghan et al (1972). The results shown above indicate that growth phase does not influence the basal level of ß-lactamase activity expressed by this strain.

5.4.2. Continuous culture.

In this investigation the effect of growth rate on β-lactamase activity was determined under more controlled conditions using continuous culture. E.cloacae E13 was grown at seven different growth rates (t_d; 0.8, 1.0, 1.1, 1.9, 2.1, 2.9 and 5.8h). The chemostats once inoculated, were allowed to stabilise through six volume changes. During this time the cultures were monitored by optical density, viable count and total count. Cultures growing at 0.8, 1.0 and 1.1h stabilised at; OD_{420} 1.2, 9 x 10^8 cfu.ml^{-1} viable count and 1 x 10^9 bacteria.ml^{-1} total count, while those growing at 1.9, 2.1, 2.9 and 5.8h stabilised at OD_{420} 2.7, 2 x 10^9 cfu.ml^{-1} viable count and 2 x 10^9 bacteria.ml^{-1} total count. After the six volume changes the whole cultures were harvested and used to make crude β-lactamase preparations. The enzyme activity from each of the seven samples did not vary significantly (0.014-0.021 μmol nitrocefin. min^{-1}. mg $protein^{-1}$; Figure 12), implying that growth rate alone did not affect β-lactamase activity.

FIGURE 12. Effect of growth rate on basal ß-lactamase activity of E.cloacae E13



E.cloacae E13 was grown in chemostat cultures in iron-deprived chemically defined medium (CDM-Fe) at seven different growth rates (t_d: 0.8, 1.0, 1.1, 1.9, 2.1, 2.9 and 5.8h). When the cultures were stable (after six volume changes of the culture vessel) bacteria were harvested and crude ß-lactamase extracts prepared from them. The specific ß-lactamase activity of each preparation was determined using the method described by O'Callaghan et al (1972) and the results are represented graphically above.

5.5. Effect of growth conditions upon the induction of β-lactamase by cefotaxime.

The effect of both growth medium and growth rate upon the induction of β-lactamase in E13 by cefotaxime was examined.

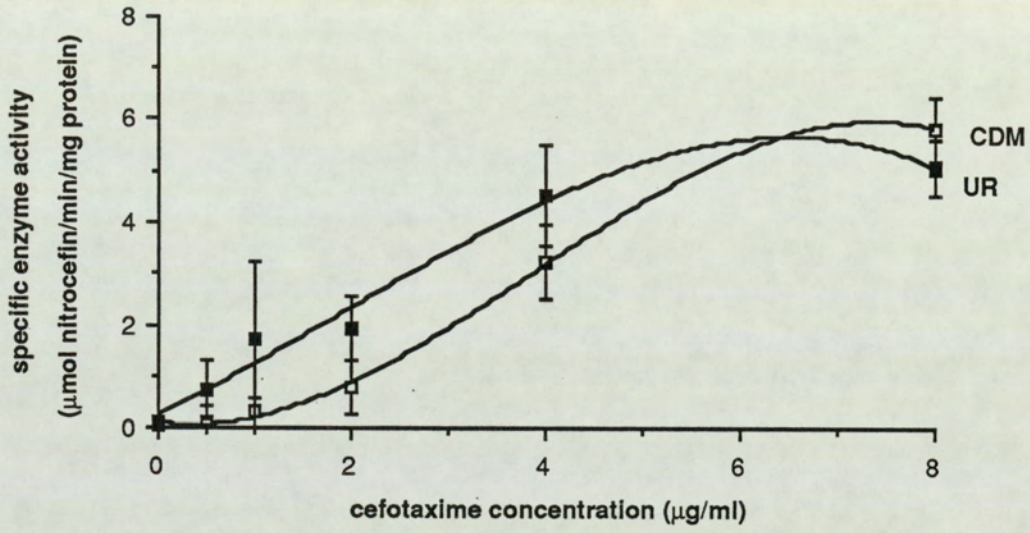
5.5.1. Growth medium.

β-Lactamase was induced in batch cultured E13 in both urine and CDM-Fe by various concentrations of cefotaxime. The specific enzyme activity of the induced enzyme was measured for each concentration of cefotaxime in duplicate. The values are plotted with standard error bars in Figure 13. These results showed no large significant difference between induction of β-lactamase in urine or CDM-Fe grown bacteria, although induction in urine did seem to peak at a lower concentration of cefotaxime in urine compared to CDM-Fe.

5.5.2. Growth rate.

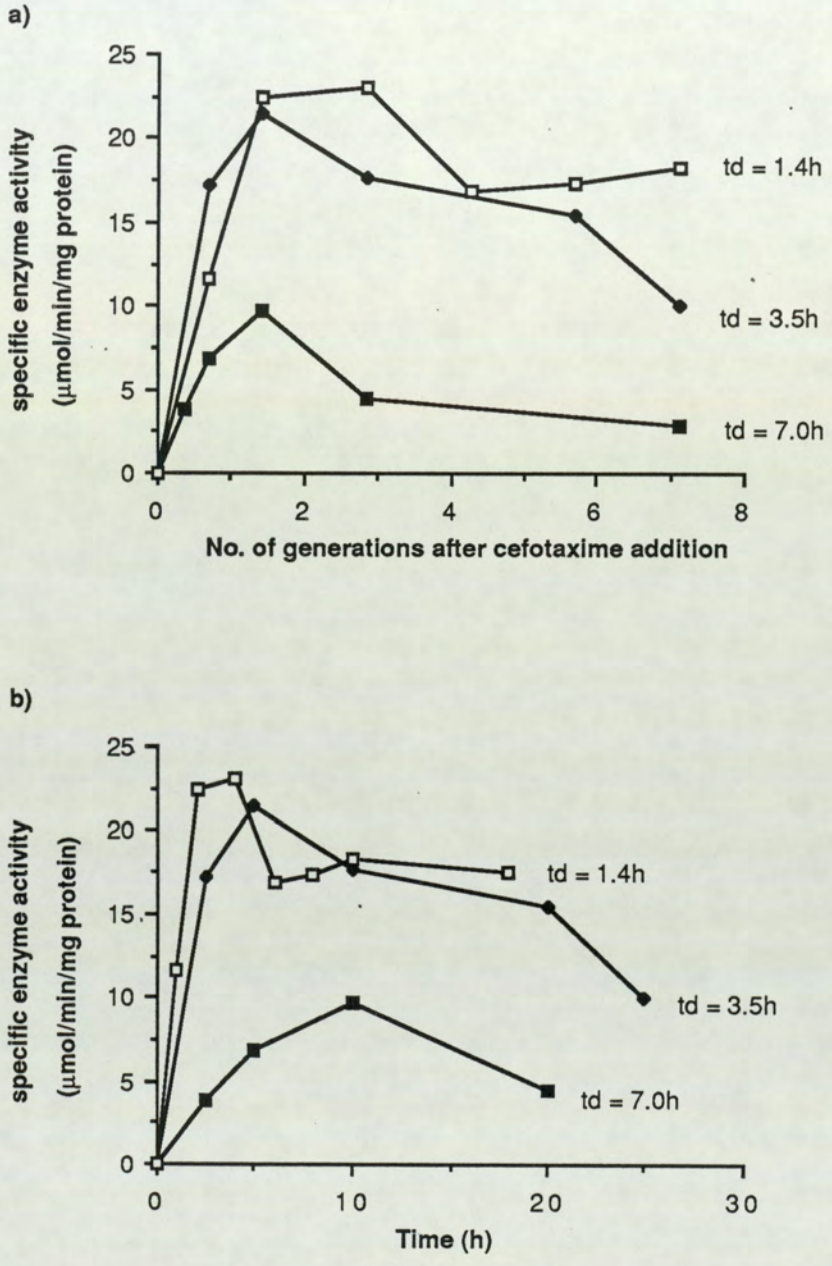
Although growth rate alone did not affect β-lactamase activity, it was found to have a profound effect upon β-lactamase induction by cefotaxime. At all three growth rates high specific activities of β-lactamase were observed within one generation time (see Figure 14). In the fast growing bacteria (t_d 1.4h) this concentration was reached at 1.4h compared to 7h in the slow growing bacteria (t_d 7h). Fast growing bacteria also produced higher levels of specific enzyme activity; 23 μmol nitrocefin. min^{-1}. mg $protein^{-1}$ at t_d 1.4h as compared to a maximum of 10 μmol nitrocefin.min^{-1}. mg $protein^{-1}$ for t_d 7h. However the value produced by fast

FIGURE 13. Effect of growth medium upon induction of ß-lacatamase in E.cloacae E13 by various concentrations of cefotaxime



E.cloacae E13 was grown in batch culture in urine (UR) or iron-deprived chemically defined medium (CDM-Fe). When the cells were growing exponentially various concentrations of cefotaxime (0, 0.5, 1, 2, 4 and 8µg.ml^{-1}) were added to induce the production of ß-lactamase. Two generations after the addition of cefotaxime, the bacteria were harvested and crude ß-lactamase extracts were prepared from them. The specific ß-lactamase activity of each extract was measured using the method described by O'Callaghan et al (1972). The results are represented graphically above. The error bars represent the standard error obtained from two separate experiments.

FIGURE 14. Effect of growth rate upon induction of ß-lactamase by cefotaxime in E.cloacae E13



E.cloacae E13 was grown in chemostat cultures, in iron-deprived chemically defined medium (CDM-Fe), at three different growth rates (td; 1.4, 3.5 and 7.0h). When the cultures were stable (t = 0h; after six volume changes of the culture vessel), the inflowing medium was changed to CDM-Fe containing 4µg.ml^{-1} cefotaxime. Simultaneously, cefotaxime was added to the culture vessel. At various time intervals after this point 5ml samples were taken from the culture vessel, harvested and used to prepare crude ß-lactamase extracts. The specific ß-lactamase activity of each preparation was measured using the method described by O'Callaghan et al (1972) and the results are represented graphically above: (a) shows the relationship between specific enzyme activity and the number of generations of growth after the addition of cefotaxime to the culture, while (b) shows the relationship between specific enzyme activity and time after the addition of cefotaxime.

growing bacteria was still approximately half that attainable by mutational derepression (see Figure 10f).

5.6. Discussion.

Inducible class I β-lactamases are prevalent among many species of enterobacteria, especially Enterobacter, Serratia, Citrobacter, indole positive Proteus and Providencia species (Livermore 1987a, Sanders and Sanders 1985, 1987). When synthesized copiously, these enzymes cause resistance to almost all β-lactams except imipenem (Livermore 1987a). Elevated β-lactamase production may arise transiently via induction when these organisms are exposed to β-lactams, or permanently via mutation in the stably derepressed mutants of these species (Livermore 1986, Phillips 1986). The presence of these enzymes in species like E.cloacae, therefore, is viewed with increasing concern as a source of resistance to the newer β-lactam antibiotics (Sanders 1983). In the clinical situation, the selection of stably derepressed mutants during treatment has proven the greater problem, rather than induction. Many reports of treatment failures through this mechanism have emerged (Sanders and Sanders 1985). However, although induction of β-lactamase is of less clinical significance than stable derepression, clinical resistance through induction has been reported (Sanders et al 1982a). In addition in certain situations induction may play an important role, for example, the strong induction of β-lactamase by cefoxitin may lead to antagonism with other β-lactams in E.cloacae (Sanders et al 1982b). At present, little is known about induction in vivo. Investigations by

Dalhoff and colleagues (Cullmann et al 1984, Dalhoff 1982, Dalhoff and Cullmann 1984) have shown that β-lactamase production by some strains of E.cloacae, Morganella morganii and P.aeruginosa was much greater in vivo than in vitro, even in the absence of β-lactam antibiotics. These workers explained this phenomenon as "non-specific" induction, since they had also observed induction of β-lactamase in vitro, to levels attainable by cefoxitin induction, by non-β-lactam compounds: the addition of certain bicyclic compounds or biological fluids to Iso-sensitest broth cultures induced β-lactamase expression (Cullmann et al 1983). It is possible, therefore, that the growth environment of bacteria may play a role in the development of β-lactam resistance through β-lactamase production. The aim of this investigation was to determine the effect (if any) of the growth environment on levels of class I β-lactamase activity, kinetic parameters and induction of β-lactamase in E.cloacae.

5.6.1. Typing of β-lactamase.

Before the investigation of the effects of the growth environment began, strains E7, E12, E13, E14 and E16 were first examined for class I β-lactamase production. These β-lactamases were analysed by isoelectric focusing. Three of the strains (E12, E14 and E16) expressed β-lactamases with the same isoelectric point (pI 7.6). E12 was a known P99 β-lactamase producer and so E14 and E16 were also thought to produce the P99 enzyme. The pI of this enzyme was very similar to values reported for P99 from other laboratories (pI 7.8; Seeberg et al 1983, pI 7.9 +/- 0.2; Then and

Angehern 1986). Strain E7 expressed β-lactamase with a very similar isoelectric point (pI 7.4) which may also be a P99-type β-lactamase. Strain E13 produced β-lactamase with a higher pI than the other strains (pI 8.8). Seeberg et al (1983) surveyed the production of β-lactamases by strains of E.cloacae. These workers concluded that 70% of E.cloacae strains possessed one of two species-specific chromosomal β-lactamases with pIs of either 8.8 or 7.8, which they designated "A" and "B" respectively. Therefore, it is probable that in this study E13 β-lactamase falls into group "A" and β-lactamases of E12, E14, E16 and possibly E7 fall into group "B". These enzymes are chromosomal class I β-lactamases (Richmond and Sykes 1973).

Once the production of chromosomal class I β-lactamases was established for each strain, the inducibility of the enzyme was determined. This was done using a simple disc diffusion technique (Sanders and Sanders 1979), referred to as an antibiogram. The test relies on the strong inducing powers of cefoxitin antagonizing the action of antibiotics in neighbouring discs. Using this test three strains (E7, E13 and E16) were shown to express inducible β-lactamase, while two (E12 and E14) expressed stably derepressed β-lactamase. The strains chosen for this study were therefore representative of those E.cloacae found clinically before treatment (low basal level, inducible β-lactamase) and those causing treatment failures (high level stably derepressed, constitutive β-lactamase) (Livermore 1987a, Sanders and Sanders 1985, 1987).

5.6.2. Stably derepressed mutants of E.cloacae.

The selection of stably derepressed mutants E13DR1 and E16DR1, from E13 and E16 respectively, served a number of purposes. Firstly to determine the levels of β-lactamase activity achievable in these strains. Secondly to examine the effect of this increase in β-lactamase activity upon the β-lactam susceptibility of these strains. Finally, these stably derepressed mutants were used as controls for their parent strains when the effect of growth medium upon specific β-lactamase activity was examined. Thus the importance of variation of specific activity of β-lactamase from each parent strain in different growth media was judged against increases of β-lactamase activity known to produce resistance. This point is discussed in section 5.6.3.

Stably derepressed mutants of E13 and E16 occurred at frequencies between 10^{-5} and 10^{-6}, which agreed with the range (10^{-5} to 10^{-8}) reported by a number of workers (Findell and Sherris 1976, Gootz et al 1982, Lampe et al 1982, Olson et al 1983). Both parent strains, E13 and E16, were sensitive to the β-lactams tested (Table 21) and both stably derepressed mutants, E13DR1 and E16DR1 were resistant. However when the mean specific β-lactamase activities of each strain grown in the nine different media (i.e. urine, serum and nutrient deprived CDM) were compared, E16DR1 was found to have much lower activity than E13DR1: E13DR1 possessed 1000-fold greater β-lactamase activity than E13 (40.0 compared to 0.04μmol nitrocefin. min^{-1}. mg $protein^{-1}$), while E16DR1 only possessed 9-fold greater β-lactamase activity than E16 (0.27 compared to 0.03μmol nitrocefin. min^{-1}. mg $protein^{-1}$). These

data suggest that either E16DR1 was only partially derepressed, or that this strain had only a limited ability for β-lactamase production. The latter point was proposed in a study by Olson et al (1983) for a clinical isolate of E.cloacae which did not develop resistance in vivo. However resistance developed in vitro was within clinically achievable drug concentrations, unlike E16DR1 which had high levels of in vitro resistance. A possible explanation for this resistance might be a decrease in permeability to the β-lactams. Increases of β-lactamase production in conjunction with a change in permeability provide an effective means of resistance to β-lactam antibiotics (Vu and Nikaido 1985) and this has previously been described for E.cloacae grown in vitro (Then and Angehern 1986, Werner et al 1985).

5.6.3. Effect of growth medium on K_m and specific β-lactamase activity of E.cloacae.

K_m: a variation in β-lactamase activities of each strain of E.cloacae under different growth conditions may be a result of either differences in rate of enzyme synthesis or through differences in properties of the enzyme. If the latter case were true, then these differences would be reflected by a change in K_m of the enzyme. The half-time analysis method was used to measure K_m values. The method was described for this purpose by Wharton and Szawelski (1982) whose studies showed it to be a statistically valid method which is simple to use, saving time materials and experimental effort when compared to other methods. Recently Nichols and Hewinson (1987)

developed the method further to give rapid automated measurement of kinetic parameters of β-lactamases. In this study the results obtained from Lineweaver-Burk plots were first compared with those obtained by half-time analysis. The results gave close agreement, hence the half-time method was adopted.

The K_m values were determined for β-lactamases of strains E13 and E16 isolated directly from in vivo infections and grown in vitro in urine, serum and nutrient deprived CDM. The K_m values were determined for the β-lactamase harvested from at least two separate cultures of bacteria for each growth medium. In each β-lactamase preparation, the K_m value was also determined at least three times. The error between different cultures was greater than the error of measurements of K_m from the same β-lactamase preparation. The error bars on graphs in Figure 9 represent the error between different cultures, hence there is no error bar on the in vivo urine value as only one sample was obtained. From these graphs the K_m did not appear to vary a great deal from the mean value (E13 63μM, E16 61μM) between the different growth media. It is therefore concluded that any change in enzyme activity observed in bacteria grown in different media will probably be due to a change in the rate of synthesis of β-lactamase and not by a change in the properties of the enzyme itself.

Specific enzyme activity: two groups of workers have reported that β-lactamase can be induced non-specifically to levels achievable by cefoxitin induction (Cullmann et al 1983, Gatus et al 1988). One group have found that glycine enhances β-

lactamase induction in E.cloacae (Gatus et al 1988) and the other found a number of bicyclic molecules (e.g. tryptophan, thiamine, folic acid and haemin) induced β-lactamase non-specifically in E.cloacae, C.freundii and P.aeruginosa (Cullmann and Dick 1985, Cullmann et al 1983, 1984, 1987, Dalhoff and Cullmann 1984). The latter observations are of greater clinical relevance, since this group have proposed that bicyclic molecules present in body fluids, such as urine and serum, can cause this induction to levels achievable by cefoxitin induction (Cullmann et al 1983). There have also been a number of reports of increased β-lactamase production in vivo, where no treatment of β-lactam or other antibiotics was given (Cullmann et al 1984, Dalhoff 1982, Dalhoff and Cullmann 1984, Dineen 1961). These workers have suggested that such cases of non-specifically induced β-lactamase could provide an explanation for therapeutic failure. However, as yet, the prevalence of these phenomena is unclear as other workers found such non-specific induction did not occur in Proteus vulgaris (Okonogi et al 1986) nor has the addition of body fluids to susceptibility test media been found to antagonise β-lactam activity against β-lactamase inducible species (Livermore 1987a).

In this study three clinical isolates of E.cloacae were obtained directly from in vivo infections. Only in one case (E13) were levels of β-lactamase elevated compared to in vitro grown E13. In the remaining two strains (E14 and E16) levels of β-lactamase from in vivo bacteria were approximately fivefold lower than from in vitro bacteria. Furthermore the levels of β-lactamase activity expressed by

bacteria grown in body fluids (urine and serum) in vitro, were not consistently higher than those of bacteria grown in CDM, which did not contain bicyclic molecules. It would seem that the phenomenon of non-specific induction did not occur with the strains of E.cloacae used in this study. Cullmann and Dick (1985) have demonstrated that this non-specific induction only occurred in resistant variants. However, in this study even strain E16DR1, a partially derepressed variant of E16, did not exhibit this non-specific induction in all cultures of urine and serum. Only one urine culture expressed levels of β-lactamase activity that were much higher than other in vitro cultures. However, this level of activity was not attained in the iron-supplemented urine culture, nor the serum grown bacteria. There was a variation in the levels of β-lactamase activity measured for all seven strains (E7, E12, E13, E13DR1, E14, E16 and E16DR1) grown in different media. However, no one growth medium consistently produced high or low levels of β-lactamase activity. The one growth medium that came close to doing this was horse serum. High levels of β-lactamase activity were observed in all strains, except E16DR1, grown in horse serum. However, this did not occur in the human serum, which is more relevant to human infections. There was no general trend in the β-lactamase activity expressed and the growth medium. In fact the variation appeared to be random. Furthermore the significance of the variation was questionable when the highest level of β-lactamase produced by basal strains (e.g. E13 0.3μmol nitrocefin. min^{-1}. mg $protein^{-1}$) was compared to the lowest level of β-lactamase produced by the stably

derepressed strains (e.g. E13DR1 18μmol nitrocefin. min^{-1}. mg $protein^{-1}$). Unless, of course, concomitant changes in the permeability barrier occurred that would have increased the impact of a small rise in β-lactamase activity. Since strains E12 and E14 were resistant to all β-lactam antibiotics tested in the different growth media (see section 4), then presumably the variation in β-lactamase activity observed here (Figure 10d and e) never fell below the level required to maintain resistance. Measurement of specific enzyme activity from the same β-lactamase preparation was found to be highly reproducible (data not shown). The greater differences were found between different β-lactamase preparations (i.e. different cultures of bacteria) even when grown in the same medium. Since stably derepressed mutants are isolated quite frequently (10^{-5} to 10^{-6})in this study, it is possible that the variation in the number of stably derepressed variants present in each population of bacteria might account for the variability in β-lactamase activity. On the other hand there may be variability at the genetic level, of the control of β-lactamase production. However, whatever mechanism is responsible, it is clear that it is not controlled by the medium in which the strains of E.cloacae used in this study have been grown in the absence of β-lactam antibiotics.

5.6.4. Effect of growth rate upon β-lactamase activity.

The growth rate of bacteria has been shown to affect their susceptibility to antimicrobial agents (Gilbert and Brown 1978, 1980, Finch and Brown 1975, Sterkenberg and Wouters

1981) including β-lactam antibiotics (Cozens et al 1986b). In general slow growing bacteria are more resistant to these agents, particularly in the case of the β-lactam antibiotics (Cozens et al 1986b, Tuomanen et al 1986). The growth rate of bacteria has also been shown to affect their production of extracellular enzymes; production of protease and lipase of P.aeruginosa decreased, while phospholipase C increased with increasing growth rate (Ombaka et al 1983). For these reasons, the effect of growth rate upon β-lactamase production was examined to determine whether the increased resistance of bacteria to β-lactam antibiotics at slow growth rates is influenced by β-lactamase. Two strains E7 and E13 were examined in batch cultures in both log and stationary phase. However, β-lactamase production remained constant at a basal level. The effect of growth rate upon β-lactamase production of E13 was then examined under more controlled conditions in a chemostat. Seven growth rates were examined. At three of the faster growth rates (t_d 0.8, 1.0 and 1.1h), the cultures stabilised at a lower optical density (OD_{420} 1.2) than the slower growth rates (t_d 1.9, 2.1, 2.9, 5.8h; OD_{420} 2.7). This decrease is partly accounted for by the different light scattering properties of the larger cells characteristic of fast growth rates (Lodge 1987). However it is mainly a consequence of the tendency to wash out as the maximum growth rate is approached. The lower OD would not affect the comparison of β-lactamase activities, since these bacteria were harvested and resuspended to the same OD before being broken. The β-lactamase activity of the bacteria at each growth rate did not vary significantly. Therefore, it

appears that growth rate alone did not affect β-lactamase activity.

5.6.5. Effect of growth conditions upon the induction of β-lactamase by cefotaxime.

The effects of growth media and growth rate upon induction of β-lactamase expression for E.cloacae strain E13 were investigated. The third generation cephalosporin, cefotaxime, was chosen as a representative of the newer β-lactams that are currently used in clinical treatment. Cefotaxime has also been responsible for the emergence of β-lactamase-mediated resistance in E.cloacae, leading to treatment failure (Bryan et al 1985, Eng et al 1987, Landesman et al 1981).

Growth medium.

The effects of two growth media upon induction of β-lactamase expression by cefotaxime were investigated. Urine was chosen, since E13 was isolated from a urinary tract infection, and CDM-Fe because previous studies from this and other laboratories provided evidence that bacteria grow in the urinary tract under iron restriction (Lam et al 1984, Shand et al 1985). Various concentrations of cefotaxime were used to induce log phase cultures of E13. As found in previous studies, the amount of β-lactamase produced related to the inducer concentration (Gootz and Sanders 1983, Livermore 1987a, Rosta and Mett in press). Thus the highest concentration of cefotaxime used ($8\mu g.ml^{-1}$) induced the highest β-lactamase activity (Figure 13). Cefotaxime is a moderate inducer of β-lactamase (Minami et al 1980, Then

1987) and this was illustrated by the fact that, at the MIC (urine 0.25μg.ml^{-1}, CDM-Fe 0.031μg.ml^{-1}, see Table 17) induction was negligible, only when concentrations much greater than the MIC were used was there a significant increase in β-lactamase activity (Figure 13). Addition of cefotaxime at the higher concentrations (up to 8μg.ml^{-1}) to log phase batch cultures of E13 did not alter their growth rate (t_d: urine 30min, CDM-Fe 40min). At these levels β-lactamase activity peaked at 5-6μmol nitrocefin. min^{-1}. mg protein^{-1}, which was approximately 8-fold less than levels achieved by stable derepression (E13DR1 mean activity 40μmol nitrocefin. min^{-1}. mg protein^{-1}) indicating that cefotaxime did not fully induce β-lactamase even at much higher concentrations than the MIC. Induction of β-lactamase at lower concentrations of cefotaxime achieved higher levels of β-lactamase activity in urine than in CDM-Fe. However, these differences were not significantly greater. The differences may be explained by the faster growth rate in urine than CDM-Fe. Such a growth rate effect of media has been described previously (Gootz and Sanders 1983). The effect of growth rate was examined under more controlled conditions in a chemostat.

Growth rate.

Growth rate showed a profound effect on the induction of β-lactamase expression (Figure 14). E13 was grown at three rates (t_d 1.4, 3.5 and 7.0h) yet high levels of β-lactamase activity were induced in all cases by the first generation, after the addition of cefotaxime. Therefore, faster growing

bacteria (t_d 1.4h) were found to respond more quickly than slower growing bacteria (t_d 7h), (i.e. a difference of 5.6 hours in the response to induction). In addition, faster growing bacteria produced higher levels of β-lactamase activity than slower growing bacteria. β-Lactamase activity peaked between the first and second generation and thereafter decreased. In slower growing bacteria this decrease occurred much more quickly than with faster growing bacteria (Figure 14). It is possible that this decrease in β-lactamase activity may be explained by the hydrolytic activity of the induced β-lactamase. The smaller decrease in activity of the faster growing bacteria could be explained by the faster supply of fresh cefotaxime-containing medium. This kept the specific β-lactamase activity at a constant high level over several generations. However, cefotaxime is a β-lactamase stable cephalosporin and its rate of hydrolysis is likely to be low, especially by the relatively low levels of β-lactamase produced in slow growing bacteria. Therefore the higher levels of β-lactamase activity achieved in faster growing bacteria are probably a result of a greater rate of protein synthesis and hence expression of β-lactamase in faster growing bacteria. These results may have important implications in determining the effectiveness of β-lactam therapy in acute infections, where bacteria are fast growing.

It has been reported that β-lactamase induction in E.cloacae may be controlled by a mechanism involving the ampD gene product, such as that described for C.freundii (Lindberg et al 1987, Nicolas et al 1987). The time lag observed for induction, especially with the slow growing bacteria (Figure

14) , might represent the time taken for the effects of an interaction of cefotaxime with the ampD product (possibly a membrane bound PBP) and consequent activation of the ampR gene product. The ampR gene product has been shown to function in both induction and repression of ampC expression in E.cloacae (Honore et al 1986).

6. EFFECT OF GROWTH MEDIUM UPON THE EXPRESSION OF OMPS OF E.CLOACAE.

E.cloacae E7, E12, E13, E14 and E16 were grown in the nine different media (i.e. urine, urine + Fe, human serum, horse serum, CDM+Fe, CDM-Fe, CDM-Mg, CDM-P and CDM-K). In addition PG-linked proteins were extracted from CDM-Fe grown bacteria, also E.coli I150 OMs were run as controls to indicate the position of OmpF, OmpC and OmpA. The OM gels of all five strains are shown in Figure 15, both the full gels (Figure 15a) and also close up pictures of the porin band regions (Figure 15b) are shown. The loading on each gel was optimised for separations of the major OMPs in the porin region. These main proteins were present in each strain in the 33-37 Kd region. Other main OMPs appeared in the 70-85 Kd region, these were the iron-regulated membrane proteins (IRMPs). If the loading of OM preparation had been increased on the gel, more proteins would have been apparent outside of these regions, some of these proteins are seen more clearly in strain E16 (Figure 15a).

6.1. Expression of IRMPs.

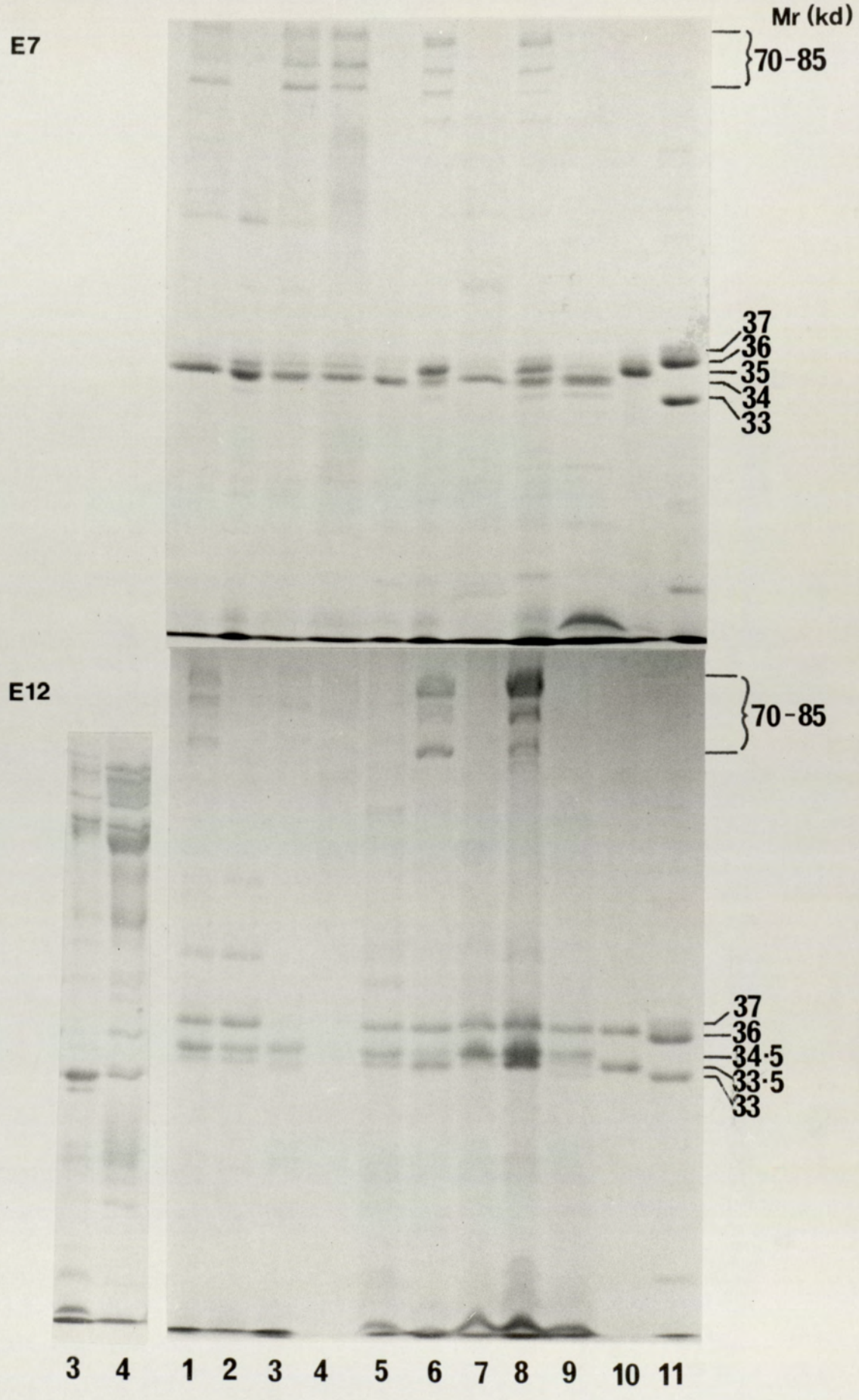
IRMPs are expressed by all five strains when grown in urine, human serum, horse serum, CDM-Fe and interestingly also CDM-P. The IRMPs expressed in CDM-P grown bacteria were not the full complement that are expressed by urine, serum and CDM-Fe grown bacteria, but included the IRMPs with higher molecular weights (Mr 73-85 Kd). The CDM-P cultures were supplemented with the same amount of iron as CDM-Mg,

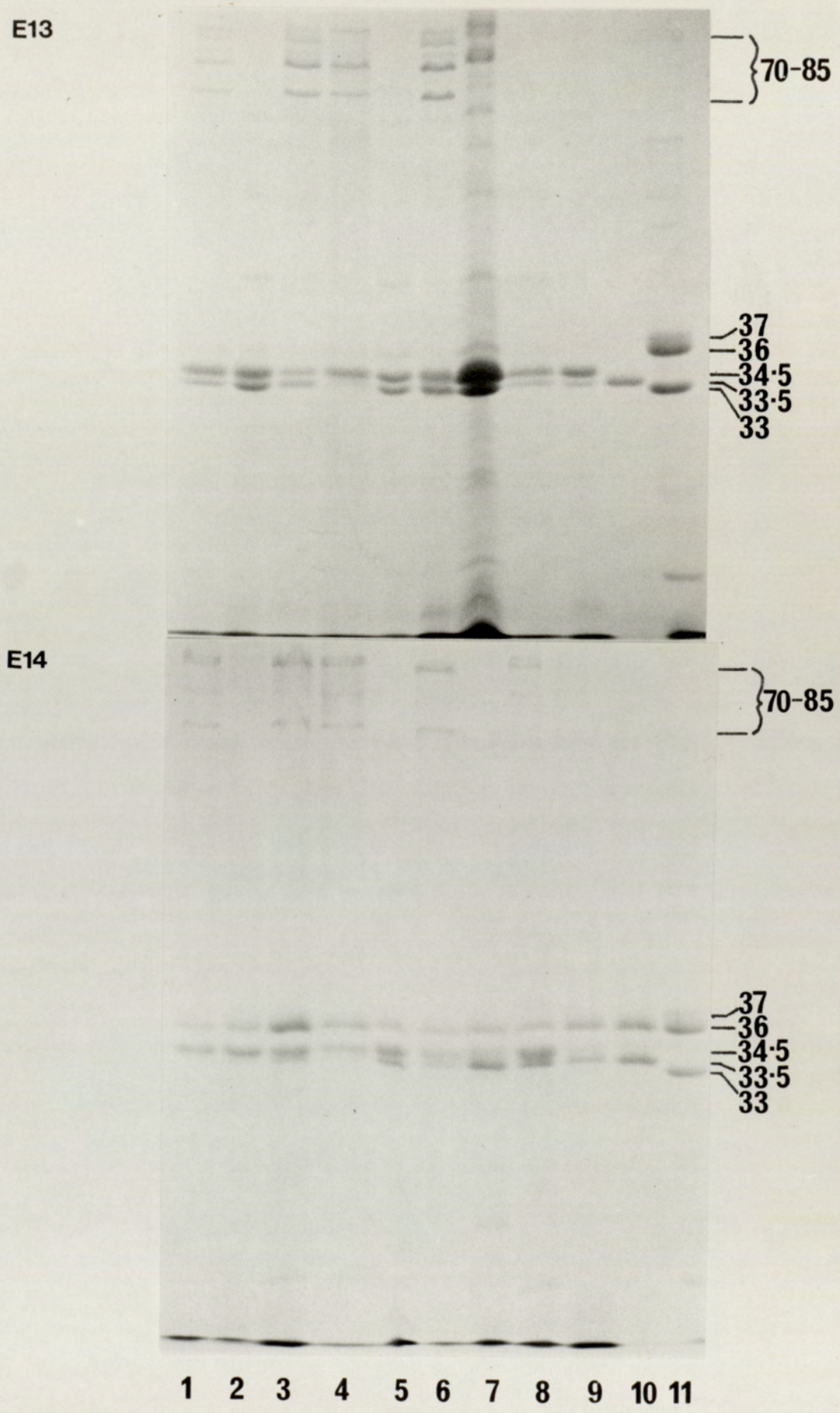
FIGURE 15.

a) Outer membrane protein profiles of E.cloacae E7, E12, E13, E14 and E16 grown in nine different media (lanes 1-9). Porin proteins were extracted using the technique of Mizuno and Kageyama (1979) (lane 10). E.coli I150 was included as a control (lane 11). SDS-PAGE was performed using the Laemmli (1970) system. Protein loading of OM preparations in each lane varied between 20 and 60$\mu g.ml^{-1}$ to optimize the separation of porin proteins of each strain. The OMP profile of strain E12 in lanes 3 and 4 is not clear on the main gel picture, hence these two lanes are inset from a previous gel (see text).

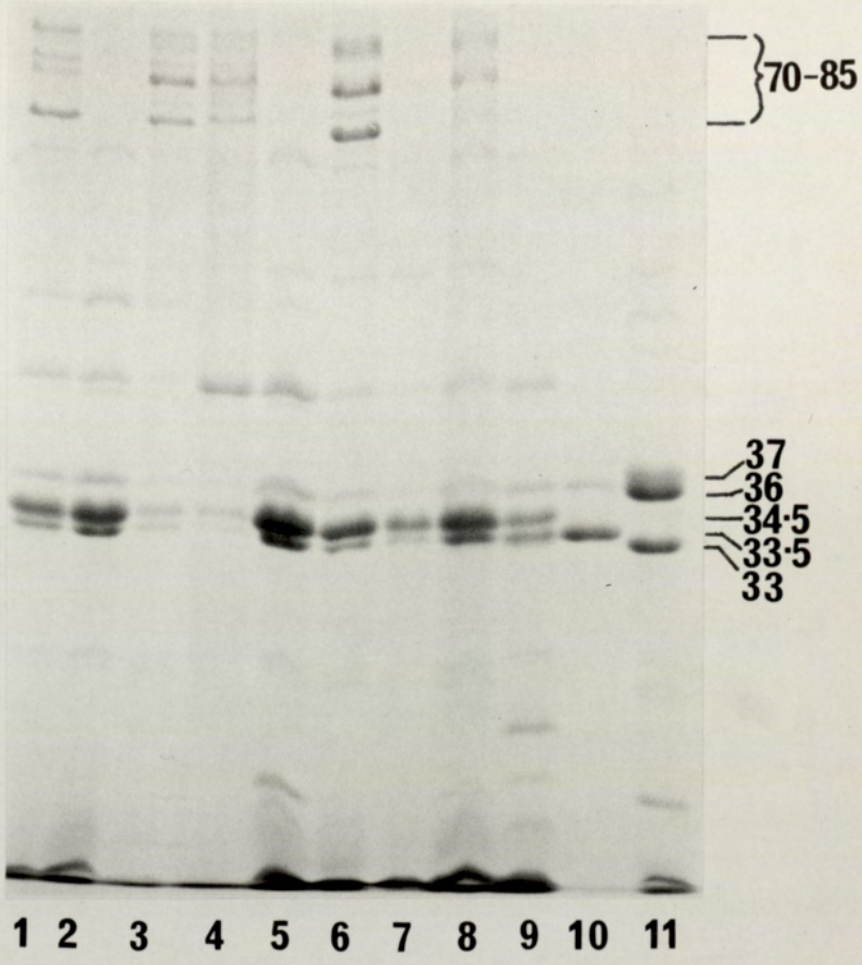
b) Close up pictures of the major OMP regions of each gel shown in (a).

FIGURE 15a Effect of growth medium on the OMP profile of E.cloacae



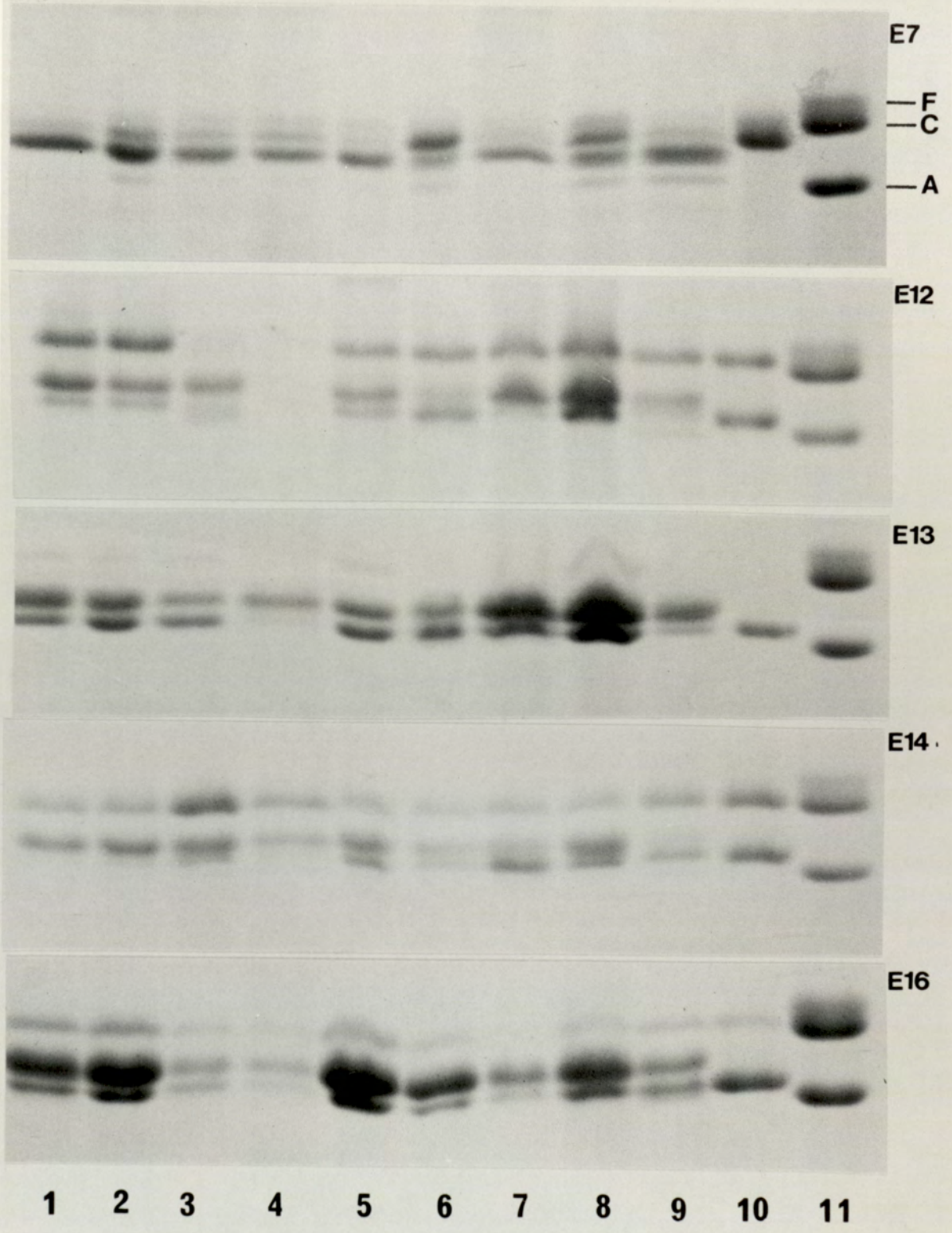

E13
70-85
37
36
34·5
33·5
33
E14
70-85
37
36
34·5
33·5
33
1 2 3 4 5 6 7 8 9 10 11

E16



1	Urine	7	CDM – Mg
2	Urine + Fe	8	CDM – P
3	Human serum	9	CDM – K
4	Horse serum	10	PG associated proteins
5	CDM + Fe	11	E.coli I 150
6	CDM – Fe		Mr= molecular weight

FIGURE 15b



Major OMP regions of previous five gels

F – OmpF C – OmpC A – OmpA

CDM-K and CDM+Fe, yet in these other nutrient deprivations the bacteria did not express IRMPs. Another interesting observation with the CDM-P cultures was that they were pink after overnight culture. This pink colouration may be seen when iron is added to the supernatant of overnight cultures of CDM-Fe grown bacteria. The pink colour is an indication of the presence of enterochelin which forms a pink-coloured complex when it binds to iron (Langman et al 1972, Rosenberg and Young 1974). The IRMPs expressed in urine grown bacteria were suppressed by the addition of 0.12mM iron.

E7: strain E7 produced four main IRMPs (Mr; 81, 80, 75 and 70 Kd) and three which could be seen only faintly on the gel (Mr; 82, 77 and 72 Kd). These proteins were expressed in the same quantities in urine, serum and CDM-Fe grown bacteria, except for the 75 Kd protein which was poorly expressed in urine grown E7 compared to the serum and CDM-Fe grown E7.

E12: strain E12 produced four IRMPs (Mr; 82, 81, 75 and 70 Kd). These were expressed well in urine and CDM-Fe, but could barely be seen in human and horse serum grown E12 on the large gel in Figure 15a. This discrepancy was caused by loading too little of the OMP preparation onto the gel. However, these proteins were visible on another gel (shown as two separate lanes beside the main E12 gel photograph in Figure 15a). From this other gel five IRMPs could be seen in the human serum grown E12. The extra protein had a molecular weight of 83 Kd and was not present in horse serum grown E12.

E13: six IRMPs could be detected in strain E13 grown in CDM-Fe (Mr; 83, 82, 81, 77, 75 and 70 Kd), the last two being

the most heavily expressed. All of these proteins were produced in equal amounts in human serum, however the 82, 81 and 77 Kd proteins appeared to be missing in horse serum and the 82 and 81 Kd proteins were missing in the urine grown bacteria.

E14: strain E14 produced four IRMPs (Mr; 81, 80, 75 and 70 Kd), the same as the four main IRMPs of strain E7. These proteins were produced in the same quantities in urine, serum and CDM-Fe, except for the 81 Kd protein in CDM-Fe grown bacteria which was poorly expressed.

E16: strain E16 produced seven IRMPs (Mr; 83, 82, 81, 77, 75, 72 and 70 Kd). Of these proteins, the 75 and 70 Kd were produced in the greatest quantity. Poorly expressed IRMPs included: the 82, 81 and 72 Kd proteins in urine; the 77Kd protein in CDM-Fe and all IRMPs other than the 75 and 70Kd proteins in horse serum. All IRMPs in human serum were heavily expressed.

The IRMPs produced by each strain are summarised in Table 24. This table shows that strains E7 and E14 expressed similar IRMPs, while strains E12, E13 and E16 expressed similar IRMPs to each other. IRMPs common to all five strains include the 81, 75 and 70 Kd proteins.

TABLE 24. IRMPs expressed by E.cloacae.

	E.cloacae				
Mr (Kd)	E7	E12	E13	E14	E16
83		+	+		+
82	+	+	+		+
81	+	+	+	+	+
80	+			+	
79					
78					
77	+		+		+
76					
75	+	+	+	+	+
74					
73					
72	+				+
71					
70	+	+	+	+	+

This table shows the total number of iron regulated membrane proteins (IRMPs) which may be expressed by each strain of E.cloacae. Note the strain to strain variation, although the 81, 75 and 70Kd proteins are produced by each one. The qualitative and quantitative expression of IRMPs in each strain is dependent upon growth medium (see text).

6.2. Expression of porin and OmpA proteins.

The best separation of major OMPs including porin proteins and OmpA was obtained using the Laemmli (1970) system. The Lugtenberg system (Lugtenberg *et al* 1975) was used at first, but did not give a satisfactory separation of these bands (see Figure 15a; compare extra two lanes of Lugtenberg system with full gel of E12 using the Laemmli system). The porin and OmpA proteins are shown in greater detail in Figure 15b. *E.coli* I150 was included as a control showing where OmpF, OmpC and OmpA were positioned in the gel in comparison to the major proteins of *E.cloacae*. The porin proteins of *E.cloacae* were identified by extracting the non-covalently linked PG proteins. The major protein band which was not present in the PG-protein extraction was assumed to be *E.cloacae* OmpA-type protein. The region of the gels shown in Figure 15b was scanned using a laser densitometer to determine the percentage intensity of each band. These results are given in Tables 25-29. Since porin expression in *E.cloacae* has been shown to affect their susceptibility to chloramphenicol and tetracycline (Sawai *et al* 1982, Werner *et al* 1985), the MIC values for these antibiotics are also given in Tables 25-29.

TABLES 25-29.

The region of the gels containing major OMPs (Figure 15b) was scanned with a laser densitometer in order to measure any variation in porin expression with respect to growth medium. A decrease in porin expression has been correlated with increased resistance to β-lactam antibiotics (Bush *et al* 1985, Marchou *et al* 1987, Sawai *et al* 1982, Werner *et al* 1985), chloramphenicol and tetracycline (Sawai *et al* 1982, Werner *et al* 1985) in *E.cloacae*. Since β-lactam antibiotic resistance is also mediated by changes in β-lactamase and PBP expression, but chloramphenicol and tetracycline are not, the latter two antibiotics were included to determine any correlation between porin expression and antibiotic susceptibility.

TABLE 25. Laser densitometer scan of OMPs of E7.

Growth medium	% Intensity of bands on gel[a]					MIC ($\mu g.ml^{-1}$)	
	Porin 36.5Kd	Porin 36Kd	OmpA 34Kd	? 33.5Kd	Total Porin	TET	CHLOR
urine	1.1	25.1	73.8	0	26.2	4	16
urine + Fe	5.5	28.9	59.7	5.9	34.4	8	8
human serum	11.8	25.2	61.0	2.0	37.0	>16	2
horse serum	12.3	25.2	59.9	2.6	37.5	0.5	2
CDM+Fe	14.8	16.6	67.2	1.4	31.4	0.5	2
CDM-Fe	7.9	58.8	26.2	7.1	66.7	0.5	2
CDM-Mg	2.3	25.8	71.9	0	28.1	0.125	2
CDM-P	16.1	38.0	37.1	8.8	54.1	0.5	2
CDM-K	5.5	17.1	64.2	13.2	22.6	0.5	2

a Intensity of 36.5 + 36 + 34 + 33.5Kd bands = 100%

TET = tetracycline, CHLOR = chloramphenicol.

TABLE 26. Laser densitometer scan of OMPs of E12.

Growth medium	% Intensity of bands on gel[a]					MIC (μg.ml^{-1})	
	Porin 36.5Kd	OmpA 34.5Kd	Porin 33.5Kd	? 32Kd	Total Porin	TET	CHLOR
urine	42.0	46.0	12.0	0	54.0	256	256
urine + Fe	49.7	36.4	13.9	0	63.6	256	256
human serum	4.6	68.4	10.5	16.5	15.1	256	128
horse serum	40.2	16.7	43.1	0	83.3	>256	128
CDM+Fe	36.9	42.8	20.3	0	57.2	128	64
CDM-Fe	37.0	19.2	43.8	0	80.8	64	64
CDM-Mg	37.7	61.6	0.7	0	38.4	64	32
CDM-P	26.9	42.7	30.4	0	57.3	64	128
CDM-K	43.0	42.8	5.6	8.6	48.6	128	64

a Intensity of 36.5 + 34.5 + 33.5 +32 Kd bands = 100%

TET = tetracycline, CHLOR = chloramphenicol.

TABLE 27. Laser densitometer scan of OMPs of E13.

Growth medium	% Intensity of bands on gel[a]				MIC (μg.ml^{-1})	
	Porin 36Kd	OmpA 34.5Kd	Porin 33.5Kd	Total Porin	TET	CHLOR
urine	4.0	59.2	36.8	40.8	256	256
urine + Fe	0.9	59.9	39.2	40.1	2	16
human serum	4.2	44.7	51.1	55.3	>256	64
horse serum	0	82.9	17.1	17.1	>16	2
CDM+Fe	5.4	50.9	43.7	49.1	0.125	2
CDM-Fe	0	58.5	41.5	41.5	0.5	2
CDM-Mg	0	61.5	38.5	38.5	4	2
CDM-P	3.7	66.1	30.2	33.9	0.125	2
CDM-K	0	84.8	15.2	15.2	0.25	2

a Intensity of 36 + 34.5 + 33.5Kd bands = 100%

TET = tetracycline, CHLOR = chloramphenicol.

TABLE 28. Laser densitometer scan of OMPs of E14.

Growth medium	% Intensity of bands on gel[a]				MIC (μg.ml^{-1})	
	Porin 36.5Kd	OmpA 34.5Kd	Porin 33.5Kd	Total Porin	TET	CHLOR
urine	36.4	62.4	1.2	37.6	256	256
urine + Fe	35.4	64.5	0.1	35.5	32	16
human serum	51.0	34.4	14.6	65.6	32	8
horse serum	52.5	38.6	8.9	61.4	256	4
CDM+Fe	25.4	42.0	32.6	58.0	0.5	4
CDM-Fe	33.0	48.7	18.3	51.3	0.5	2
CDM-Mg	26.0	29.7	44.3	70.2	0.5	2
CDM-P	18.4	49.1	32.5	50.9	0.5	4
CDM-K	47.3	19.8	32.9	80.2	0.5	4

a Intensity of 36.5 + 34.5 + 33.5Kd bands = 100%

TET = tetracycline, CHLOR = chloramphenicol.

TABLE 29. Laser densitometer scan of OMPs of E16.

Growth medium	% Intensity of bands on gel[a]				MIC ($\mu g.ml^{-1}$)	
	Porin 36.5Kd	OmpA 34.5Kd	Porin 33.5Kd	Total Porin	TET	CHLOR
urine	18.6	58.3	23.1	41.7	4	16
urine + Fe	11.4	66.5	22.1	43.6	2	16
human serum	11.6	60.6	27.8	39.4	4	8
horse serum	10.9	61.6	27.5	38.4	16	8
CDM+Fe	11.5	63.3	25.2	36.7	0.25	2
CDM-Fe	7.7	78.2	14.1	21.8	0.5	4
CDM-Mg	1.4	85.5	13.1	14.5	0.25	2
CDM-P	4.9	65.2	29.9	34.8	0.25	8
CDM-K	12.6	45.1	42.3	54.9	0.25	2

a Intensity of 36.5 + 34.5 + 33.5Kd bands = 100%

TET = tetracycline, CHLOR = chloramphenicol.

E7: strain E7 produced two porin proteins (Mr; 36 and 36.5). Both porins were present in all growth media. However their quantitative expression varied between the different growth media. The 36Kd porin was present in greater quantities when E7 was grown in CDM-Fe and CDM-P and the 36.5 Kd porin was expressed weakly when E7 was grown in urine, urine + Fe, CDM-Fe, CDM-Mg and CDM-K compared to other growth media. Strain E7 produced two other major proteins (Mr; 34 and 33.5 Kd). The former was expressed in the greatest quantity and was assumed to be the OmpA-type protein. The total quantity of porin, expressed as percentage intensity of the major protein bands, varied between 22.6 and 66.7% being lowest in CDM-K and highest in CDM-Fe. There was no correlation between changes in porin expression and sensitivity to tetracycline or chloramphenicol

E12: strain E12 also produced two porins (Mr; 36.5 and 33.5 Kd). Both porins were present in all growth media, however, the 36.5 Kd porin was only just detectable on the gel for the human serum grown E12. The poor expression coincided with the appearance of a 32 Kd protein. A 32 Kd protein also appeared in potassium-deprived bacteria, however, in this case it was the 33.5 Kd porin which was weakly expressed. It is possible that the 32 Kd protein represents modified forms of the poorly expressed porins with different electrophoretic mobility, or this may be a completely new protein. In comparison to other growth media, the 33.5 porin was expressed in greater quantities in horse serum and CDM-P grown bacteria. Strain E12 also possessed a 34.5 Kd protein which was assumed to be the OmpA-type

protein. The total quantity of porin expressed varied between 15.1 and 83.3%, being lowest for human serum and highest for horse serum grown bacteria. There was no correlation between changes in porin expression and sensitivity to tetracycline or chloramphenicol.

E13: strain E13 possessed two porin proteins (Mr; 36 and 33.5 Kd). However, the 36 Kd porin was very weakly expressed and even then was only present in E13 grown in urine, urine + Fe, human serum, CDM+Fe and CDM-P. The 33.5 Kd porin on the other hand was present in much greater quantities in most of the growth media except for human serum and CDM-K. Strain E13 expressed an OmpA-type protein (Mr 34.5). Total porin expression varied between 15.2 and 55.3%, being lowest for CDM-K and highest for human serum grown bacteria. There was no correlation between changes in porin expression and sensitivity to tetracycline or chloramphenicol.

E14: strain E14 possessed two porin proteins (Mr; 36.5 and 33.5 Kd) which were present in all growth media. The 36.5 Kd porin was expressed in the greatest quantities when bacteria were grown in human serum, horse serum and CDM-K relative to the production of the other major proteins, but was weakly expressed in CDM-P grown bacteria. The 33.5 Kd porin was produced in small quantities in urine, urine + Fe and horse serum grown bacteria. Strain E14 possessed an OmpA-type protein (Mr 34.5 Kd). The total porin expression varied between 35.5 and 80.2%, being lowest for urine + Fe and highest for CDM-K grown bacteria. There was no correlation between changes in porin expression and sensitivity to tetracycline or chloramphenicol.

E16: strain E16 possessed two porin proteins (Mr 36.5 and 33.5 Kd). Compared to other growth media the 36.5 Kd protein was expressed poorly in CDM-Mg and CDM-P grown bacteria. The 33.5 Kd porin was produced in the greatest quantity in CDM-K, but was rather weakly expressed in CDM-Mg grown bacteria. Strain E16 possessed an OmpA-type protein (Mr 34.5 Kd). The total porin expression varied between 14.5 and 54.9%, being lowest for CDM-Mg and highest for CDM-K grown bacteria. There was no correlation between changes in porin expression and sensitivity to tetracycline or chloramphenicol.

From the data shown for the five strains of E.cloacae used in this study, it appeared that each strain had two porins. In three of these strains (E12, E14 and E16) these porins had identical molecular weights. Both E7 and E13 shared one porin in common with E12, E14 and E16. The expression of individual porins varied with the growth medium, however no particular growth medium consistently produced greater or lesser amounts of porin. Also the variation in porin expression did not correlate with any variation in sensitivity to tetracycline or chloramphenicol. All five strains produced a major protein with a molecular weight of approximately 34.5 Kd and this was assumed to be the E.cloacae equivalent of the E.coli OmpA protein.

6.3. Discussion.

The OMP profiles of E.cloacae strains E7, E12, E13, E14 and E16 were examined after these strains had been grown in a variety of media (i.e. urine, serum and nutrient deprived CDM). Two major groups of proteins were of interest; those corresponding to porin proteins and the high molecular weight IRMPs. Porin expression has been implicated in resistance of E.cloacae to β-lactam antibiotics (Bush et al 1985, Marchou et al 1987, Sawai et al 1982, Werner et al 1985). In addition a catechol substituted cephalosporin (E-0702) has been described (Watanabe et al 1987). Resistance to this antibiotic occurred in E.coli mutants lacking functional high affinity iron uptake systems (Watanabe et al 1987).

6.3.1. Expression of IRMPs.

An essential part of high affinity iron uptake systems based on siderophores is the production of OMP receptors and enzymes which are involved in the uptake and release of iron from the iron-chelator complex (Neilands 1982). The IRMPs of enteric bacteria are generally in the range 74-84Kd (Griffiths 1987b). IRMPs have previously been reported for E.cloacae (Oudega et al 1979, van Tiel-Menkveld et al 1981, 1982). One of these proteins (Mr 85Kd) has been identified as the aerobactin receptor (also the receptor for cloacin DF13; Oudega et al 1979, van Tiel-Menkveld et al 1982). Three other IRMPs were described by van Tiel-Menkveld et al (1981) with apparent Mr; 70, 75 and 83Kd. These proteins were induced in cultures of E.cloacae grown in minimal medium in the presence of the iron chelator α,α'-dipyridyl (van Tiel-Menkveld et al

1981). The aerobatin receptor did not seem to be present in E.cloacae cultured under these conditions (van Tiel-Menkveld 1981). However, this was probably a result of the iron chelator used, since Chart et al (1986) demonstrated both qualitative and quantitative differences in expression of IRMPs in E.coli, depending on which chelator was used. In their study E.coli O164 grown in the presence of α,α'-dipyridyl only produced one IRMP (76Kd) compared to expression of five IRMPs when EDDA or desferal were used (83, 81, 78, 76 and 74Kd) or expression of four IRMPs when ovotransferrin was used (83, 81, 76 and 74Kd). In this study iron deprivation in CDM in vitro was achieved by the elimination of iron from the medium and the reduction of contaminating iron by the use of chemicals of high purity (i.e. ARISTAR, see section 3.2.) and by cleaning glassware with EDTA (see section 2.6.1.). No chelator was used. In the E.cloacae strains grown in CDM-Fe, the number of IRMPs produced varied: E7 produced seven (Mr 82, 81, 80, 77, 75, 72 and 70Kd); E12 produced four (Mr 82, 81, 75 and 70Kd); E13 produced six (Mr 83, 82, 81, 77, 75 and 70Kd); E14 produced four (Mr 81, 80, 75 and 70Kd) and E16 produced seven (Mr 83, 82, 81, 77, 75, 72 and 70Kd). These results demonstrate strain to strain variation in expression of IRMPs even when grown in the same medium. This variation was also seen in E.coli and Chart et al (1988) have suggested the possibility of exploiting this fact to provide a new set of markers to characterize groups of pathogenic E.coli. Three IRMPs were common to each strain (Mr 81, 75 and 70Kd) and these are similar to those described by van Tiel-Menkveld et al (1981)

(Mr 83, 75 and 70Kd) implying that the 75 and 70Kd IRMPs at least, may be well conserved between different strains of E.cloacae. The 85Kd aerobactin receptor was not present in any of the strains examined in this study, assuming of course that this receptor has an apparent molecular weight of 85Kd in all strains of E.cloacae.

IRMPs were also expressed by E.cloacae grown in urine and serum. In these cultures iron was probably restricted by the presence of transferrin and/or lactoferrin. Both qualitative and quantitative differences were observed in IRMP expression in the same strain of E.cloacae grown in urine, serum and CDM-Fe. Even differences between the two sera used were apparent. Between the different strains of E.cloacae no one IRMP was influenced in the same way by urine, serum or CDM-Fe, implying that the control of IRMP expression may be a complex process subject to variation both between and within strains of E.cloacae.

In addition to IRMP expression in E.cloacae grown in urine, serum and CDM-Fe, growth in CDM-P also induced the high affinity iron uptake system. This observation was interesting, since CDM-P contained excess iron in the same quantity as CDM+Fe, CDM-Mg and CDM-K, yet IRMPs were not expressed in the latter growth media. A possible explanation for this observation might be the solubility of iron in CDM, which in turn would affect its availability to E.cloacae. In highly aerated conditions in aqueous solution at physiological pH, such as those conditions present in in vitro CDM cultures Fe^{2+} is oxidised to Fe^{3+}, the process being driven by the hydration of Fe^{3+} to form insoluble

ferric hydroxide polymers (Fenchal and Blackburn 1979). Therefore the amount of free ionic iron in solution will be extremely low. The solubility of iron in a simple salts medium (e.g. CDM) may also be dependent on other factors, such as the amounts of anions and cations present in the medium. In all CDM, except CDM-P, there was an excess of phosphate ions present in the medium, which are negatively charged. It is possible that the presence of excess negatively charged phosphate groups helps to keep the positively charged iron in a soluble form, available to E.cloacae. In the absence of excess phosphate, as in CDM-P, it is feasible that iron might come out of solution as an insoluble hydroxide. In this case E.cloacae would need to produce siderophores to chelate, and hence solubilise, iron making it available for growth. The presence of IRMPs would therefore be induced for the uptake of iron from the iron chelator complex. Since enterochelin is pink when complexed with iron (Langman et al 1972, Rosenberg and Young 1974), and the CDM-P cultures of E.cloacae were pink, it would seem that E.cloacae was producing enterochelin to solubilise iron. The production of enterochelin and its receptor protein are generally considered to be coordinately regulated (Fleming et al 1983, Klebba et al 1982, M^{c}Intosh and Earhart 1977). It is probable, therefore, that one of the IRMPs expressed in CDM-P grown E.cloacae was the enterochelin receptor. The 81Kd IRMP was the prime candidate for this role, since it was present in all five strains of E.cloacae grown in CDM-Fe and CDM-P. E.cloacae grown in CDM-Fe were also shown to produce enterochelin; the addition of iron to overnight culture

supernatant resulted in a strong pink colour. The enterochelin receptor of E.coli also has a molecular weight of 81Kd (Griffiths 1987b).

The expression of the 81Kd IRMP varied between different growth media (e.g. urine, serum and CDM). It would be interesting to determine the expression of this receptor in vivo, in the light of the possible development of catechol-substituted cephalosporins (e.g. E-0702). Unfortunately in this study the quantity of OMs obtained from in vivo E13, E14 and E16 were insufficient for such analysis. In vivo enteric bacteria from UTIs have previously been shown to express IRMPs (Lam et al 1984, Shand et al 1985) therefore it is highly probable that E.cloacae also produce IRMPs in vivo. If the development of catechol-substituted cephalosporins is to provide an alternative route of entry for β-lactams other than porins, then it is important to determine the presence of this receptor in vivo. An important factor to consider also is the expression of the aerobactin receptor in vivo, as this system is thought to be more efficient in vivo than the enterochelin system (Griffiths 1987b): resistant strains could arise through the loss of the enterochelin iron-uptake system while maintaining iron uptake through the aerobactin system.

6.3.2. Expression of porin and OmpA proteins.

Sawai et al (1982) described the major OMPs of E.cloacae 206: Mr 35, 37 and a doublet 39-40Kd. These proteins were later purified and characterised by Kaneko et al (1984) and the 37 and 39-40Kd proteins were shown to be porins, forming

channels with radii of 0.6 and 0.8nm respectively. The 35Kd protein may be the OmpA equivalent protein of E.cloacae. Other groups have since described major OMPs of different strains of E.cloacae, each expressing different OMPs to those of E.cloacae 206. Werner et al (1985) described three major OMPs in two strains of E.cloacae: strain 55ML expressed 37,39 and 63Kd proteins and strain P99⁻ expressed 37, 39 and 43Kd proteins. Marchou et al (1987) described four strains of E.cloacae expressing 35, 37, 38 and 42Kd proteins. The 35Kd protein was heat modifiable and probably was OmpA. The 37 and 38Kd proteins formed a doublet and therefore may be comparable to the 39-40Kd porin described by Sawai et al (1982). Gutmann et al (1985) described a strain of E.cloacae with only two major OMPs; 37 and 38Kd proteins. The 38Kd protein was heat modifiable and probably was OmpA. Therefore there appears to be considerable strain to strain variation in the major OMPs of E.cloacae. In this study, the five strains of E.cloacae examined, each produced three major OMPs. Three of these strains (E12, E14 and E16) produced major OMPs with identical molecular weights (36.5, 34.5 and 33.5Kd). E7 produced 36.5, 36 and 34Kd proteins and E13 produced 36, 34.5 and 33.5Kd proteins. In each case two of these proteins remained bound to PG after incubation in 2% w/v SDS at 60°C, but were released after heating in denaturing buffer (containing 2% w/v SDS) for 10 minutes at 100°C prior to electrophoresis. These proteins are therefore tightly, although not covalently bound to PG (Mizuno and Kageyama 1979) and therefore are probably porin proteins. The one major OMP that did not remain bound to PG after the 60°C

incubation had a molecular weight of 34Kd (E7) or 34.5Kd (E12, E13, E14 and E16) and was therefore probably the OmpA protein. These molecular weights for OmpA agree well with those observed in other strains of E.cloacae (Marchou et al 1987, Sawai et al 1982).

The expression of the porin and OmpA proteins of each strain of E.cloacae was measured using a laser densitometer. The decreased expression or loss of porin proteins in E.cloacae has previously been correlated with a decrease in susceptibility to β-lactam antibiotics (Bush et al 1985, Marchou et al 1987, Sawai et al 1982, Werner et al 1985), tetracycline and chloramphenicol (Sawai et al 1982, Werner et al 1985). Resistance to β-lactam antibiotics is also affected by the expression of β-lactamase and PBPs. Since these factors do not influence resistance to tetracycline or chloramphenicol, these antibiotics were used to determine any correlation between porin expression and antibiotic resistance. The expression of porin proteins did vary with respect to growth medium. However, there was no general trend of porin expression (i.e. no one growth medium induced high, or low, levels of porin expression consistently in each strain). In addition there was no correlation between the variation in porin expression and sensitivity to tetracycline or chloramphenicol. It could be that these particular antibiotics do not permeate through porins in these strains of E.cloacae. However, there was no correlation between the variation in porin expression and the sensitivity to β-lactam antibiotics either (the MIC values to β-lactam antibiotics are given in Table 17). It may be, therefore,

that the lowest expression of total porin (i.e. 14.5% for E16 in CDM-Mg, see Table 29) was sufficient to allow entry of these antibiotics, such that resistance to these antibiotics was not caused by the changes in porin expression observed.

7. EFFECT OF GROWTH MEDIUM ON PBP EXPRESSION IN E.CLOACAE.

7.1. Determination of the saturating concentration of [^{14}C] penicillin G.

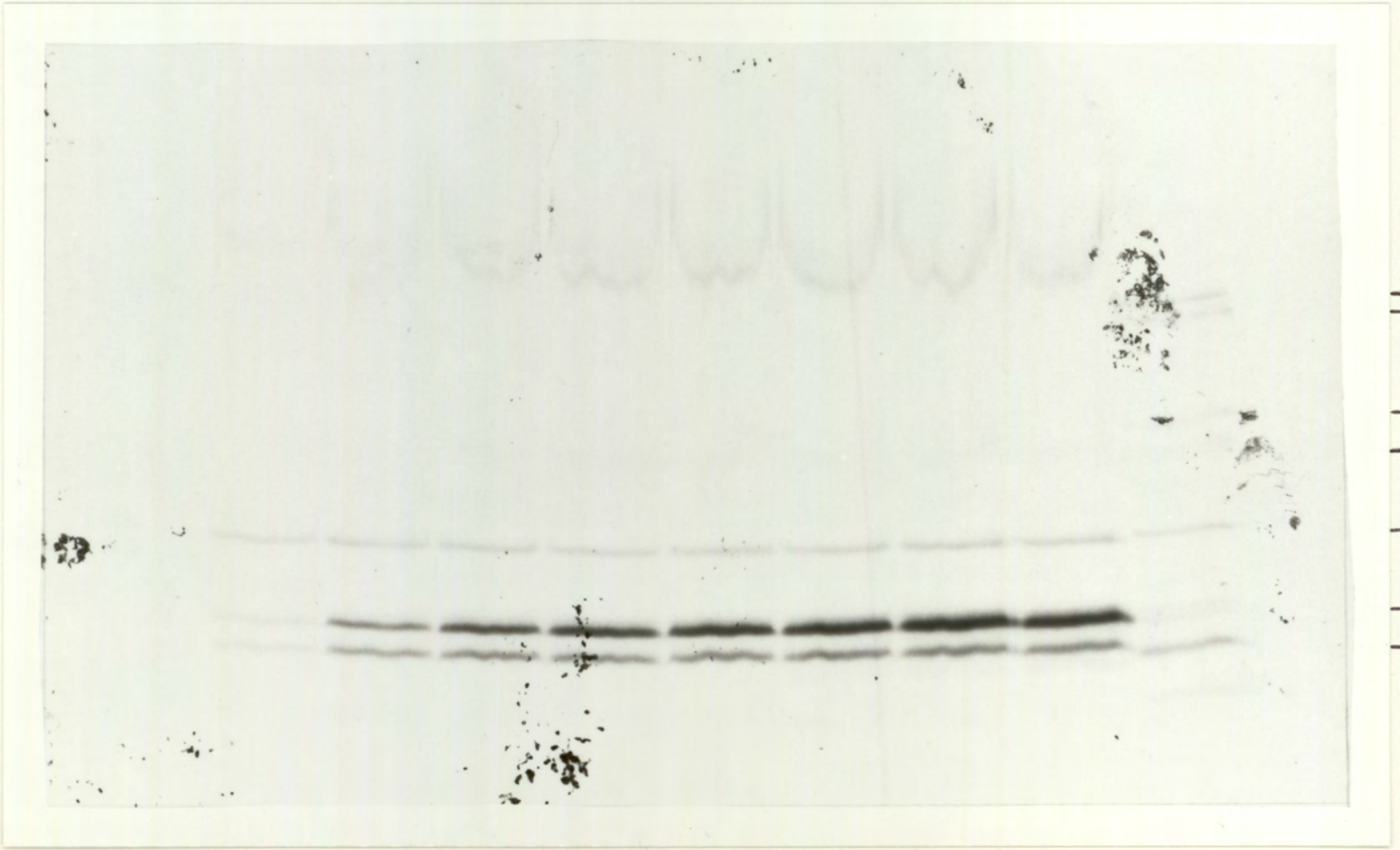
PBP preparations were labelled with varying concentrations of [^{14}C] penicillin G to determine the concentration required to saturate all the E.cloacae PBPs. The results are shown in Figure 16. Only strains E12 and E16 displayed the full complement of PBPs (i.e. 1A, 1B's, 2, 3, 4, 5 and 6), E13 only expressed PBP2 very faintly and also PBPs 3, 4, 5 and 6. E7 and E14 only displayed PBPs 4, 5 and 6. The concentration of [^{14}C] penicillin G at which all bands were visualised (i.e. all that were present) and after which no further bands were visualised was taken as the saturating concentration. This value was 30$\mu g.ml^{-1}$ for strains E7, E13 and E16, but was 75$\mu g.ml^{-1}$ for strains E12 and E14 due to the presence of β-lactamase in the PBP preparations, which could not be removed by washing. This contaminating β-lactamase can be seen on the fluorograph as a band just above PBP5 (Figure 16).

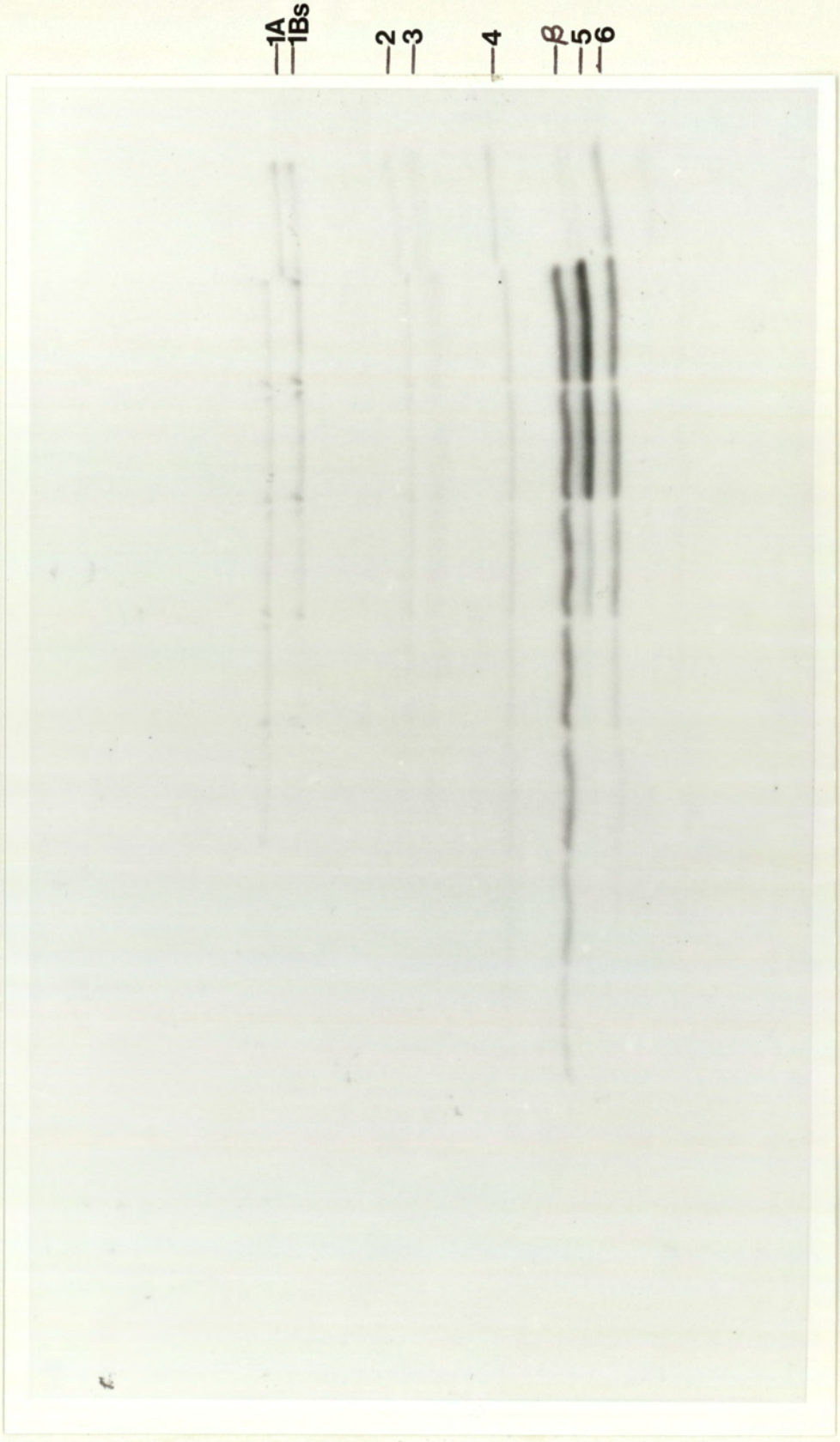
FIGURE 16.

The concentrations of [^{14}C] penicillin G required to saturate all PBPs of the five strains of E.cloacae (E7, E12, E13, E14 and E16) were determined. For each strain, ten aliquots of membrane preparation (10mg.ml^{-1}) were labelled with varying concentrations of [^{14}C] penicillin G (0.1, 1, 5, 10, 20, 30, 50, 75 and 100µg.ml^{-1}; lanes 1-9 respectively). E.coli I124 was run as a control to indicate the position of PBPs 1A, 1B's, 2, 3, 4, 5 and 6 on the gel (lane 10).

Lane	[^{14}C] penicillin G (µg.ml^{-1})
1	0.1
2	1
3	5
4	10
5	20
6	30
7	50
8	75
9	100
10	30 (E.coli I124)

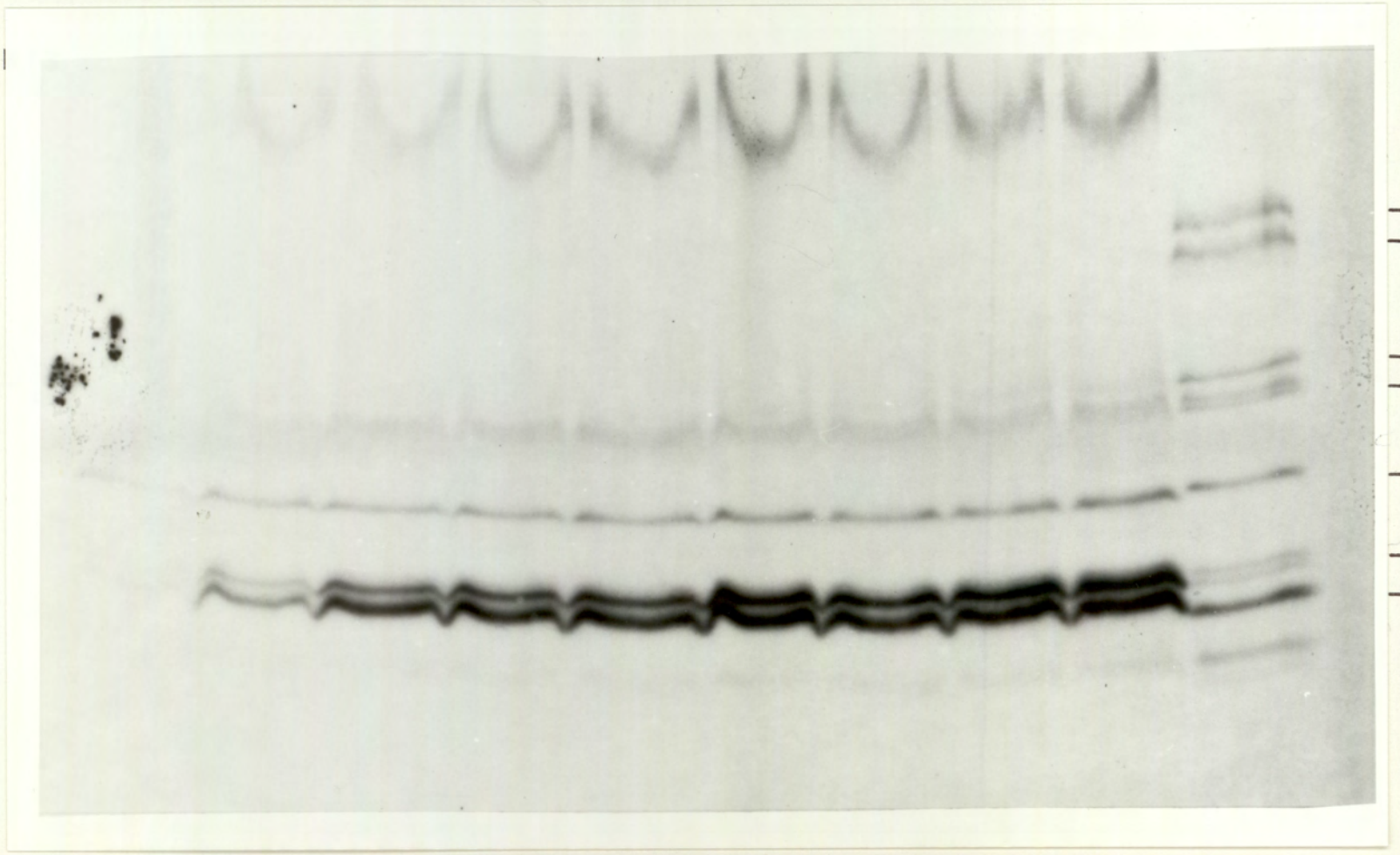
FIGURE 16 . Determination of the saturating concentration of ^{14}C penicillin G for E.cloacae PBPs



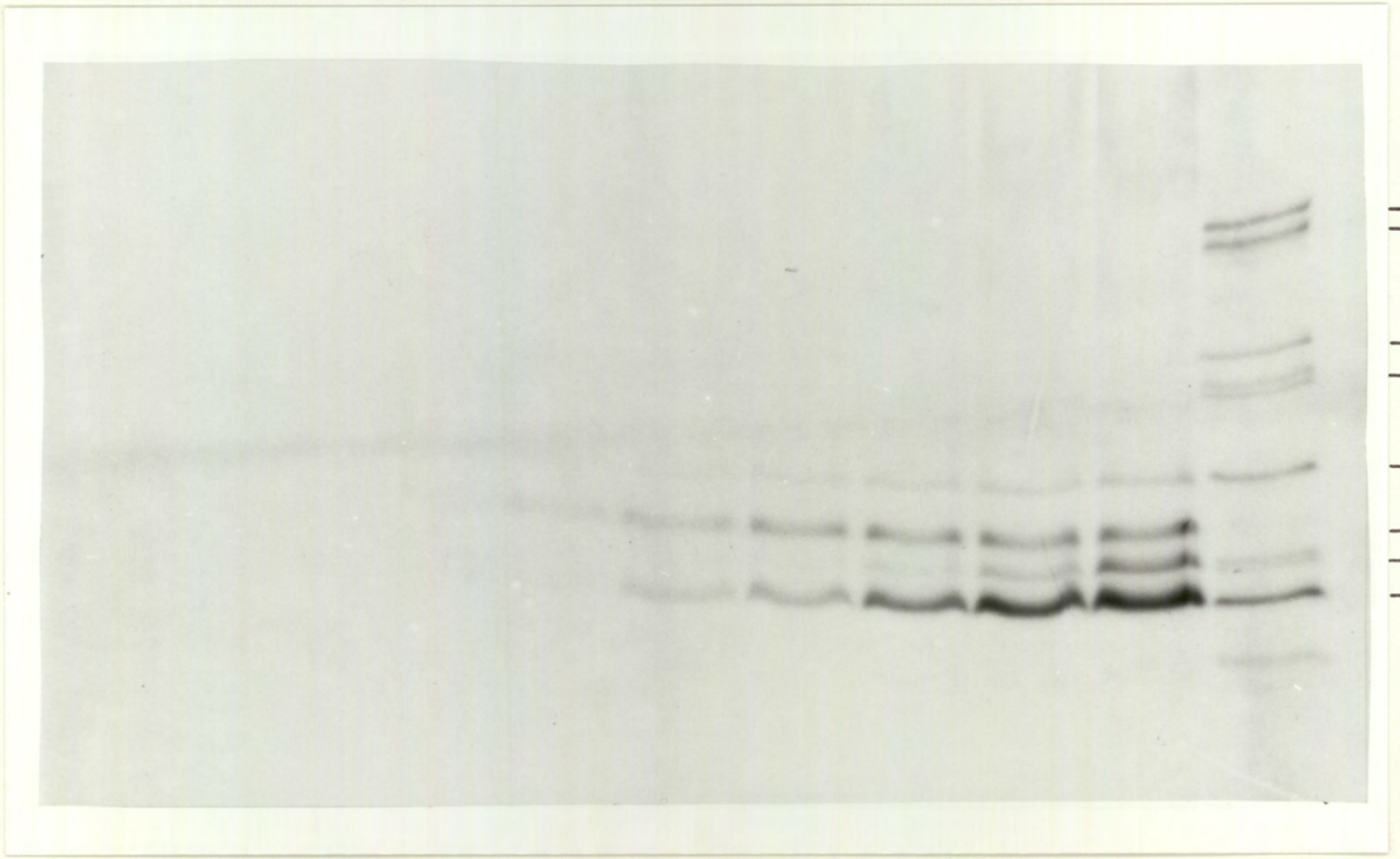


E12

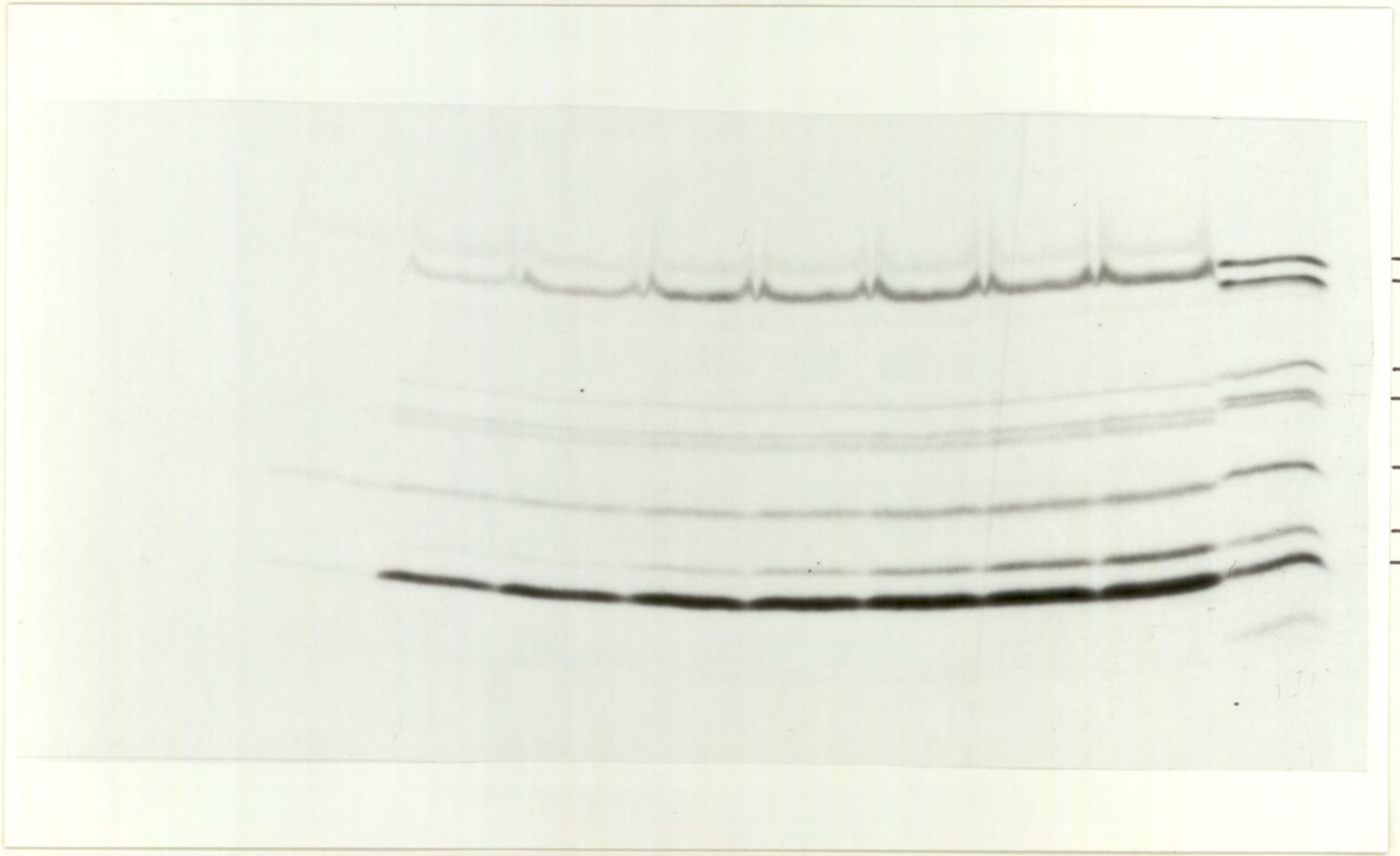
E13



E14



E16



7.2. Effect of growth media on PBP production.

The fluorographs of the PBPs from the five strains of E.cloacae grown in the various media are shown in figure 17.

E7: as with the above experiment, only PBPs 4, 5 and 6 were visualised in this strain. The expression of these PBPs was the same in all growth media except for human serum, where PBP5 was not detected.

E12: the full complement of PBPs were visualised with this strain. Immediately obvious was the failure to detect of any PBP in the preparation from human serum grown bacteria and the presence of a diffuse band between the PBP1B's and PBP2 positions. Other differences to note were: the presence of a weakly expressed PBP immediately below PBP1B's; a slight decreased production of PBP1B's in CDM-P grown bacteria; increased expression of PBP4 in urine + Fe and CDM-P accompanied by a reduction in PBP5 and in the case of urine + Fe also a reduction in PBP6. β-Lactamase was present on this fluorograph as a band situated just above PBP5.

E13: only PBPs 4, 5 and 6 were detected on this fluorograph. As with strain E7, the expression of PBPs 4, 5 and 6 was the same for each growth media, except that PBP5 was not detectable in serum grown bacteria.

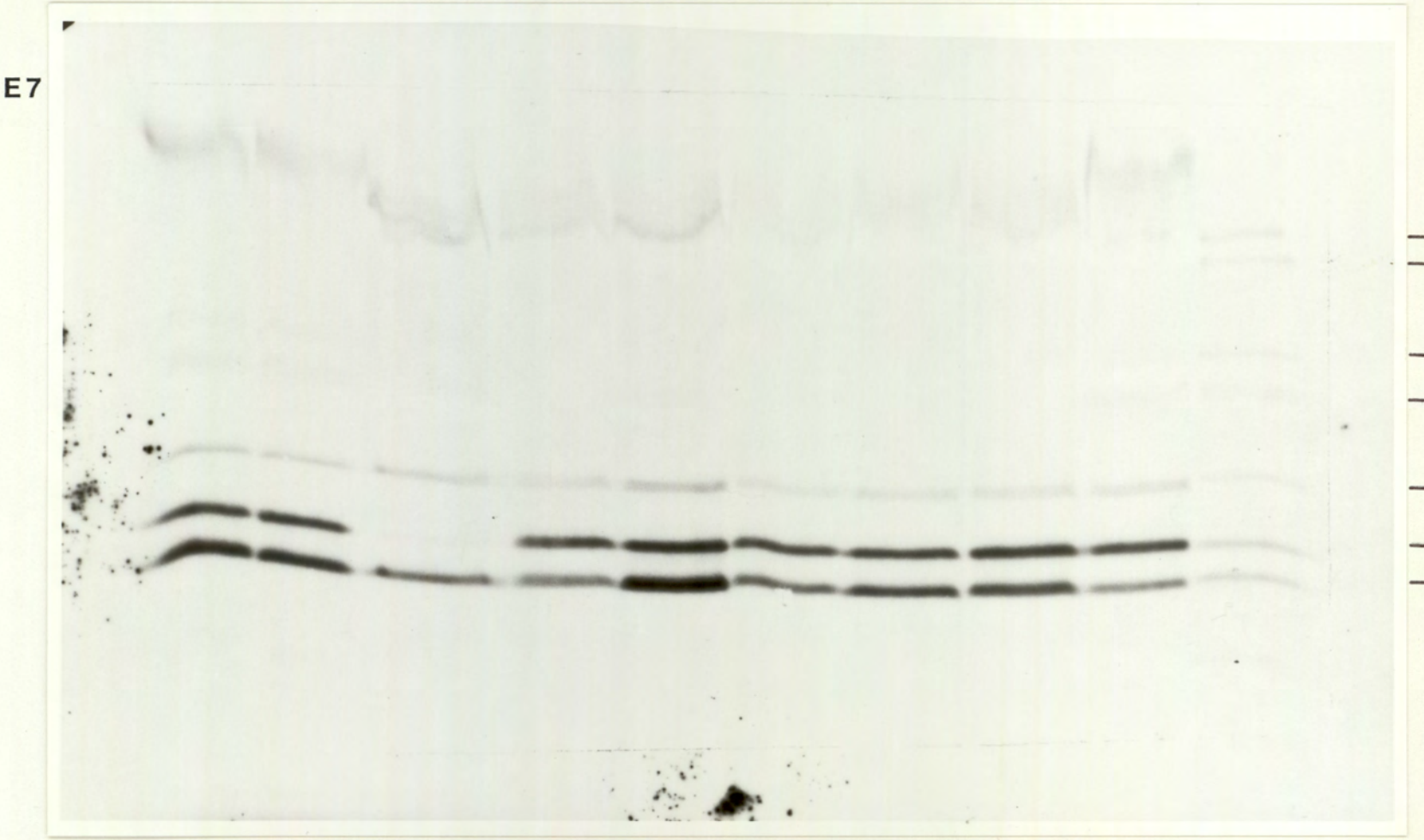
E14: in this strain again only the lower molecular weight PBPs were detected. As with strain E12 contaminating β-lactamase was detected as a band situated just above PBP5. The only major change in PBP expression between the different growth media was the absence of PBP5 in human serum and CDM-P grown bacteria.

E16: again in this case only PBPs 4, 5 and 6 were visualised clearly, although there were some traces of the higher molecular weight PBPs. In the lower molecular weight PBPs the human serum grown bacteria were somewhat different; PBP5 appeared to have an altered molecular weight.

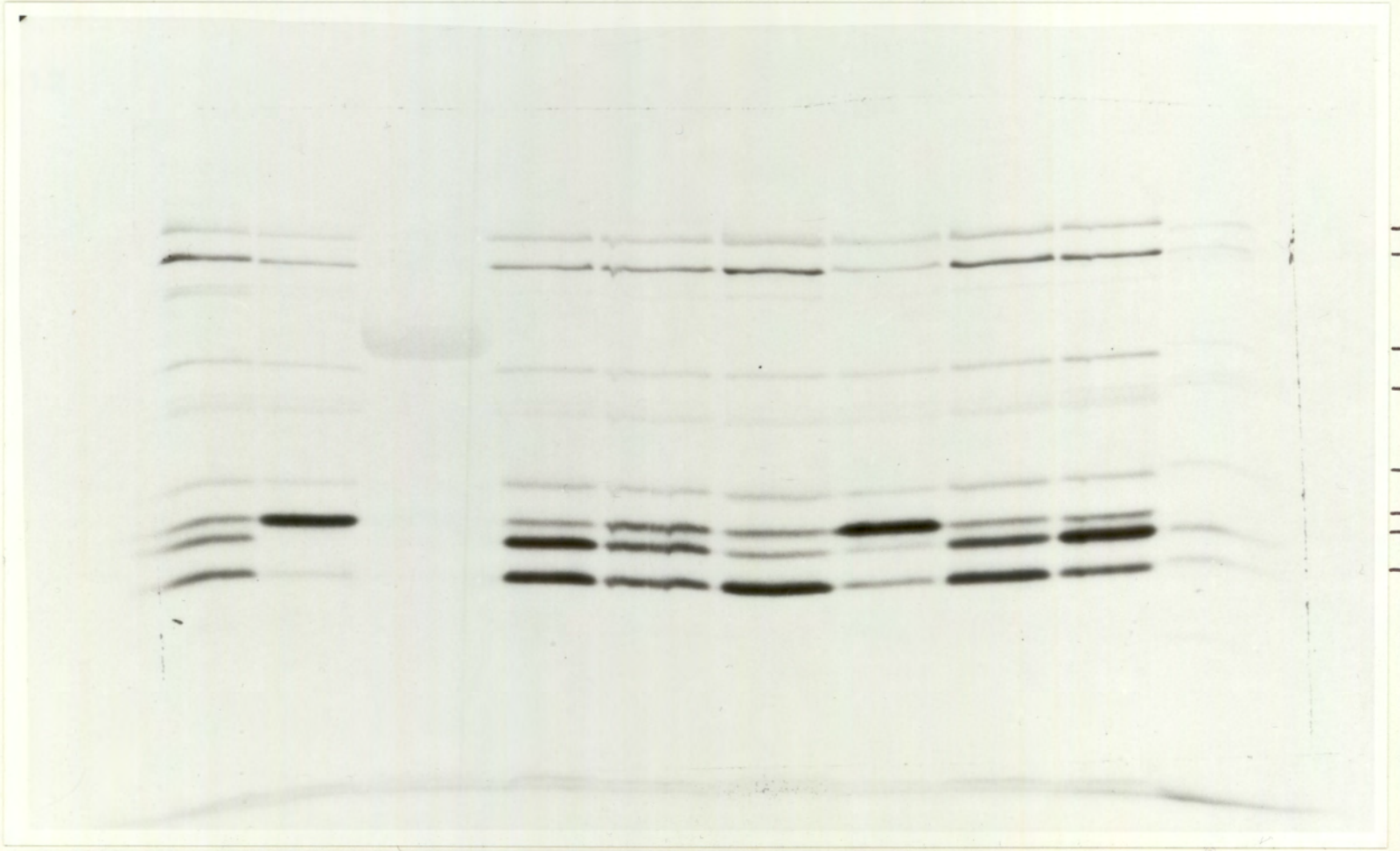
FIGURE 17.

E.cloacae E7, E12, E13, E14 and E16 were grown in: urine (UR), iron-supplemented urine (UR+Fe), human serum (Hu), iron replete CDM (CDM+Fe), iron-deprived CDM (CDM-Fe), magnesium-deprived CDM (CDM-Mg), phosphate-deprived CDM (CDM-P), potassium-deprived CDM (CDM-K) and Iso-sensitest broth (ISB). Their PBP profiles were examined to determine any effect of growth medium. E.coli I124 was run as a control strain. Each membrane preparation of E7, E13 and E16 (standardised to 10mg.ml^{-1}) was labelled with 30µg.ml^{-1} [^{14}C] penicillin G, while those of E12 and E14 were labelled with 75µg.ml^{-1} [^{14}C] penicillin G. The higher quantity of label required for the latter two strains was due to β-lactamase contamination of the membrane preparations, since these strains were stably derepressed mutants. This contaminating β-lactamase was visualised as a band just above PBP5 on the respective fluorographs.

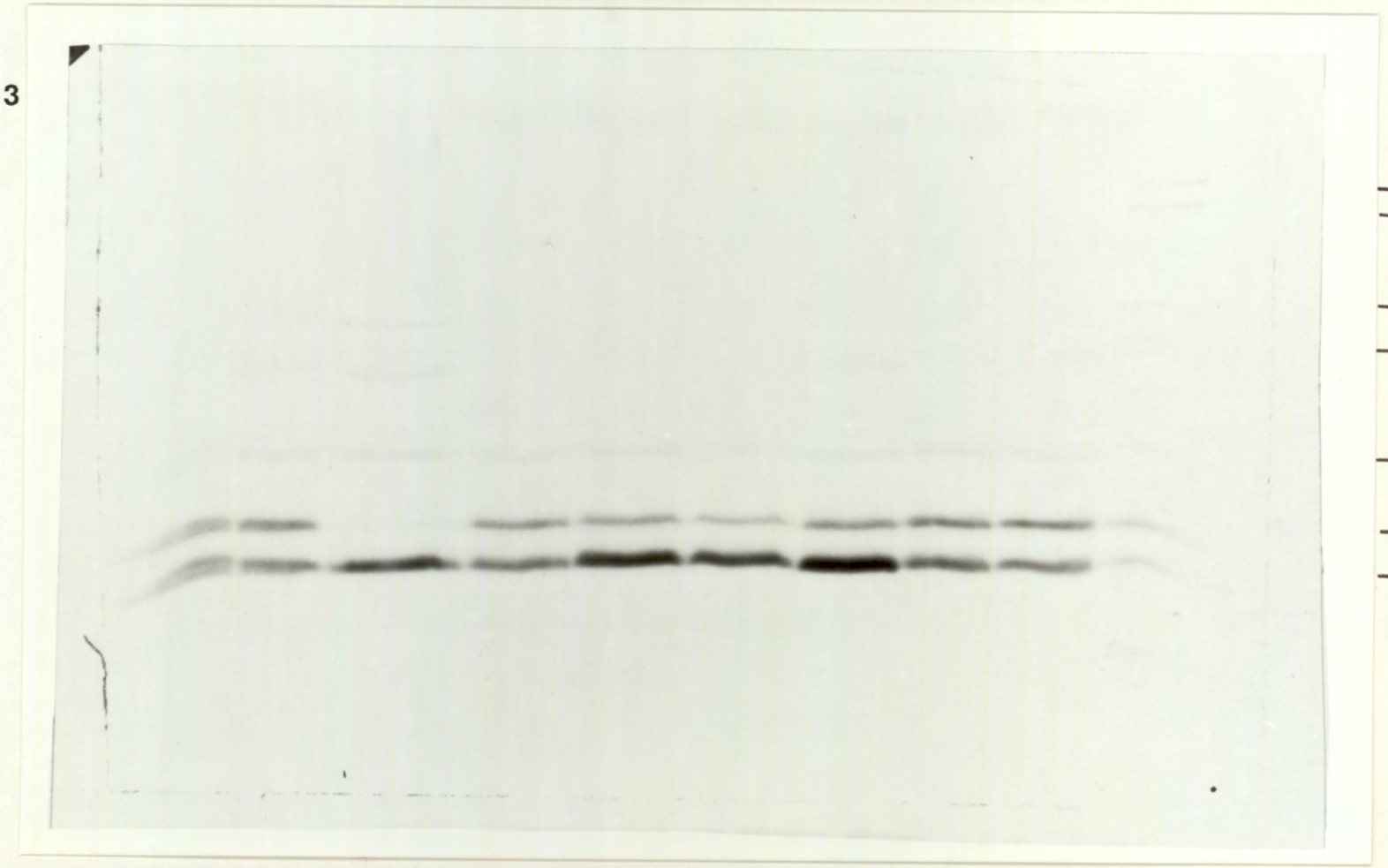
FIGURE 17 . Effect of growth medium on the PBP profile of E.cloacae



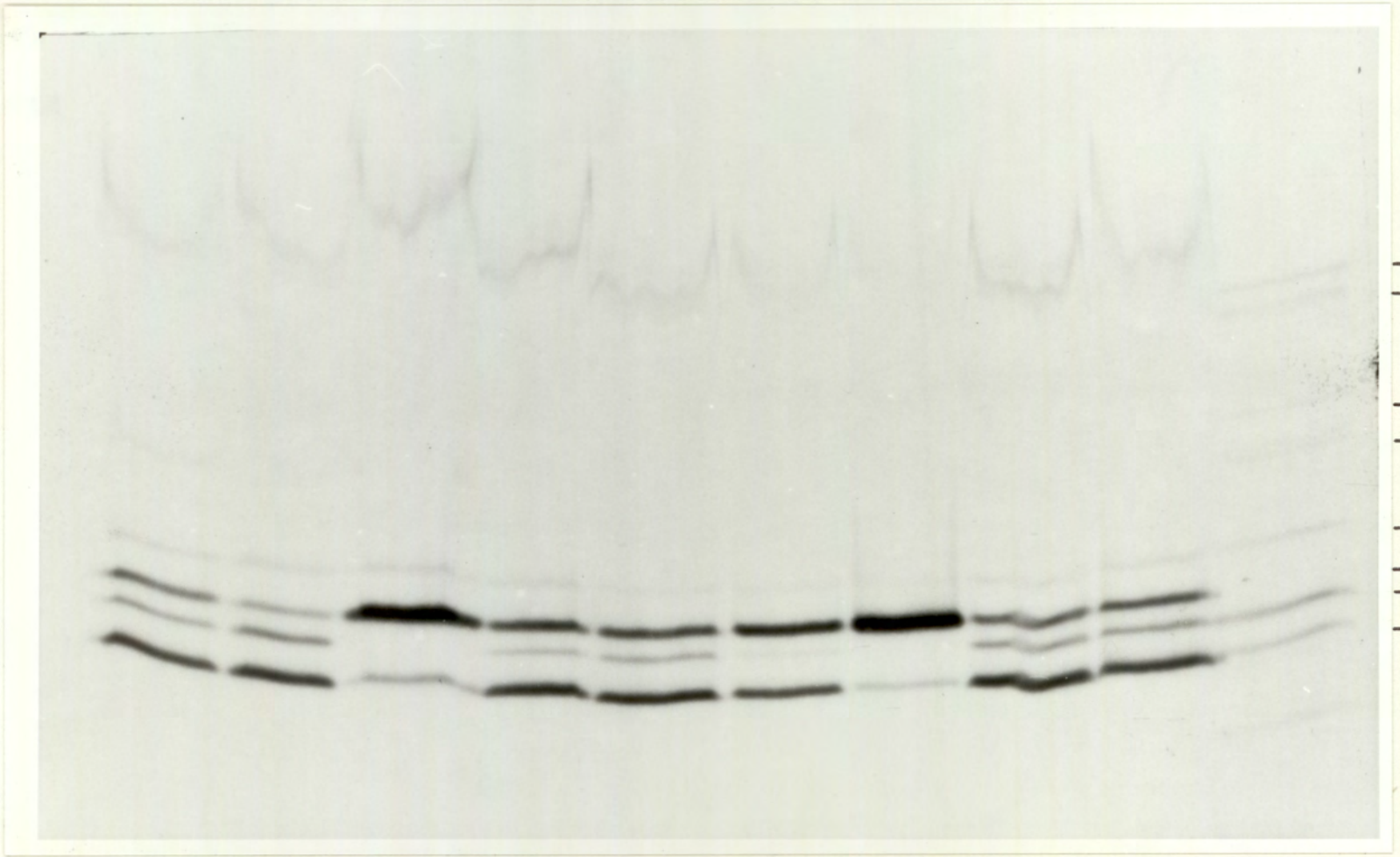
E12



E13

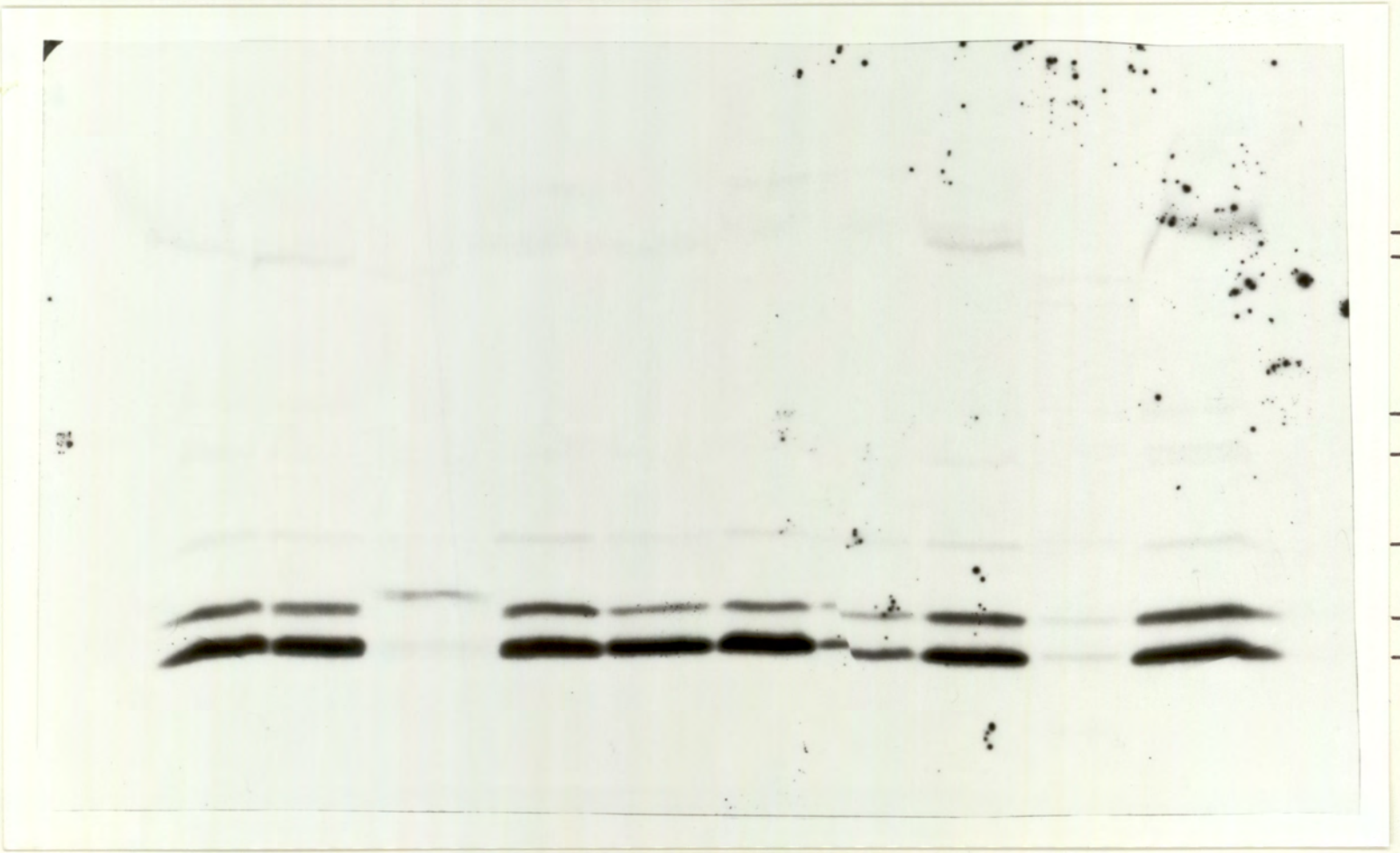


E14



UR | UR +Fe | Hu | CDM +Fe | CDM -Fe | CDM -Mg | CDM -P | CDM -K | ISB | E.coli

E16



7.3. Discussion.

β-Lactam antibiotics exert their lethal effects on bacteria by binding to one or more essential PBPs, thereby inhibitng their respective functions in bacterial morphogenesis (Spratt 1975, 1977a).

Resistance to β-lactam antibiotics can be acquired by alterations in these targets, resulting in a decreased amount or loss of affinity of one or several crucial PBPs (Malouin and Bryan 1986). This mechanism of resistance has been involved in the emergence of resistance in strains of P.aeruginosa during therapy (Godfrey et al 1981).

The expression of PBPs has been shown to vary depending on both the composition of the growth medium and the growth rate (Driehuis and Wouters 1987, Tuomanen et al 1986, Turnowsky et al 1983). Therefore the purpose of this study was to investigate the effect of medium composition upon the expression of PBPs in E.cloacae E7, E12, E13, E14 and E16.

Initially the concentration of [^{14}C] penicillin G required to saturate all the PBPs was determined for each strain. Strains E12 and E14 were found to require greater quantities of [^{14}C] penicillin G due to the presence of contaminating β-lactamase in the membrane preparations. These strains were stably derepressed mutants which produced high levels of β-lactamase constitutively. β-Lactamase contamination persisted even in washed membrane preparations. Then and Angehern (1986) found that PBPs in β-lactamase overproducing variants could not be reliably visualised with [^{14}C] penicillin G because of this residual β-lactamase in the membrane fraction. However, this was overcome to a

certain extent by the addition of greater quantities of [^{14}C] penicillin G. In fact the contaminating β-lactamase was detected as a band on the fluorographs between PBPs 4 and 5 (see Figure 16; E12 and E14).

Curtis et al (1979a) examined the PBP expression of P.aeruginosa 18sSA1$^-$, E.cloacae P99$^-$, K.pneumoniae K1$^-$, Proteus rettgeri 17/1 and E.coli DC0 and found that they exhibited similar, but not identical PBP patterns. By examining the effects of cephaloridine, amdinocillin, cefuroxime and cefoxitin on morphology of these species, they determined that their PBPs performed the same roles as shown for E.coli K12. E.coli I124 used in this study (Figure 16, lane 10) is E.coli DC0 and was obtained from Dr. N.A.C.Curtis (see Table 5). E.cloacae E12 (P99$^+$) was also obtained from Dr. N.A.C.Curtis (see Table 5) and is an isogenic mutant of E.cloacae P99$^-$. The only difference between these strains being that P99$^-$ lacks the ability to produce β-lactamase, while P99$^+$ overproduces β-lactamase. The molecular weights of PBPs of E.coli DC0 and E.cloacae P99$^-$ are given in Table 30; these molecular weights also apply to E.coli I124 and E.cloacae E12.

Only two strains of E.cloacae used in this study (E12 and E16) exhibited the full complement of PBPs (Figure 16). E16 had an identical PBP pattern to E12 (of course in E16 the extra band due to contaminating β-lactamase was missing). The lower molecular weight PBPs visualised in strains E7, E13 and E14 were also identical to E12. It is possible that there was very little qualitative strain to strain variation. However, there was an apparent quantitative difference between the

TABLE 30. Molecular weights of E.coli and E.cloacae PBPs

	Molecular weight (Kd)	
PBP	E.coli DC0	E.cloacae P99⁻
1A	91	92.5
1B's	86.5	
	84	86.5
	81.5	82
2	66	65.5
3	60	59
4	49	48
5	42	42
6	40	39

Molecular weights of PBPs of E.coli DC0 and E.cloacae P99⁻ as determined by Curtis et al (1979a). In this study E.coli DC0 is referred to as I124. E12 is an isogenic mutant of E.cloacae P99⁻. Therefore the molecular weights of PBPs of both strains given above will apply to I124 and E12 used in this study.

expression of PBP5 and PBP 6: in strains E7 and E12 expression of PBP5 was greater than PBP6; in strains E14 and E16 expression of PBP6 was greater than PBP5 and in strain E13 their expression was equal. The significance of this observation is unclear, since PBP5 and 6 appear to have a redundancy of function (at least in E.coli), the loss of one being compensated for by the presence of the other (Tipper 1985).

The interpretation of the effect of the growth medium on expression of PBPs was complicated by the lack of detection of most of the high molecular weight PBPs, except in strain E12. In this strain all six PBPs were present in each growth medium, except for human serum. This result appears to be an anomaly since none of these essential PBPs were detected. It is possible that the membrane preparation was contaminated with a large amount of serum protein, thus giving a falsely high protein estimation. The result would be too little membrane loaded on the gel and hence no visualisation of PBPs on the fluorograph. This may also have occurred in strain E16 since detection of PBPs in the human serum culture was very poor. In strain E12 besides the six PBPs present in each growth medium, two other bands were detected. The first of these was easily visualised immediately above PBP5 this was probably the contaminating β-lactamase. The second band was very weakly expressed just below PBP1B's, it is possible that this may be a new PBP. The appearance of new PBPs has been implicated as a mechanism of resistance in S.pneumoniae (Hakenbeck et al 1980, Zighelboim and Tomasz 1980) and S.aureus methicillin resistance (Brown and Reynolds 1980,

Reynolds and Brown 1985). However the appearance of these new PBPs is usually accompanied by a decrease in at least one of the lethal target PBPs, and this did not occur with strain E12. Furthermore it is not logical that a strain producing high levels of β-lactamase constitutively, sufficient to provide resistance to most β-lactam antibiotics, would also alter its target proteins. It was most probably an artefact caused by the presence of contaminating β-lactamase in the membrane preparation, since E.cloacae P99⁻, the isogenic mutant, was not found to produce this extra PBP and the only difference between these strains was the production of β-lactamase.

Other changes observed in PBPs were limited to the low molecular weight PBPs. In particular PBP5 was not expressed in human serum in E7, E13 or E14. In E16 also, this PBP appeared to have an altered molecular weight. PBP5 was also missing in CDM-P and CDM-Mg. In addition PBP6 was weakly expressed in: E12 in iron-supplemented urine and CDM-P; E14 in human serum and CDM-P and was missing altogether in E16 in human serum. Thus it appears that PBPs 5 and 6 were sensitive to changes in the growth medium, particularly in serum and when limited by phosphate. Unfortunately, the lack of detectable high molecular weight PBPs in most strains makes it impossible to comment on the effect of PBP changes on the β-lactam resistance of these strains, since the high molecular weight PBPs are the lethal targets. The high molecular weight PBPs are particularly heat labile, therefore, it is possible that during the preparation of the membranes these PBPs underwent degradation. However all

membrane preparations were treated in exactly the same way, so it is strange that the high molecular weight PBPs remained intact for strains E12 (Figure 16 and 17) and E16 (Figure 16) and yet not for others. In addition there was a variation in the quality of detection of E.coli PBPs, yet these membrane preparations were made in one batch. It is possible that had the fluorographs been exposed for a longer period of time before developing, more PBP bands would have been visualised. An alternative solution might be to use a label with a shorter half-life than [^{14}C] penicillin G, for example [^{3}H] penicillin G. Clearly further work is required before adequate conclusions can be drawn about the effect of growth medium on PBP expression.

8. CONCLUDING REMARKS.

As with other Gram-negative bacteria, the mechanisms of resistance of Enterobacter cloacae to β-lactam antibiotics operate in the envelope. The composition and structure of the envelope are profoundly influenced by the growth environment (Brown and Williams 1985a, b). Therefore when investigating properties of the envelope which affect β-lactam susceptibility, it is crucial to define the growth environment (Gilbert et al 1987). To this end, this study involved the investigation of β-lactamase, porin protein and penicillin-binding protein (PBP) production of E.cloacae grown in media chosen to mimic conditions which may exist in vivo. The media used included human urine and serum and also a chemically defined medium (CDM) in which the effects of iron-, magnesium-, phosphate- and potassium-deprivation were investigated.

Investigations by Dalhoff and colleagues have shown that β-lactamase production by some strains of E.cloacae, Morganella morganii and Pseudomonas aeruginosa is much greater in vivo than in vitro, even in the absence of β-lactam antibiotics (Cullmann et al 1984, Dalhoff 1982, Dalhoff and Cullmann 1984). This phenomenon was explained as "non-specific" induction, since they had also observed induction of β-lactamase in vitro (to levels achievable by cefoxitin) by non-β-lactam compounds including: tryptophan, thiamine, haemin, folic acid and certain biological fluids (urine, serum, cerebrospinal fluid and pleural fluid), (Cullmann et al 1983). Other workers, however, have looked

for and not observed this phenomenon (Livermore 1987a, Okonogi et al 1986). The results of this study demonstrated a variation in β-lactamase production in different growth media. However, this variation did not correlate with a particular growth medium, such as urine or serum. Hence it was concluded that non-specific induction did not occur with the strains of E.cloacae used in this study. It is clear that non-specific induction cannot be assumed to be a widespread phenomenon. In addition to the lack of non-specific induction by the growth media used in this study, "specific" induction of β-lactamase by cefotaxime in E.cloacae E13 also was not affected by growth medium. Thus β-lactamase production by the strains of E.cloacae used in this study was not influenced by the medium in which they were grown either in the presence or absence of a β-lactam antibiotic.

Investigations into the effect of growth medium on porin production demonstrated that each strain produced two porins and that their expression was variable in different growth media, both within and between strains. However, this variation could not be correlated with either growth medium or β-lactam susceptibility. It is therefore unlikely that growth medium influences β-lactam susceptibility, through porin expression alone, in E.cloacae.

In the future, there is a possibility that iron-regulated membrane proteins (IRMPs) may be exploited for the uptake of certain β-lactam antibiotics (e.g. E-0702 via the enterochelin receptor; Watanabe et al 1987). Thus it is important to investigate IRMP expression by in vivo bacteria. Although in vivo outer membrane protein profiles were not

obtained in this study, preliminary investigations demonstrated that E.cloacae produced IRMPs when grown in human serum or urine. In the light of these observations and previous investigations showing that Gram-negative bacteria grow under iron restriction in vivo (Brown et al 1984, Lam et al 1984, Shand et al 1985), it is likely that in vivo E.cloacae would also express IRMPs. The expression of IRMPs by E.cloacae in vitro varied between serum, urine and iron-deprived CDM, including the expression of an 81Kd protein, which was possibly the enterochelin receptor. If future β-lactam treatment by compounds taken up by this receptor, such as E-0702, is to be successful, it is vital that the prevalence of this receptor in vivo is first established.

Preliminary investigations into the expression of PBPs by E.cloacae in different growth media demonstrated that PBPs 5 and 6 were sensitive to changes in the growth medium. Changes in expression of these PBPs may affect the extent of peptidoglycan cross-linking in these cells. However, both PBPs were not missing at the same time and since they are individually dispensable (Broome-Smith and Spratt 1982, Spratt 1980) the variation caused by the growth medium was not lethal to these strains. Further investigations are required to determine the effect of growth medium on expression of the higher molecular weight PBPs, the lethal target enzymes of β-lactam antibiotics.

The growth rate of bacteria has been shown to influence their resistance to β-lactam antibiotics (Cozens et al 1986b, Tuomanen et al 1986). In particular slow-growing or non-growing bacteria express altered PBP profiles and are

resistant to nearly all β-lactam antibiotics except the carbapenems (Tuomanen and Schwartz 1987). In this study, the effect of growth rate on β-lactamase production was investigated. Faster growing bacteria demonstrated superior β-lactamase induction potential than slower growing bacteria. This observation has important implications for the β-lactam therapy of fast-growing (fulminating) infections. It is possible that treatment of such infections with β-lactam antibiotics may induce levels of β-lactamase high enough and rapidly enough to influence their efficacy (especially if the β-lactamase were acting in conjunction changes in porin expression). However, in the treatment of slow-growing (chronic) infections, then changes in PBP expression or β-lactam tolerance (Tuomanen 1986, Tuomanen and Schwartz 1987) may play more important roles than β-lactamase.

Overall, the results presented in this thesis suggest that growth medium and specific nutrient deprivation do not affect the susceptibility of E.cloacae to β-lactam antibiotics. However, growth rate has a significant effect upon the induction of β-lactamase by β-lactam antibiotics. The results suggest that future work should be targeted on the control of β-lactamase induction and the role that growth rate plays in its control.

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APPENDIX 1. Data from *in vivo* urine samples.

Examination	Strain		
	E13	E14	E16
Microscopic:			
Gram-negative rods	+ + +	+ +	+ +
White blood cells	+	-	+
Red blood cells	+ + +	-	-
Epithelial cells	-	+	+
crystalline deposit	+	+	-
other	one budding yeast	filamented bacteria	Gram +ve bacilli
Viable count	3.5×10^8	1.1×10^6	1.0×10^6
Culture: MacConkey and Nutrient agar			
No. colony types	1	2	1
colony diameter (mm)	2 - 3	1, 2-3	2 - 3
appearance	glossy	glossy	glossy
ferment lactose	+	+	+
API identification	*E.cloacae* 3105573	*E.cloacae* (2) 3105573 3305573	*E.cloacae* 3305573
Other			70-75 $\mu g.ml^{-1}$ trimethoprim [a]

a This patient was given a 200 mg dose of trimethoprim before the urine sample was collected. After a normal oral dose of 160 mg, the levels expected in the urine are approximately 100 µg. This value is greater than the normal MIC for *E.cloacae* (< 32 $\mu g.ml^{-1}$).