Influence of sub-MICs of β-lactam antibiotics, growth rate, and iron limitation on the surface structures of *Klebsiella pneumoniae*.

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Doctor of Philosophy

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Chemostat culture makes it possible to control growth rate and nutrient availability. It was used in this study to mimic some of the conditions which are characteristic of bacterial growth *in vivo*.

The pattern of expression of one of the porin proteins of *K. pneumoniae* was shown to vary with changes in growth rate and iron availability. This could have important implications in antibiotic resistance.

High molecular weight iron regulated outer membrane proteins (IRMP's) were expressed in iron limited chemostats at both slow and fast growth rates but the amount of the bacterial iron chelator enterochelin detected in the media was reduced at the slow growth rate. When iron was not the growth rate limiting nutrient but was reduced almost to these levels the IRMP's were expressed only by fast growing cells. The evidence suggests that acquisition of iron is less physiologically stressful in slow compared to fast growing cultures.

The amount both of cell free and of cell associated capsular polysaccharide produced by slow growing iron limited chemostat cultures of *K. pneumoniae* was significantly greater than in faster growing cultures. Slow growth rates are characteristic of bacteria adapted to persistence *in vivo* and encapsulation may be of particular importance in protecting these bacteria from clearance by host defence mechanisms. There was little effect of growth rate on the lipopolysaccharide ladder pattern.

At concentrations of 1/5 MIC two β-lactam antibiotics Ceftriaxone and Cefotaxime were shown to affect both enterochelin and capsule production. The production of enterochelin was reduced in fast growing cultures where it's production is maximal and where iron acquisition is critical to the bacteria. Capsular polysaccharide was conversely affected most in slow growing cells for which, in the *in vivo* situation, encapsulation is likely to be of importance.

Growth rate and nutrient availability were shown to influence two virulence factors of *K. pneumoniae* in ways which may be of importance for survival *in vivo*. Sub-MIC concentrations of β-lactam antibiotics were shown to have effects which may act synergistically with host defence mechanisms to clear bacteria from infections.

Key words: capsular polysaccharide, growth rate, iron limitation, siderophore, sub-MIC.

Acknowledgements

To build a hut
One needs mud
and sissal
And friendly
Neighbours.

Alice Walker

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Abbreviations

CAS	chrome azurol.
DBS	dihydroxybenzoylserine.
EDTA	ethylenediaminetetra-acetic acid.
HDTMA	hexadecyltrimethylammonium bromide.
IRMP	iron regulated membrane protein.
MBC	minimum bactericidal concentration.
MIC	minimum inhibitory concentration.
PAGE	polyacrylamide gel electrophoresis.
PBP	penicillin binding protein.
R_f	retardation factor.
SDS	sodium dodecyl sulphate.
t_d	doubling time.

1. Introduction

1.1. Introductory remarks.

It is becoming clear that bacteria used for the development of antibiotics and vaccines and which are grown under standard laboratory conditions are in some very important ways different from those which are found causing infection *in vivo*. Factors such as nutrient limitation, especially iron, growth rate (Brown and Williams 1985a,b) and the presence of sub-MIC concentrations of antibiotics (Lorian 1980) affect the bacterium as well as influence it's interaction with the host. The cell envelope is an important interface between the bacterium and the host organism (Smith 1977), and is affected by the conditions in which the bacterium is grown (Brown and Williams 1985a,b). The purpose of this study is to use the chemostat to mimic some of the factors of importance *in vivo*, to study the effect on the envelope of *K. pneumoniae* and discuss the importance of these effects in the host parasite interaction.

1.2. Nomenclature

The genus Klebsiella contains *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* which are three closely related bio-sub-pathogroups of one genetic species (Brenner *et al* 1969). According to Bergeys Manual of systematics (1984) *K. aerogenes* is a biogroup of *K. pneumoniae* and although it does not have standing in the literature it is used clinically in Great Britain. The name *K. aerogenes* is never used in the United States of America leading to the same organism being classified both as *K. pneumoniae* and as *K. aerogenes*. Throughout the experimental part of this thesis the two strains of Klebsiella used have are referred to as *K. pneumoniae*. In references to other work the name used in the

original paper is used but it should be born in mind that *K. pneumoniae* and *K. aerogenes* are the same organism.

1.3. The importance of *K. pneumoniae* as a pathogen.

Infections occurring during hospitalisation are an increasing clinical problem. The occurrence of these infections can be correlated with the increase in the use of instrumentation and the great variety of drugs which are used in hospitals today (Lorian and Topf 1972). These nosocomial infections are seldom the sole cause of death but in many cases are contributory factors. The concentrated use of antibiotics in the hospital environment inevitably leads to the emergence of resistant strains of bacteria.

A study of the cases of clinically significant bacteraemia reported to the PHLS between the years 1975 and 1980 (Young 1982) found that 42% of organisms isolated were Gram negative and that *K. pneumoniae* was the second most common after *E. coli*; a similarly high incidence was found in the USA (Bryan *et al* 1984). *K. pneumoniae*, however, is not a common cause of infection in the community (Montgomerie and Ota 1980) and has only emerged as an important nosocomial pathogen after the introduction of antibiotics (McGowan 1985), it shows a particularly high incidence of antibiotic resistance among the enterobacteraceae (Ullmann 1978).

Infection with *K. pneumoniae* is associated with a number of predisposing factors. The PHLS report showed that 49% of patients with *K. pneumoniae* infections were elderly and that 30% had concurrent urinary tract infections (Young 1982). *K. pneumoniae* is an important pathogen in neonates where it causes sepsis, meningitis and necrotising enterocolitis (Hill *et al* 1974). It is also often found colonising the

respiratory tract especially in patients with preexisting respiratory diseases (Johanson *et al* 1972). Patients who are immunocompromised by trauma, disease or immunosuppressive chemotherapy are also particularly susceptible to *K. pneumoniae* infections (Montgomerie and Ota 1980).

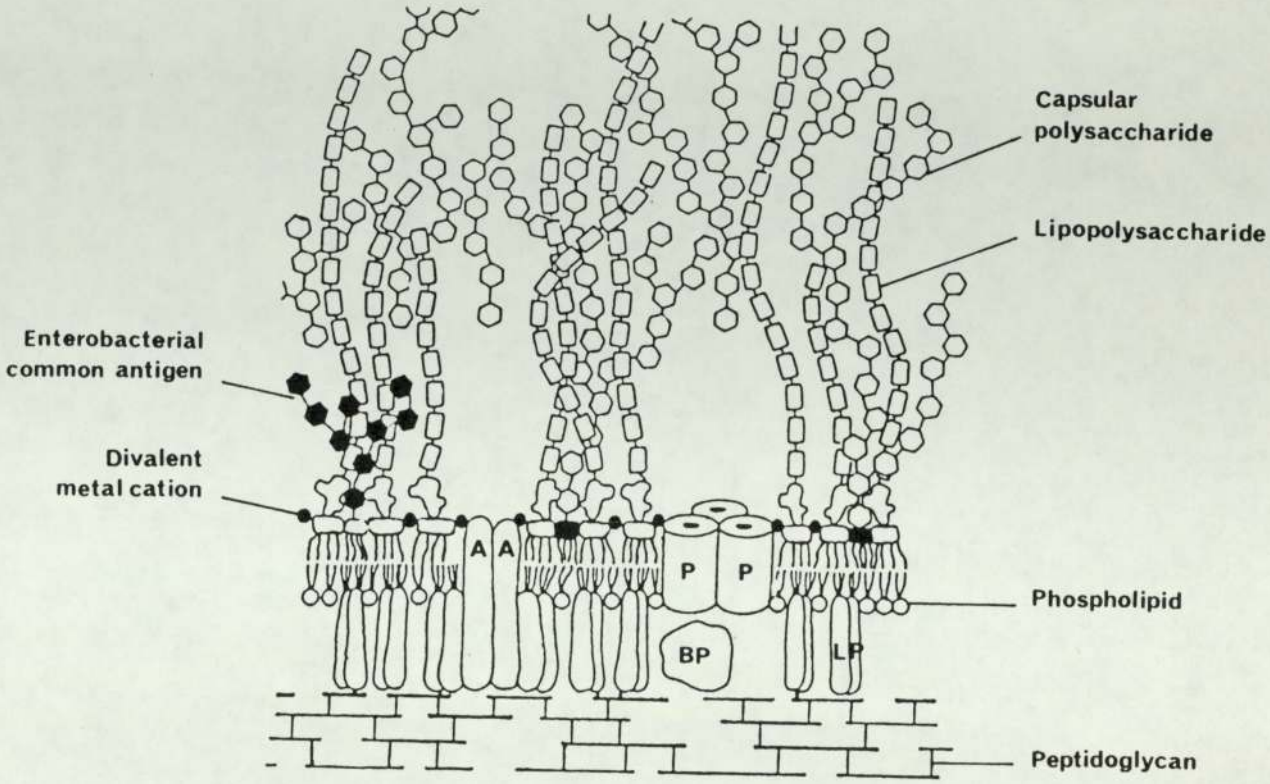
1.4. The Gram negative outer membrane and capsule.

The envelope of Gram negative bacteria is a complex structure consisting of: the periplasm, a compartment with a composition distinct from that of the cell interior and the external environment, the outer membrane, and in many cases a layer of capsular polysaccharide. The outer membrane is composed of 20 to 25% phospholipid, 30% lipopolysaccharide and 45 to 50% protein (DiRienzo *et al* 1978). The lipid components form a bilayer structure in which the protein components are embedded. It forms a selective barrier around the bacteria through which it is possible for hydrophobic molecules to pass but which excludes hydrophilic molecules with a molecular weight above 650 daltons (Decad and Nikaido 1976). Fig 1.1. shows a diagramatic representation of the gram negative outer membrane.

1.4.1. The Periplasm.

The envelope of Gram negative bacteria has been shown by electron microscopy to consist of an inner and an outer membrane separated by a periplasmic space which contains: the peptidoglycan, the membrane derived oligosaccharides and a number of different proteins. These proteins are periplasmic enzymes, including antibiotic inactivating enzymes (Curtis *et al* 1972, Neu and Heppel 1965), nutrient scavenging enzymes (Malamy and Horecker 1961) and nutrient binding proteins (Heuzenroeder and Reeves 1980). The membrane derived oligosaccharides are water soluble oligosaccharides consisting of 8 to 10 glucose

Fig.I.1. Schematic representation of the outer membrane of Gram negative bacteria.



Adapted from Lugtenberg and van Alphen (1983), showing the capsular polysaccharide and possible anchorage in the outer membrane (Gotschlich *et al* 1980, Schmidt and Jann 1982) and the enterobacterial common antigen (Kunin and Beard 1963).

P Porin protein
LP Lipoprotein
BP Nutrient binding protein
A Outer membrane protein A

residues linked in a highly branched fashion. The membrane derived oligosaccharides are multiply substituted with *sn*-phosphoglycerol residues which are derived from the cytoplasmic membrane phospholipid (Kennedy *et al* 1976) hence their name. These molecules, which comprise up to 1% of the cell dry weight are either free in the periplasm or loosely associated with the outer membrane (Schulman and Kennedy 1979). Membrane derived oligosaccharides have a net negative charge and their synthesis is regulated in response to changes in the osmolarity of the medium so it seems likely that they perform a role in osmoregulation (Kennedy 1982).

The peptidoglycan forms a rigid and highly structured layer around the cytoplasmic membrane. This layer has high mechanical strength and is responsible for maintaining the cells shape. The chemical composition of peptidoglycan varies between species but there is a basic underlying structure. It consists of linear glycan strands of up to 200 disaccharides in length which are cross-linked by short peptides (Schleifer and Kandler 1972). It is thought that the peptidoglycan layer of a single bacterium consists of a single bag shaped macromolecule (Weidel and Pelzer 1964). Electron microscopy of purified peptidoglycan sacculi after partial degradation of peptide bonds with endopeptidase suggests that the glycan strands are orientated perpendicular to the long axis of the cells (Verwer *et al* 1978). The degree of cross linkage of the glycan chains varies from one organism to the next. A relatively low degree of cross-linkage is characteristic of Gram negative bacteria; in *E. coli* there is only 20% cross linkage (Schleifer and Kandler 1972). The traditional model of the Gram negative cell envelope locates the peptidoglycan as a "central line" in the periplasm with the periplasmic space lying between this and the cytoplasmic membrane (DePetris 1967,

Murray *et al* 1965). Recent work using a low temperature embedding technique, which causes less damage than conventional techniques, for the preparation of specimens for electronmicroscopy has proposed an alternative model (Hobot *et al* 1984). These workers propose that the space between the inner and outer membranes is filled with a periplasmic gel of extended peptidoglycan. They propose that the peptidoglycan would form a frame filled with an aqueous solution of the periplasmic proteins and oligosaccharides. Which ever of these models is correct it is clear that the periplasm is more than an empty space between two membranes, it is a dynamic structure with distinct enzyme and osmoregulatory properties and is probably involved in the maintenance of the peptidoglycan and of both membranes (Beveridge 1981).

1.4.2. Phospholipids.

Both the cytoplasmic and outer membranes of Gram negative bacteria contain a mixture of phospholipids: phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. It has been shown in *Salmonella typhimurium* and *E. coli* that the outer membrane is enriched for phosphatidylethanolamine (Osborn *et al* 1972, Lugtenberg and Peters 1976). This asymmetric distribution of phospholipids in the membranes may be explained by the fact that phosphatidylethanolamine forms stable bilayers with lipopolysaccharide (Fried and Rothfield 1978). The phospholipid composition of the outer membrane is not greatly influenced by growth conditions other than phosphate limitation (Minnikin *et al* 1971) but there is an important adjustment of the proportions of saturated to unsaturated fatty acids in the membrane in response to temperature changes. The fluidity of the outer membrane is very important for it's correct functioning and is affected by temperature. Bacteria respond very rapidly to the increased fluidity

caused by a rise in temperature with an increase in the proportion of saturated to unsaturated fatty acids (Cronan and Gelmann 1975).

1.4.3. Lipopolysaccharide.

The outer membrane of Gram negative bacteria contains a unique lipopolysaccharide. The molecule consists of three covalently linked portions: the lipid A, the core region and the polysaccharide side chain (Wilkinson 1977). The lipid A regions of lipopolysaccharide from a wide range of Gram negative bacteria show a great degree of structural similarity (Wicken and Knox 1980). This portion of the molecule is hydrophobic and is inserted in the phospholipid membrane. The core region is less highly conserved between species. It is a complex oligosaccharide containing the sugar acid 3-deoxy-D-*manno*-2-octulosonate (KDO) through which it is linked to the lipid A region (Lüderitz *et al* 1982). The O-side chain polysaccharide is the portion which gives the characteristic appearance to smooth strains of Gram negative bacteria (Wicken and Knox 1980). There is a wide variation in the composition and branching structure of the polysaccharide side chains, which are composed of a repeating oligosaccharide units, within species (Lugtenberg and van Alphen 1983). A preparation of lipopolysaccharide from a single organism will contain molecules of lipid A and the core region and a series of molecules with different numbers of polysaccharide repeating units (Wicken and Knox 1980), giving rise to the characteristic ladder pattern seen in PAGE.

Most of the lipopolysaccharide is situated in the outer leaf of the outer membrane bilayer, with the antigenic O polysaccharide side chain extending outwards from the cell. It has been suggested that the O polysaccharide side chain may adopt a coiled configuration (Labischinski

et al 1985). The asymmetrical distribution of the lipopolysaccharide has been demonstrated by Mühlradt and Golecki (1975) using a ferritin labelled antibody against the polysaccharide region of the lipopolysaccharide molecule and by Funahara and Nikaido (1980) who showed that treatment of lipopolysaccharide with galactose oxidase resulted in the same extent of oxidation whether the enzyme acted on isolated lipopolysaccharide or on the lipopolysaccharide of intact cells. This asymmetrical distribution required an intact peptidoglycan layer for it's maintenance (Mühlradt and Golecki 1975).

There is a body of evidence to suggest that the lipopolysaccharide is not freely moving within the outer face of the outer membrane. Schindler *et al* (1980) studied the movement of lipopolysaccharide from *Salmonella typhimurium* and *E. coli* in reconstituted membranes containing phospholipid and outer membrane proteins. They showed that as the concentration of protein increased the mobility of the lipopolysaccharide was reduced while that of the phospholipid was largely unchanged. They suggest that the association between the matrix proteins and lipopolysaccharide (Datta *et al* 1977, Schindler and Rosenbusch 1978) go part of the way to explain their findings and propose that the underlying peptidoglycan layer, which is covalently linked to the outer membrane (Braun 1975), could also control the diffusion of outer membrane components. *E. coli* lipopolysaccharide consists of two populations, up to half of the lipopolysaccharide can be released into the medium by the action of EDTA (Leive 1974). This presumably is a result of the removal of divalent cations which are involved in lipopolysaccharide lipopolysaccharide interactions and are important in maintaining the molecular organisation of the outer membrane. The remaining portion of the lipopolysaccharide interacts

hydrophobically or ionically with the other molecules of the membrane (Freid and Rothfield 1978, Datta *et al* 1977, Schindler and Rosenbusch 1978).

The Fluid Mosaic membrane model (Singer and Nicolson 1972) goes only part of the way to explaining the structure of the bacterial outer membrane. The function of the outer membrane requires a degree of structural organisation which is brought about by the various interactions between the membrane constituents; divalent cations are of particular importance in these interactions. The underlying rigid network of peptidoglycan to which the outer membrane is anchored also plays a role in this structural organisation.

1.4.4. Enterobacterial common antigen.

Enterobacterial common antigen has been found in almost all of the Enterobacteriaceae but not in other Gram negative organisms (Kunin and Beard 1963). It is a linear polymer of *N*-acetyl-D-glucosamine and *N*-acetyle-D-mannosaminuronic acid. The molecule also contains a lipid moiety (Mäkelä and Mayer 1976). The molecule exists in two forms, a free form which is present in most strains and an immunogenic form which is found in rough strains (Rinno *et al* 1980) and is anchored in the outer membrane by linkage to the core lipid A moiety (Kiss *et al* 1978).

1.4.5. Outer membrane proteins.

The outer membrane of the members of the enterobacteriaceae contain one or more protein which remain strongly but not covalently bound to the peptidoglycan fraction after extraction in 2% sodium dodecyle sulphate (SDS) at 60°C (Lugtenberg *et al* 1977). This association with the peptidoglycan, while characteristic of these proteins, is probably an *in*

vitro artefact (Lugtenberg and van Alphen 1983). These proteins form non specific diffusion channels through which hydrophilic solutes of less than 600 daltons can pass (Decad and Nikaido 1976) and are termed porins. Protein monomers each with a molecular weight of 35 to 38 kDa associate into functional units consisting of three identical molecules (Schindler and Rosenbusch 1978, Steven *et al* 1977). Three stain filled indentations have been demonstrated by electronmicroscopy, on the outer face of the outer membrane which apparently coalesce to a single pore at the inner face (Dorset *et al* 1984). Conductance measurements using conditions under which a single triplet could be examined confirmed the model of three porin channels within a single trimeric unit (Engel *et al* 1985).

The outer membrane of *E. coli* contains two peptidoglycan associated porin proteins, known as OmpF and OmpC. They are immunologically related to each other as well as to the phosphate inducible pore protein PhoE (Overbeeke *et al* 1980). *K. pneumoniae* exhibits two peptidoglycan associated outer membrane proteins (Hofstra *et al* 1980, Lugtenberg *et al* 1977, Williams et al 1984), which cross react immunologically with the OmpF and OmpC proteins of *E. coli* (Hofstra and Dankert 1979, Hofstra *et al* 1980) and to which they are probably functionally related.

The outer membrane of *K. pneumoniae* contains a third major protein which is not peptidoglycan associated and which cross reacts immunologically with the OmpA protein of *E. coli* (Hofstra and Dankert 1979). The OmpA protein is heat modifiable and because it acts as a receptor for phages (Skurray *et al* 1974) it is assumed to be exposed at the envelope surface. This protein plays a role in F-pilus mediated conjugation (Havekes and Hoekstra 1976, Skurray *et al* 1974) and is involved in the

maintenance of the structure and rod shape of the bacterium (Sontag *et al* 1978).

The envelope of Gram negative bacteria contains a large number of molecules of a lipoprotein. This protein does not serve as a receptor for any phages and antibodies raised against isolated protein do not bind to intact cells (Bosch and Braun 1973) which suggests that the protein is deeply buried within the envelope. The *N*-terminus of the protein is linked to the lipid portion, the fatty acids of which are similar to those found in phospholipids from the same organism (Hanke and Braun 1973). The molecule is located in the inner face of the outer membrane via the lipid portion. About one third of the cell's complement of lipoprotein is covalently bound to the peptidoglycan through the *C*-terminal lysine (Braun and Bosch 1972). This peptidoglycan linked lipoprotein acts as a link which anchors the outer membrane to the rigid peptidoglycan layer (Braun and Rehn 1969). Mutants of *E. coli* which are defective in synthesis of both forms of the lipoprotein grow and divide normally but are highly sensitive to EDTA and detergents (Hirota *et al* 1977). Electronmicroscopy of these mutants shows surface blebbing and also indicates that the outer membrane is no longer attached to the peptidoglycan (Sontag *et al* 1978).

The outer membrane of Gram negative bacteria also contain a number of "minor" proteins which in conventional laboratory conditions are present only in very low copy numbers. Alterations in the growth conditions can result in derepression of these proteins to much the same extent as the "major" outer membrane proteins; many of these proteins may be important *in vivo* (Hammond Lambert and Rycroft 1984). Growth of *E. coli* and *K. pneumoniae* in iron deficient conditions results in the induction of a

Table 1.1. Functions or well-characterised iron-regulated outer membrane proteins of *E. coli*. Taken from Griffiths (1987b).

Protein	Apparent molecular mass	Receptor for phage/ colicin	Function
Cir protein	74KDa	Colicin I	Unknown
ColV-aerobactin receptor	74KDa	Colicin DF13	Uptake of Fe^{3+} aerobactin
Fhu protein	78 KDa	Phages T1, T5 and ø 80 Colicin M	Uptake of ferrichrome
Fec A protein	80.5 KDa		Uptake of Fe^{3+} citrate
Fep A protein	81 KDa	Colicin B	Uptake of Fe^{3+} enterochelin
83 KDa protein	83 KDa		Unknown

series of high molecular weight proteins (Klebba *et al* 1982, Williams *et al* 1984). The role of these proteins in iron uptake and their importance in the *in vivo* environment will be discussed in detail in section 1.5.2. Table 1.1 shows the functions of these proteins in *E. coli*. The outer membrane of *K. pneumoniae* possess up to six high molecular weight iron regulated outer membrane proteins (IRMP's) one of which has been shown to cross react with the ferric enterochelin receptor of *E. coli* (Chart and Griffiths 1985, Williams *et al* in press). The other IRMP's of *K. pneumoniae* presumably will be shown to serve similar functions to those of *E. coli*.

Other minor outer membrane proteins include those induced by specific nutrient limitations: the D1 protein of *Pseudomonas aeruginosa* (Hancock and Carey 1980), and the MalB protein of *E. coli* (Nakae and Ishii 1980)

which are induced by glucose limitation, PhoE the phosphate limitation specific protein of *E. coli* (Argast and Boos 1980, Overbeeke and Lugtenberg 1980), and several nutrient limitation specific proteins of *K. pneumoniae* (Sterkenberg *et al* 1984).

1.4.6. Exopolysaccharides.

Many Gram negative bacteria have associated exopolysaccharide which may take the form of a discreet capsule or of a more diffuse slime layer (Costerton *et al* 1981, Sutherland 1977); soluble exopolysaccharide may also be detected in the growth medium (Ehrenworth and Baer 1956). The capsule of *K. pneumoniae* is sufficiently structured to exclude particles of indian ink (Duguid and Wilkinson 1953) and is intimately associated with the cell surface (Dudman and Wilkinson 1956); large amounts of soluble polysaccharide are detected in the culture supernatant (Domenico *et al* 1985, Duguid and Wilkinson 1953, Ehrenworth and Baer 1956). There is wide degree of antigenic variation among the capsular types of *K. pneumoniae* and it is these which are used for serotyping the organisms (Jann and Jann 1977).

The cell associated capsular polysaccharide and the cell free soluble exopolysaccharide for a given serotype are immunologically identical. It is not clear what the difference is between the exopolysaccharides which form a capsule and those which form slime (Sutherland 1977). It has been suggested that the capsular polysaccharides of *Klebsiella* strains were linked in some way to lipopolysaccharide because of the traces of lipopolysaccharide derived sugars found in exopolysaccharide preparations but similar amounts of lipopolysaccharide are detected in preparations of slime so it seems unlikely that this accounts for the structure of the capsule (Sutherland 1977). Severe treatment such as

phenol extraction is necessary to remove the capsule from *E. coli* while slime is relatively easy to extract. It has been suggested that this is a result of alkali labile linkages in the capsular polysaccharide which result in a polymer composed of polysaccharide chains. (Hungerer *et al* 1967). Small amounts of lipid material which are covalently linked to the polysaccharide chain of the capsular material of *E. coli* and the group specific meningococcal polysaccharides have been proposed as membrane anchorages a portion of the exopolysaccharide in these organisms (Gotschlich *et al* 1981, Schmidt and Jann 1982).

The exopolysaccharides of *K. pneumoniae* are heteropolymers composed of repeating units comprising two to six monosaccharide units. The capsule of *K. pneumoniae* DL1 consists of fucose, glucose, glucuronic acid and pyruvate (Erbing *et al* 1976) while the capsule of strain 5055 is composed of mannose, glucose and glucuronic acid (Sutherland 1971).

1.5. Factors important in the interaction between host and pathogen.

1.5.1. Introductory remarks.

A bacterial infection is an interaction between two organisms. The host organism has a series of defensive mechanisms against infection which range from simple mechanical barriers such as the skin (Smith 1978) to complex physiological mechanisms such as the withholding of essential nutrients (van Asbeck and Verhoef 1983). The bacterium in turn adapts to the environment of the host and adopts characteristics which protect it from or enable it to overcome the hosts defences. The interaction between these two organisms is further complicated by a third element which may be present in the interaction, namely antibiotics (Brown and Williams 1985b). The outcome of an infection is determined by the various interactions between these three elements.

1.5.2. The role of iron in infection.

Iron is an essential nutrient which is involved in the catalysis of a wide variety of biochemical processes in microorganisms and in higher animals (Griffiths 1987a, Hubbard *et al* 1986). It has been shown for a wide variety of infecting organisms, including *K. pneumoniae*, that the injection of iron causes an increase in susceptibility to infection (Kochan *et al* 1977, Miles *et al* 1979, Payne and Finkelstein 1975). Iron is a highly insoluble element commonly existing in oxidised, hydrolysed or polymerised states under physiological conditions, and can also be highly toxic because of it's ability to form free radicals (Griffiths 1987a). Animals possess a variety of iron binding proteins which serve to keep iron in a soluble form, and hence available for the biochemical pathways it catalyses, and at the same time control it's toxic effects (Bezkorovainy 1987). Serum iron is found bound to ferritin, haemosiderin or haem and in other body fluids it is bound to transferrin and lactoferrin (Bullen 1981). These iron binding proteins have a very high affinity for iron and combine effectively to reduce the level of free iron to negligible amounts (Bullen *et al* 1978). The physiological importance of these proteins can be deduced from the fact that transferrin is found early in the evolutionary process among the hag fish and that the protein is very similar to that found in mammals (Aisen *et al* 1972). These iron binding proteins are not normally fully saturated. Human serum transferrin is only about 30-40% saturated (Weinberg 1984), giving the body a capacity for further reducing the iron level.

The amount of iron available in the body to invading bacteria is very low; in response to infection there is a further reduction in serum iron

levels referred to as hypoferraemia (Cartwright *et al* 1946, Kluger and Rothenburg 1979). The mechanism producing hypoferraemia is not clearly understood, it has been suggested that lactoferrin released from polymorphs at the site of inflammation removes iron from transferrin and that this is then taken up by macrophages and removed from the circulation by reticuloendothelial system (van Snick *et al* 1974). Alternatively it has been suggested that alterations in the processing of iron limits the supply of the metal to the intracellular pool and hence causes hypoferraemia (Letendre and Holbein 1984).

In response to the low level of available iron *in vivo* bacteria induce high affinity iron uptake systems which consist of a low molecular weight iron chelator and an outer membrane receptor protein. Species from the genera *Salmonella, Escherichia* and *Klebsiella* secrete an iron chelating compound enterochelin (also known as enterobactin), under iron restricted conditions *in vitro* (O'Brien and Gibson 1970, Perry and San Clemente 1979, Pollack and Neilands 1970, Williams *et al* 1984). This compound is a cyclic trimer of 2,3-dihydroxy-*N*-benzoylserine (DBS) (O'Brien and Gibson 1970) (Fig. 1.2.), and has a very high affinity for iron; the formation constant for ferric enterochelin is 10^{52} (Raymond and Carrano 1979). By virtue of it's high affinity for iron, enterochelin is capable of removing iron from host iron binding proteins (Carrano and Raymond 1979). This process can occur across a dialysis membrane so direct contact between the bacteria and host iron compound is not necessary (Tidmarsh and Rosenberg 1981). Enterochelin is not recycled by the cells; it is broken down into it's DBS constituents (O'Brien *et al* 1971). It has been suggested that hydrolysis is necessary to liberate the iron from the compound inside the cell (Cooper *et al* 1978, Raymond and Carrano 1979).

Fig.I.2. Structural formula of enterochelin.

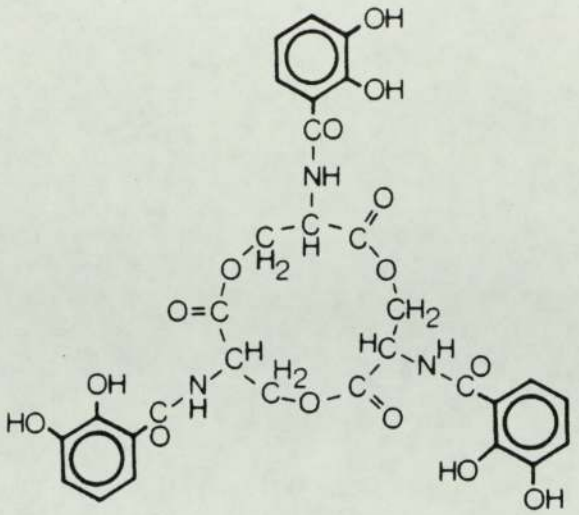
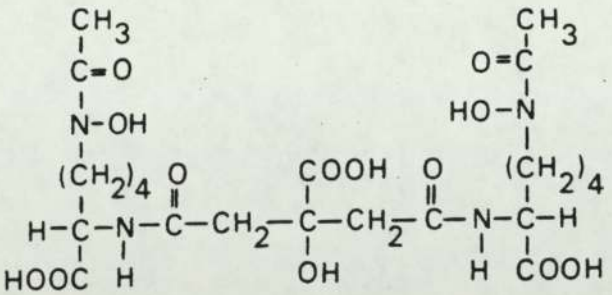


Fig.I.3. Structural formula of aerobactin.



The hydroxamate siderophore aerobactin is also produced by members of the *Enterobacteriaceae* (Braun 1981, Payne 1980, Williams 1979, Williams *et al* in press). This compound is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid (Fig. 1.3.), it forms an octahedral complex with ferric iron (Harris *et al* 1979). *E. coli* also poses a citrate mediated iron uptake system but this does not appear to be widely distributed among the *Enterobacteriaceae* (Griffiths 1987b).

In addition to these siderophores members of the *Enterobacteriaceae* derepress a series of IRMP's in response to iron restriction *in vivo*. These are receptor proteins involved in the uptake across the outer membrane of the iron siderophore complexes (Neilands 1982). It was shown that the synthesis of three of the IRMP's and the phenolate compounds of *E. coli* were coordinately regulated (Klebba *et al* 1982, McIntosh and Earhart 1977); the 81 kDa protein has since been identified as the enterochelin receptor (Hollifield and Neilands 1978). The receptor for aerobactin has been identified as a 74 kDa (Krone *et al* 1985) or a 76 kDa (Griffiths *et al* 1985a) protein, and is essential for ferric aerobactin uptake (Williams and Warner 1980). The 80.5 kDa Fec A protein is involved in iron citrate uptake (Hussein *et al* 1981).

The need for specific outer membrane receptor proteins as part of the high affinity iron uptake system is in part due to the molecular weight of the iron siderophore complex exceeding the limit for diffusion through the pores although in the case of the iron citrate complex it is small enough to gain access through this route. Griffiths (1987b) suggests that the concentration of iron at the cell surfaces is raised

relative to the surrounding concentration by binding of the loaded siderophores onto the cell surface.

There is evidence that the bacterial iron sequestering systems operate *in vivo*. Both the IRMP's and enterochelin have been demonstrated in the peritoneal washings of guinea pigs infected with *E. coli* (Griffiths and Humphreys 1980, Griffiths *et al* 1983). The presence of IRMP's has also been demonstrated in *K. pneumoniae* and *Proteus mirabilis* recovered without subculture from infected urine (Lam *et al* 1984b, Shand *et al* 1985) and in *P. aeruginosa* from the sputum of a patient with cystic fibrosis (Brown *et al* 1984). Bacteria isolated directly from the *in vivo* situation do not express all of the high molecular weight proteins expressed under iron restriction *in vitro* although both the enterochelin and the aerobactin receptor proteins are expressed in the guinea pig model (Griffiths *et al* 1983). It is clear that bacteria growing *in vivo* are growing under iron restricted conditions but that other factors are also operating *in vivo*.

The ability to produce siderophores is a virulence factor for members of the *Enterobacteriaceae*. Enterochelin production is commonly associated with clinical isolates of both *E. coli* and *K. pneumoniae* (Chart *et al* 1985, Williams *et al* in press). Aerobactin synthesis is coded for on the ColV virulence plasmid which suggests that it is important in virulence (Braun 1981, Crosa 1984) although it can also be chromosomal (Montgomerie *et al* 1984). It is the only factor which correlates with virulence in a mouse peritoneal model *K. pneumoniae* infection (Nassif and Sansonetti 1986). Aerobactin is only produced by some clinical isolates of *E. coli* and *K. pneumoniae* and it's distribution may be

associated with the type infection from which it was isolated (Chart *et al* 1985, Williams *et al* in press).

Aerobactin is 29 orders of magnitude less efficient at binding iron than enterochelin and marginally less efficient than transferrin with which it must compete *in vivo* (Harris *et al* 1979). It is not clear why the ability to synthesise aerobactin should be a selective advantage to bacteria already capable of synthesising enterochelin. Unlike enterochelin, aerobactin is recycled by the cell (Braun *et al* 1984) which might be of particular importance *in vivo*. Most surveys of the distribution of these two siderophores are performed in laboratory media, the relative efficiencies of the two siderophores could be substantially different in body fluids. Aerobactin has been shown to remove iron from transferrin more efficiently than enterochelin in serum (Braun *et al* 1984, Williams and Carbonetti 1986) and in the presence of anions other than iron in the media (Konopka *et al* 1982). It has been shown that normal human serum contains an antibody to ferricenterochelin which inhibits the uptake of this complex (Moore and Earhart 1981) and also antibodies directed against the receptor protein (Griffiths *et al* 1985b). Serum albumen binds enterochelin and taken with the above factors may reduce the efficiency of enterochelin *in vivo* sufficiently to make aerobactin mediated uptake of iron an important virulence factor. It has also been shown that while enterochelin releases iron into an intracellular pool aerobactin delivered the iron directly to where it was required for growth (Williams and Carbonetti 1986). In one study aerobactin synthesis was found to be characteristic of 75% of *E. coli* isolates from patients with bacteraemia (Montgomerie *et al* 1984). It has also been suggested that aerobactin may be of importance when the infecting dose is low (Williams 1979).

The effects of a low iron environment of bacteria are major, involving production of new metabolic products, the siderophores, and new outer membrane proteins. This in turn effects the interaction with the host organism and the susceptibility to antibacterial agents. It has been shown that iron limitation alters the penicillin binding protein profile of *P. aeruginosa* in chemostat culture (Turnowsky *et al* 1983), which could alter susceptibility to ß-lactam antibiotics. The minimum bactericidal concentration (MBC) of penicillin G for *K. aerogenes* was found to be four times higher in iron limited than in ammonia limited chemostat culture (Sterkenberg 1984b). The IRMP's of gram negative bacteria have been shown to be recognised by serum samples from patients from whom the organism was originally isolated (Anwar *et al* 1984, Shand *et al* 1985). It is not yet clear what part these antigens play in the clearance of the bacteria from an infection. Growth under iron restriction has been shown to alter the expression of a variety of other virulence factors in pathogenic bacteria (Griffiths and Bullen 1987), the implications of iron restricted growth for the interaction between host and bacteria have not yet been fully elucidated.

1.5.3. The interaction between bacteria and the host immune system.

The host response to bacterial invasion involves a complex series of interrelated defence mechanisms which combined make up the immune response. These include the action of various classes of phagocytic cells which ingest the bacteria and specific antibodies and complement which make the bacteria more susceptible to phagocytosis (Stanier *et al* 1980). The initial response is non specific with inflammation, often in response to bacterial endotoxin, playing an important role in mobilisation of phagocytes (Spector and Willoughby 1963). At this stage numbers of bacteria are usually small and they are often effectively

eliminated (Smith 1978). As the infection progresses a specific response to the invading organism develops including specific opsonising antibodies and specifically primed T lymphocytes. A series of non specific responses such as the action of complement and of lysozyme act to enhance this response (Stanier *et al* 1980). Bacteria respond to the *in vivo* environment with a series of strategies which enable them to avoid or interfere with the host immune response.

1.5.4. The role of growth rate in the interaction between host and pathogen.

The growth rate of an organism in vivo is dependant on a number of factors and is part of the response of the organism to the precise conditions it encounters. Measurement of growth rate *in vivo* is complicated by the effect of the host immune system which, by removing bacteria can lead to an underestimation of growth rate (Meynell 1959, Morris Hooke 1985) and by the fact that bacteria often persist in a semidormant state in the *in vivo* situation and are not easy to detect (Durak and Beeson 1972b). Several elegant techniques have been developed to take into account these factors (Baselski *et al* 1978, Meynell 1959, Morris Hooke *et al* 1985) however each of these techniques is open to criticism. Baselski *et al* (1978) used radioactively labelled cells and took into account the decrease in specific activity as a measure of clearance. It has been suggested that leakage of the label out of the bacteria could lead to an overestimation of the efficiency of the host clearance mechanisms (Freter and O'Brien 1981). Meynell (1959) used a superinfecting phage which does not replicate during bacterial multiplication. The proportion of superinfected bacteria bears a known relationship to the number of generations of growth, the growth rate can therefore be calculated. Morris Hooke (1985) used a mixed population of

cells containing a temperature sensitive mutant and a wild type. At non permissive *in vivo* temperatures only the wild type was capable of replication; the number of wild type generations can be calculated from the declining ratio of mutant to wild type. Both of these techniques rely on two distinct populations of cells being cleared at the same rate by the body's defence mechanisms. Cozens *et al* (1986a) described a method which is particularly applicable to measurement of growth rate in long term infections. A positive correlation between growth rate and the proportion of cells just beginning to divide (showing invagination of the cell envelope) had been reported (Woodringh 1976). Cozens *et al* (1986a) used chemostats to construct standard curves correlating frequency of division with growth rate, and used these to calculate the growth rate in cells recovered from a variety of experimental infections.

In some animal models bacterial division rates approach *in vivo* rates, *Pseudomonas aeruginosa* has been shown to achieve division rates of 30 min in the lung and 20 min in the peritoneal cavity (Morris Hooke *et al* 1985, Sordelli *et al* 1985). They suggest that the difference in the growth rates of the organism in the two different sites may be due to differences in pH and nutrient availability. A study of intravascular replication rates of *Haemophilus influenzae* reported doubling times of 47 min after intravascular inoculation, and 50 to 60 min after intranasal inoculation, this compares with *in vitro* doubling times of 28 min but takes no account of the effects of clearance (Rubin *et al* 1985). Similarly *Streptococcus viridans* exhibited growth rates similar to those found *in vitro* in the early stages of colonisation of a non-bacterial thrombotic endocarditis (Durak and Beeson 1972a). The ability to replicate rapidly *in vivo* may be an important virulence factor for

some bacteria and may be of particular importance in the early stages of infection when there is a relatively small population of bacteria and the host parasite interaction is weighted in favour of the host.

In many experimental infections the bacterial doubling time increases as the infection proceeds. Durak and Beeson (1972a) report that after 24 h the bacteria in an endocarditis vegetation enter a comparatively stationary phase. The rate of increase in the magnitude of the bacteraemia caused by *Haemophilus influenzae* decreased after 6 to 8 h probably indicating a decrease in the growth rate (Rubin *et al* 1985). A study of generation times of *E. coli* and *Proteus mirabilis* in experimental kidney infections reported doubling times of 0.9 and 2.3 h respectively in the first seven hours, 3 to 5 and 5 to 7 h in the first 24 h and in both cases as slow as once every 20 h in the 24 to 48 h period (Eudy and Burrous 1973). The generation time of *E. coli* in a rat meningitis model was shown to increase from 1.27 h at 3 h post infection to 12 h at 29 h post infection and to as much as 16.9 h in experimental pyelonephritis 16 days post infection (Cozens *et al* 1986a). It is difficult to determine wether the reduction in growth rate over the course of an infection is a result of growth conditions such as nutrient limitation and the presence of host antibodies or whether it is a strategy for survival.

ß-lactam antibiotics are effective only against actively growing bacteria; bacteriostatic agents such as sulfadiazine were found to interfere with bacterial killing by penicillin (Hobby and Dawson 1944). Slow growing as well as non growing bacteria are more resistant to antibacterial agents (Brown 1977). Chemostat culture, which allows specific growth rate to be controlled over a range of growth rates has

been used to study the effect of growth rate on antibiotic sensitivity *in vitro* . It has been shown that *Pseudomonas aeruginosa* is more susceptible to the action of halogenated phenols (Gilbert and Brown 1978) and EDTA (Finch and Brown 1975) at fast growth rates under certain conditions of nutrient limitation and that the sensitivity of *Bacillus megaterium* to the action of chlorhexidine and 2-Phenoxyethanol in carbon and magnesium limited cultures increases with growth rate (Gilbert and Brown 1980). Increased resistance of slow growing chemostat cultured cells has also been demonstrated in *Yersinia enterocolitica* and *K. pneumoniae* (Dean *et al* 1976, Harakeh *et al* 1985).

Sterkenberg (1984a) used the MBC, the lowest concentration of antibiotic at which the number of CFU ml^{-1} decreased at a rate faster than the theoretical washout rate, to investigate the effect of growth rate on susceptibility to β-lactams of *K. aerogenes* in chemostat culture. The MBC of Penicillin G at a fast growth rate D=1.0 h^{-1} was comparable with that found in batch culture, this increased four fold with cells growing at D=0.1 h^{-1}, the same increase in MBC in response to reduced growth rate was found with cephalothin. Chemostat studies with a number of Gram negative bacteria have shown that the rate of kill per generation is constant at all growth rates for a given antibiotic (Cozens *et al* 1986b, Tuomanen *et al* 1986). This represents a marked decrease in rate of kill, in terms of absolute time, as the growth rate decreases.

Growth rate has been shown to affect many properties of the bacterial cell: permeability of the *Pseudomonas aeruginosa* outer membrane to nitrocefin, a β-lactam compound is decreased at slow growth rates (Broxton and Brown 1984), the structure of peptidoglycan and the PBP's have both been shown to vary with growth rate (Buchanan and Sowell 1982,

Pisabarro *et al* 1985, Turnowsky *et al* 1983), as have many components of the cell wall (Ellwood and Robinson 1981, Dodds *et al* 1987, Sterkenberg *et al* 1984). These factors all potentially play a role in the decreased susceptibility of the slow growing cell to antibacterial agents. In addition slow growing bacteria will manufacture cell wall at a reduced rate and the enzymes involved in the process will be operating at sub maximal rates, it may be true that in these circumstances a greater concentration or a longer exposure time may be necessary to affect the cell.

The effect of growth rate on susceptibility to antibiotics *in vivo* has been less thoroughly investigated. Durak and Beeson (1972a,b) suggested that the relatively dormant *Streptococci* found in the vegetations in endocarditis were resistant to the effects of antibiotics but were still viable and could act as the source of bacteria for remission after completion of a course of antibiotics. An examination of serial sections of a consolidated rat lung after infection with pneumococci showed a gradient of growth rates from rapid multiplication at the edges of the infected area to slow in the core region. Treatment with penicillin had little effect on the cells in the core region which remained intact but reduced those at the periphery to lysed remnants (Wood and Smith 1956).

Growth rate affects the susceptibility of bacteria both to serum killing and to phagocytosis. *E. coli* strains grown under carbon and magnesium limitation in chemostat culture were examined for sensitivity to serum killing (Taylor *et al* 1981). Serum sensitivity was shown to be strain dependant and to be effected by the growth limiting nutrient. Under conditions where the bacteria were serum sensitive the degree of serum sensitivity was increased with increasing growth rate. In one case a

serum resistant organism became serum sensitive in the carbon limited chemostat at growth rates approaching the maximum. It is proposed that the amount and composition of the lipopolysaccharide, capsule and envelope proteins in different combinations in the different strains are responsible for serum resistance; all of these are affected by growth rate and these alterations could mediate the effect of growth rate. *P. aeruginosa* grown slowly under magnesium limitation became resistant to the killing action of intact rabbit polymorphonuclear phagocytes and cationic proteins isolated from them (Finch and Brown 1978).

1.5.5. The role of bacterial exopolysaccharides in the interaction between host and pathogen.

Bacterial polysaccharides play an important role in resistance to host defence mechanisms in many bacteria. Possession of a capsule correlates with virulence in many organisms (Cross *et al* 1984, Domenico *et al* 1982, Ehrenworth and Baer 1956, Masson and Holbein 1985, Schweinle 1986), and anti exopolysaccharide antibodies have proved successful in protection against various bacterial infections (Cryz *et al* 1986b, Fenwick and Osburn 1986). Both capsule and lipopolysaccharide play important roles in serum resistance (Cross *et al* 1986, Tomás *et al* 1986, Williams *et al* 1983) and in the prevention of phagocytosis (Cryz *et al* 1986a, Williams *et al* 1983).

Since the early work of Ehrenworth and Baer (1956) the importance of encapsulation in the virulence of *K. pneumoniae* has been recognised. They reported a correlation not only between encapsulation and virulence but between the degree of encapsulation and virulence. This has more recently been confirmed by Domenico *et al* (1982). The importance of capsular polysaccharide *in vivo* is emphasised by the ability of anticapsular

antibodies to protect against challenge with *K. pneumoniae* in experimental infections (Cryz *et al* 1986b, Robert *et al* 1986). Although possession of a capsule is an essential virulence factor for *K. pneumoniae* it is not the sole determinant of virulence; avirulent encapsulated strains have been reported (Mizuta *et al* 1983); other factors such as the lipopolysaccharide also play a role.

The role of capsular polysaccharide and lipopolysaccharide in resistance to phagocytosis and serum killing is complex and in some cases strain specific. In a series of experiments with two strains of *K. aerogenes* with immunologically identical lipopolysaccharide but different capsular types and a series of exopolysaccharide mutants the role of these two exopolysaccharides was investigated (Williams *et al* 1983). Both encapsulated strains were found to be resistant to serum killing; serum was bacteriostatic for a nonencapsulated mutant and bactericidal for a mutant lacking both lipopolysaccharide and capsule. In this instance both exopolysaccharides can be seen to play a role in serum resistance. In another study serum resistance was found to reside in the lipopolysaccharide and in particular in the O side chain (Tomás *et al* 1986). Encapsulation has been shown to be important in serum resistance in *E. coli* only in strains which have a lipopolysaccharide type which is incapable of protecting them from the effect of serum (Cross *et al* 1986). Capsular polysaccharide and not lipopolysaccharide were found to be antiphagocytic in a whole blood assay for two strains of *K. aerogenes* (Williams *et al* 1983). Opsonisation by anticapsular antibodies is an important factor in phagocytosis (Cryz *et al* 1986a) and also opsonisation by other, probably lipopolysaccharide specific, antibodies in the case of strains with a smaller capsules (Williams *et al* 1983).

K. pneumoniae produces large amounts of soluble polysaccharide which is released into the medium (Ehrenworth and Baer 1956). It has been suggested that this plays a role in the virulence of the organism (Domenico *et al* 1982). Circulating capsular antigen has been detected by immunoelectrophoresis in the serum of patients with klebsiella infections and has been found to be correlated with the severity of the disease (Pollak 1976). Soluble capsular polysaccharide has been shown to have a number of deleterious effects on the host immune system. It has been shown to paralyse the immune system and to inhibit the action of lysozyme (Kato *et al* 1979). Very small quantities of exopolysaccharide were shown to inhibit macrophage maturation (Yokochi *et al* 1977). It has also been suggested that circulating capsular antigen, by reacting with capsule specific antibody could effectively reduce the opsonic potential of the serum consequently reduce phagocytosis (Cryz *et al* 1986a, Pollak 1976).

K. pneumoniae produces an extracellular toxic complex with lipopolysaccharide and protein components as well as capsular polysaccharide (Straus *et al* 1985) which was capable of producing a lung pathology similar to that caused by *K. pneumoniae* (Straus *et al* 1985). Straus and his group identified the lipopolysaccharide portion of the complex as being the active part using sophisticated purification procedures but reported that isolated lipopolysaccharide was not as effective as the complex (Straus *et al* 1985). They propose that the capsular polysaccharide portion which is a relatively large molecule may play a role in reducing excretion of the extra cellular toxic complex from the blood stream and hence enhance it's virulence properties. In studies into the immunoprotective properties of a cell surface preparation containing protein, lipopolysaccharide and capsular

material, Robert *et al* (1986) report that the purified capsular portion is the protective constituent in mice but that the combined cell surface preparation is more effective. They suggest that the lipopolysaccharide plays a role in forming the capsular material into aggregates. It is probable that both circulating capsular polysaccharide and lipopolysaccharide play important roles in combating the host defence mechanisms and although their precise roles are not yet clear, interactions between the two types of exopolysaccharide are of significance.

1.5.6 The role of β-lactam antibiotics in the interaction between host and pathogen.

As outlined previously the way in which bacteria grow *in vivo*, at a slow division rate (Eudy and Burrous 1973, Meynell 1959) and under conditions of iron limitation for instance (Brown *et al* 1984, Griffiths et al 1983, Lam *et al* 1984b), influences their susceptibility to the action of antibiotics (Brown and Williams 1985a,b). At the same time the presence of antibiotics must be considered as part of the host environment as they affect the bacteria in ways which influences their interactions with the host defence mechanisms in many cases rendering them more vulnerable to clearance from the body.

The β-lactam group of antibiotics includes the penicillins and the cephalosporins. These two groups of compounds have similar structures in that they have in common the β-lactam ring structure, which in the cephalosporins this is fused to a six membered dihydrothiazine ring and in the penicillins to a five membered thiazolidine ring (Greenwood 1982).

This group of antibiotics includes many derivatives of the early β-lactams with improved antimicrobial properties. These antibiotics have increased resistance to β-lactamases, and a broader spectrum of activity as well as better penetration to the PBP's. The pharmacological properties such as rate of breakdown in the gut, clearance from the blood stream and degree of penetration into the target tissues are also improved (Greenwood 1982).

Simplistically penicillin has structural similarities to the terminal amino acid residues on the short peptides involved in cross linking peptidoglycan, to give the cell wall it's rigidity. It acts by selectively inhibiting the trans peptidase enzyme involved in this reaction. The cell loses it's structural integrity as it takes up water by osmosis and eventually the cells lyse.

In fact different β-lactam antibiotics have different effects on the cell, suggesting that the mode of action is more complex and involves more than one site of action. Certain of these antibiotics cause filament formation (Georgopapadakou *et al* 1982) indicative of a failure of cell division, while others cause spheroplast formation (Greenwood and O'Grady 1973). Gram negative bacteria possess up to eight penicillin binding proteins, which are penicillin sensitive enzymes catalysing the final stages of the synthesis of the peptidoglycan network of bacterial cell walls, but each with a different role in the process. The number and size of these PBP's varies between species but the pattern reflects the taxonomy of the organisms (Georgopapadakou and Liu 1980). This relationship is particularly marked within the enterobacteria. Together with the similarity of biochemical properties of the PBP's within the

group, this suggests that the extensive work on the PBP's of *E. coli*, can be extrapolated to the other enterobacteria (Curtis *et al* 1979).

The interaction between the enzyme and the antibiotic involves the breaking of the very reactive β-lactam bond by the enzyme leading to the formation of a stable complex between the two. While the nature of the reactions mediated by the β-lactam targets, the PBP's, and the effect of inhibition of these enzymes on the bacterial cell are well understood the mechanism by which cell death occurs is not. Penicillins of inhibitory concentrations and above cause a complete cessation of cell growth in cultures where peptidoglycan synthesis can still be detected (Williamson and Tomasz 1985). Lack of growth can not therefore simply be a result of an inability to synthesise peptidoglycan. It is possible to uncouple the inhibition of cell growth by the antibiotic from cell death and lysis in *Bacillus subtilis* mutants which have a normal complement of PBP's (Tomasz 1978). Cell death is brought about by the action of naturally occurring autolytic enzymes. Analogous phenomena have also been observed in *E. coli* (Tomasz 1981). The mechanism by which penicillins trigger the action of these enzymes is not understood but it seems to be an indirect regulatory consequence of the action of the antibiotic (Tomasz 1986).

Although the antibiotic dose given to a patient is based on the MIC the concentration of antibiotic to which bacteria are exposed *in vivo* depends on many factors including the site of the infection and on the duration of the treatment. The antibiotic dose is usually calculated to give serum levels of the same order as the MIC. Infections, however, are usually localised outside the vascular bed and the degree of partitioning into extravascular compartments may result in the bacteria

being exposed to reduced concentrations (Norrby *et al* 1978). The degree of binding of the antibiotic to serum proteins (Wise 1986) and it's rate of clearance (Meinardi *et al* 1978) are thought to be important factors in determining the concentration of antibiotic in tissue. In urinary-tract infections the rate of excretion is important in determining the concentration of antibiotic in the urine, which is more important in determining the effectiveness of the treatment than the serum level (Stamey *et al* 1974). Diseased tissue is often poorly penetrated by antibiotics. The concentration of the Cephalosporin cephacetrile in lung tissue after repeated intravenous administration was measured, the concentration in inflamed tissue was found to be less than in normal tissue and that in tumorous tissue still less (Kiss *et al* 1978). Bacteria growing as adherent microcolonies, surrounded by a polysaccharide layer (Costerton *et al* 1981) or inside a well-established endocarditis vegetation (Sullman *et al* 1985) may be relatively protected from antibiotics (Nickel *et al* 1985a,b, Slack and Nichols 1982). Iron limitation which is important *in vivo* (Sect. 1.5.2.) has been shown to increase the MBC of Penicillin G for *K. aerogenes* four fold (Sterkenberg 1984b), if this is a general phenomenon it could lead to a general underestimation of the level of antibiotic required to kill bacteria *in vivo* and to bacteria routinely being exposed only to sub-MIC concentrations. In the early stages of treatment and in situations where penetration is poor the bacteria are likely to be exposed to a sub-MIC concentration of an antibiotic. These low concentrations of antibiotics have effects on bacteria "which are not milder than those seen at the MIC, but which are different in kind" (Lorian 1980). An understanding of the effects of sub-MIC's of antibiotics is important to our understanding of the way antibiotics work *in vivo*.

Sub-MIC levels of β-lactam antibiotics have a spectrum of effects on bacteria *in vitro* which correlate with the kinetics of their binding to specific PBP's. For instance, a sub-MIC concentration of Ceftriaxone, which binds preferentially to PBP3 (Cleeland and Squires 1984), will cause filament formation *in vitro*, only causing inhibition of growth and cell lysis at higher concentrations (Ullman and Hammer-Uschtrin 1984).

Filamentous forms of bacteria have been found *in vivo* after exposure to sub-MIC levels of β-lactams. In model infections of rabbits with a range of gram negative bacteria, including *K. pneumoniae*, which received infusions of antibiotics to give steady state levels of 1/3 and 1/6 MIC, filaments of up to 50μm in length were found. After six hours there was a loss of viability of at least two logs and an increase of survival over untreated animals (Zak 1978). The differing responses of bacteria to sub-MIC's of β-lactams can explain the relative efficacies of these drugs *in vivo*. Amoxycillin is significantly more effective in the treatment of experimental mouse infections than is Ampicillin. It was shown that in response to the relatively low concentrations of the antibiotics present in the blood and tissues Amoxycillin caused rapid spheroplast formation followed by rapid lysis, in contrast to the filament formation and slower lysis seen *in vivo* with Ampicillin treatment (Comber *et al* 1977).

Patients admitted to hospital with an undiagnosed infection are often started on an antibiotic course before a specimen is taken for culturing. The bacteria seen in these specimens, which have been subjected to a sub-MIC level of antibiotic show that the the abnormal forms of bacteria seen *in vitro* are not artefacts. For example a blood culture obtained from a 66 year old man with a peptic ulcer and diabetes

mellitus treated one hour previously with penicillin showed filaments of *K. pneumoniae* up to 200μm long (Lorian 1978). This, taken together with the animal experiments, demonstrates the importance of understanding the effects of sub-MIC concentrations of β-lactam antibiotics. It would be interesting to know whether these abnormal forms are also found in tissues where antibiotic penetration is low and where bacteria are growing protected by a glycocalyx or a vegetation.

Sub-MIC doses are rarely used therapeutically because of the fear of selecting for resistant bacteria although single dose therapy, which amounts to a sub-MIC, with Amoxicillin has been found to be as effective as conventional treatment in urinary tract infections (Fang *et al* 1978). The emergence of resistance to β-lactam antibiotics after exposure to sub-MIC concentrations has been investigated *in vitro*. The MIC of a range of β-lactams for *E. coli* does not increase after exposure to a sub-MIC for up to 30 subcultures, nor was there any case of resistant mutants being selected (Grassi 1978). The situation in an infection *in vivo* may be different; there is evidence that plasmid transfer is enhanced *in vivo* by exposure to sub-MIC's (Barr *et al* 1986).

That sub-MIC's of β-lactam antibiotics have marked effects on bacteria *in vitro* and *in vivo* is well established. By definition these levels of antibiotic are not bactericidal but their effect on the cell will affect it's interaction with the host. Exposure of bacteria to a sub-MIC concentration of antibiotic may influence the expression of virulence factors as well as their susceptibility to the host immune system (Ahlstedt 1981, Lorian and Atkinson 1984).

The first step in the course of many infections is the attachment of the bacteria to the host mucosal surfaces. In urinary tract infections the ability of a strain of *E. coli* to adhere correlates with it's ability to cause infection (Sandberg *et al* 1979). Several authors have shown that sub-MIC's of β-lactams reduce the ability of *E. coli* to adhere to monolayers of human uroepithelial cells *in vitro* (Sandberg *et al* 1979, Ofek *et al* 1979). This decrease in adhesion could be a direct result of the abnormal shape of the bacteria or an effect on the pili, which mediate attachment. *E. coli* grown in the presence of 1/4 MIC of Ampicillin were found to be mostly free of pili when examined by electron microscopy (Svanborg-Edén *et al* 1978). Similar observations have been made on the reduced attachment and piliation of gonococci and meningococci subjected to sub-MIC's of penicillin (Stephens and McGee 1985). The action of the antibiotic may be on the process of synthesis of the pili or on the insertion of the pili into the membrane which would lead to aberrant functioning of the organelle (Sandberg *et al* 1979, Stephens and McGee 1985). The reduction of adhesion by sub-MIC's of β-lactams has not been universally demonstrated. Vosbeck *et al* (1979) suggested that their failure to demonstrate this effect with a range of β-lactams and *E. coli* may be an effect of growth conditions, incubation times or strain variation.

An important condition bacteria encounter *in vivo* is iron stress and the ability to acquire iron from host binding proteins by the secretion of siderophores is a virulence factor (Sect. 1.5.2.). Sub-MIC's of ceftriaxone reduced by 25% the amount of enterochelin, and by nearly half the amount of aerobactin produced by *E. coli* grown in iron depleted conditions (Kadurugamuwa *et al* 1985c).

The presence of a polysaccharide capsule has been shown to be a virulence factor for *K. pneumoniae* (Domenico *et al* 1982). *K. pneumoniae* grown in the presence of sub-MIC's of a range of cephlosporins showed a reduced capsule under india ink staining and pellets of the organisms lost their gel-like viscosity (Kadurugamuwa *et al* 1985a, Gemmell *et al* 1983)

There have been various reports of the ways in which the effects of serum, both complement and specific antibody, are enhanced by the presence of sub-MIC's of β-lactams. In strains of *K. pneumoniae* which are encapsulated and consequently resistant to the action of complement it was shown that treatment with a sub-MIC of Cefuroxime, resulted in the deposition of complement, and an increase in cell surface hydrophobicity. It was suggested that this is a result of a reduction of the capsule, exposing complement activating outer membrane components (Williams 1987). It was suggested that the increased susceptibility of spheroplast forms of *Proteus mirabilis* to the action of complement is a result of a decrease in the amount of peptidoglycan in spheroplasts. This would result in areas completely devoid of peptidoglycan and increase the accessibility of the cytoplasmic membrane to complement (Siegmund-Schultze *et al* 1985). Complement acts further to disrupt the bacterial outer membrane decreasing it's effectiveness as a permeability barrier. This may result in an increase in the concentration of the antibiotic at it's active site at the cytoplasmic membrane, and a consequent increase in it's effect on the bacteria. Thus complement and antibiotic reinforce each other to the detriment of the bacteria.

Sub-MIC treatment of *K. pneumoniae* with a range of cephlosporins exposed a number of protein antigens to the action of antibodies which are not

normally accessible in *in vitro* grown cells (Kadurugamuwa *et al* 1985b). This may have the effect of amplifying the host response to the bacteria *in vivo*. An increased number of antibody forming cells was observed in the mouse spleen after an injection of bacteria, pretreated with a sub-MIC of Cyclacillin (Friedman and Warren 1977). It was suggested that sub-MIC treated bacteria were more readily broken down by the macrophages into immunogenic moities and that this would be particularly important in initiating the the immune response during the early stages of infection, when the levels of antibiotic are likely to be subinhibitory (Friedman 1985). Like the effect on adhesion properties the synergistic effect of sub-MIC's of β-lactams and serum has not been universally demonstrated (Lorian and Atkinson 1979, Wiemer *et al* 1985).

Pre incubation of gram negative bacteria with sub-MIC concentrations of β-lactam antibiotics has been shown to have an effect on chemotaxis, phagocytosis and killing by host phagocytic cells. Cefotaxime enhanced each of these processes with *K. pneumoniae* and *E. coli* (Bassaris *et al* 1984). Ceftazidime, which was shown to have no significant effects on phagocytic cells alone, enhanced granulocyte and lymphocyte function against *E. coli* and *P. aeruginosa* treated with sub-MIC concentrations (Gnarpe *et al* 1984). Sub-MIC's of β-lactams appear to act directly on the bacteria to render them more susceptible to cell mediated host defence mechanisms.

Some strains of *E. coli* are resistant to the action of lysosyme allowing them to proliferate inside macrophages eventually causing degeneration and killing of the cell (Iida-Tanaka *et al* 1986). The presence of sub-MIC's of monobactams significantly enhanced killing of *E. coli* by macrophages. Electron micrographs showed a disorganisation of the

bacterial envelope which may have allowed lysosyme access to the cell and facilitated killing (Iida-Tanaka *et al* 1986). Dalhoff (1986) showed a correlation between augmentation of intra leucocyte killing of *E. coli*, and the degree of filament formation caused by a range of β-lactams at sub-MIC levels. He suggested that a filament, which can have up to 100 times the surface area of a single cell, has a proportional increase in fimbriae and a consequent increase in adhesiveness for PMN's. Lorian and Atkinson (1984) pointed out that not only are PMN's more efficient at engulfing filaments than single organisms, but also that the likelihood of a phagocyte killing a number of organisms is increased if they are grouped together in a filament. Filament formation alone is probably not the complete explanation for the increased susceptibility to phagocytosis of sub-MIC treated bacteria, as a reduced but still significant effect could be observed at concentrations which did not cause filamentation (Andreana *et al* 1984). Other factors such as the breakdown of the peptidoglycan and capsule probably also play a role.

None of the effects of sub-MIC's of β-lactam antibiotics on the various interactions between the host and bacteria are found consistently among gram negative bacteria. Conflicting results are even reported by different authors using the same species, this is probably a result of the different strains and techniques employed. Growth rate and nutrient limitation cause phenotypic changes in bacteria which affect their response to antibiotics (Brown and Williams 1985a,b). Nonetheless it is clear that these effects are real, and variation in type and degree between organisms is not surprising. Although some of the effects may be minor, taken together they are of significance in the eradication of bacteria in infections. This may be of particular importance in the

early stages of infection and in the immunesuppressed host (Friedman 1985, Lam *et al* 1984a, Oleske 1984)

1.5.7. Conclusion.

There are many factors which play an important part in the host bacteria interaction. Nutrients other than iron may be in short supply *in vivo* (Broughton *et al* 1968, Brown and Williams 1985b, Kilbourn 1984, Weinberg 1978) and temperature is an important factor in the survival of pathogenic bacteria (Bennett and Nicastri 1960, Roberts 1979). The importance of antibiotics, particularly at sub-MIC concentrations, as part of the host environment must not be overlooked (Lorian 1980). In *in vivo* situations bacteria may be growing as an adherent biofilm or protected by a vegetation (Durack and Beeson 1972a,b) forms of growth which confer resistance to host defences (Costerton *et al* 1981) and to antimicrobial agents (Costerton and Marrie 1983, Slack and Nichols 1982). No single virulence factor can be distinguished for *K. pneumoniae*. Capsule and lipopolysaccharide are important as is the ability to produce siderophores capable of competing for iron in physiological conditions. Although growth rate may be rapid in the early stages of an infection, doubling times as long as 20 h are common in chronic infections. It is not clear whether this is a strategy for survival or simply a response to adverse conditions such as nutrient deprivation but slow growing bacteria are known to be resistant to host defence mechanisms (Taylor *et al* 1981, Brown and Williams 1985b) and to antibiotics (Brown and Williams 1985a).

2. Materials and Methods.

2.1. Equipment and chemicals.

Addresses of suppliers and manufacturers are given in the appendix to this section.

All chemicals were of analytical grade and supplied by BDH unless otherwise stated.

Complex media, nutrient agar and nutrient broth were supplied by Lab M

The antibiotics used in this study were Ceftriaxome (Hoffmann La Roche, Basel, Switzerland) and Cefotaxime (Roussel, Dublin, Ireland).

Equipment not otherwise specified in text:

Balances	Oertling HC 22
	Sartotious 1702
Centrifuges	Beckman J2-21 High speed
	Beckman L8-M Ultracentrifuge
	Eppendorf 5412
Freeze Dryer	Modulyo EF4
Incubators	Mickle shaking waterbath
Microscope	Wild Model M20
pH meter	FSA Model PT1-15
Pipettes	Gilson Pipetman, P200, P1000, P5000
Power packs	Bio-Rad Model 3000/300
	Bio-Rad Model 250/2.5
Rotary Evaporator	Buchi Model R
Spectrophotometers	LKB Ultrospec 4050
	Cecil CE292

2.2. Bacterial strains.

K. pneumoniae DL1 is a clinical isolate donated by Dr R Jones. This isolate has a K1 type capsule which is copious when grown on nutrient agar and type 01 lipopolysaccharide. NCTC 5055 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Collingdale Avenue, London NW6 5HT, U.K. It has a type K2 capsule but an immunologically identical lipopolysaccharide type to that of DL1. Mutants lacking capsule (M10) and lacking both capsule and O-antigen polysaccharide (M10B), selected after nitrosoguanidine mutagenesis of NCTC 5055, were donated by Drs I.R. Poxton and I.W. Sutherland (1976).

2.3. Growth conditions.

2.3.1. Preparation of glassware.

All glassware was soaked overnight (18 h) in EXTRAN 100 (BDH) and rinsed six times in single distilled and three times in double distilled water. For iron limitation experiments it was additionally immersed in a 0.01% solution of ethylenediaminetetra-acetic acid di-sodium salt (EDTA) (BDH) for 18 h. The glassware was then rinsed six times in double distilled water.

2.3.2. Simple Salts Medium.

All chemicals were of analytical grade except glucose, ammonium chloride and in some cases the sodium phosphate salts which were 'ARISTAR' grade (BDH). The SSM was made in one of three ways depending on the purpose of the media and the quantity to be prepared. Stock solutions containing combinations of the constituent salts were made (Table 2.1. 1-6). These were autoclaved separately and combined in sterile glassware for each experiment. For larger quantities the constituents excluding the buffer and the iron sulphate (4 and 5) were combined, either from the stock

Table 2.1. Composition of full strength Simple Salts Medium (SSM).

	Constituent	Concentration in medium	Iron contribution to medium
1	Glucose	35.0 mM	0.19 μM
2	Ammonium chloride	25.0 mM	0.0032 μM
	Magnesium sulphate	0.4 mM	
	Potassium chloride	1.5 mM	
3	Sodium chloride	45.2 mM	0.004 μM
4	Sodium phosphate buffer pH7.4	66.67 mM	0.465 μM
5	Iron sulphate	20.0 μM	
6	Calcium chloride	0.5 μM	None detected
	Boric acid	0.5 μM	
	Cobalt chloride	0.05 μM	
	Copper sulphate	0.05 μM	
	Zinc sulphate	0.05 μM	
	Manganese sulphate	0.05 μM	
	Ammonium molybdate	0.005 μM	

solutions or as powders with the appropriate amount of water, and autoclaved. The buffer and iron solutions were sterilised separately and added before use.

For limitation experiments the concentrations were altered as follows:
Carbon limited SSM contained only 3.5 mM glucose.
The iron sulphate solution was omitted from all iron limited media. The phosphate buffer was found to be the component contributing the most contaminating iron to the media (Table 2.1.). This was reduced either by substituting the anhydrous Artistar grade for the dihydrated 'AnalaR' grade sodium phosphate salts comprising the buffer or by chelex treatment of the buffer.

Chelex 100 (Bio.Rad) a chelating ion exchange resin was used to reduce the level of iron in the phosphate buffer. Two columns were prepared; the first contained 200 ml and the second 100 ml of chelex. The chelex was regenerated by passing 2 bed volumes of 1M HCl at 80°C down the column, this was then washed with at least 5 bed volumes of double distilled water. 2 bed volumes of 1M NaOH were then passed down the column and it was washed again with double distilled water until the pH began to fall. The chelex was then washed with phosphate buffer and samples were periodically tested until pH7.4 was obtained. The flow rate was then reduced to 4 ml min^{-1} cm^{-2} and the buffer collected into EDTA treated glassware. The buffer was passed once down the 200 ml column and once down the 100 ml column.

2.3.3. Batch Culture.

Batch culture was carried out in Erlenmeyer flasks containing not more than 1/4 of their volume of media. These were incubated at 37°C either in a shaking water bath or in a warm room on a shaking platform shaking at 180 rpm.

2.3.4. Continuous culture.

The chemostat makes it possible to grow bacteria at a constant and controllable growth rate in closely defined nutritional conditions. In batch culture, as the number of organisms increases, the concentration of any given nutrient decreases and the concentration of metabolic products in the media increases; the bacteria are subjected to a constantly changing environment which is difficult to define. Bacteria grow at the maximum rate (μ_{max}) possible in the prevailing conditions and growth is eventually curtailed by a single nutrient becoming scarce.

Monod (1949) defined the relationship between growth rate(μ) and the concentration of an essential nutrient (S):

$$\mu=\mu_{max}\left(\frac{S}{Ks+S}\right)$$

where Ks is the saturation constant. The culture grows at the maximum rate until the substrate concentration is no longer saturating, it then enters stationary phase. It is possible, within a fairly narrow range, to vary growth rate in batch culture by altering temperature, oxygenation or pH but it is difficult to distinguish in these circumstances between the effects of the alteration of growth conditions and the intrinsic effect of growth rate. Continuous culture comprises an open system where fresh media is being constantly supplied and spent media and cells are constantly removed (Herbert *et al* 1956), the culture is mixed thoroughly and the volume of the culture (V) is kept constant (Fig. 2.1.). The residence time of any given cell in the culture vessel is a function of the dilution rate (D):

$$D=\frac{F}{V}$$

where F is the rate of flow of fresh media into the culture vessel. In the chemostat the substrate concentration is kept constant by the flow of fresh media, any increase in growth rate results in a fall in the substrate concentration resulting in a decrease in growth rate. Conversely any decrease in growth rate results in a rise in substrate concentration and a consequent increase in growth rate. A steady state is reached where growth rate is controlled by the rate of supply of an essential nutrient. Growth rate can be calculated from the equation:

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

where t_d is the doubling time.

Continuous culture in a chemostat makes it possible to study the intrinsic effects of growth rate on organisms grown under closely defined conditions nutrient availability.

Jacketed 50 ml glass chemostats (Fig 2.2.) at 37°C were used in these experiments. The design was based on that of Gilbert and Stuart (1977) with some modifications. *K. pneumoniae* was found to grow as a film on the sampling port tube. This was removed and sampling accomplished by removing the top of the chemostat and drawing up a sample with a sterile glass pipette. The culture was stirred and aerated by passing sterile air through a warmed humidifier and into the culture vessel through a glass sinter at the bottom at a rate of 160 ml min^{-1}. A flowstat (G.A.Platon Ltd.) was introduced before the humidifier to compensate for the effect of the exopolysaccharide produced by *K. pneumoniae* blocking the sinter and prevent any subsequent reduction in the rate of aeration. Media was added to the culture vessel at a constant rate using a peristaltic pump (Watson Marlow 501S) and excess media passed via the overflow to a sterile collecting vessel thus maintaining a constant volume in the culture vessel. Where possible experiments were carried out on a sample taken directly from the culture vessel. When large volumes were required samples were collected from the overflow at 4°C to maintain steady state in the culture vessel.

Fig. 2.I. Schematic representation of chemostat culture.

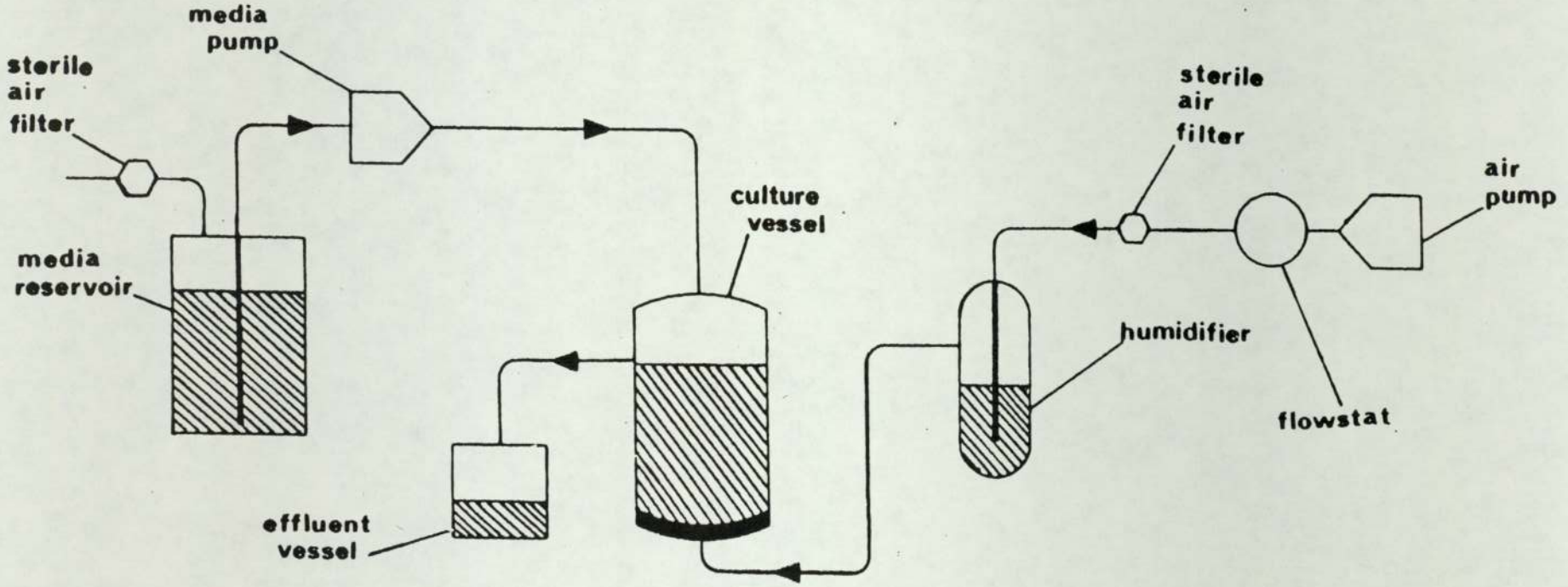
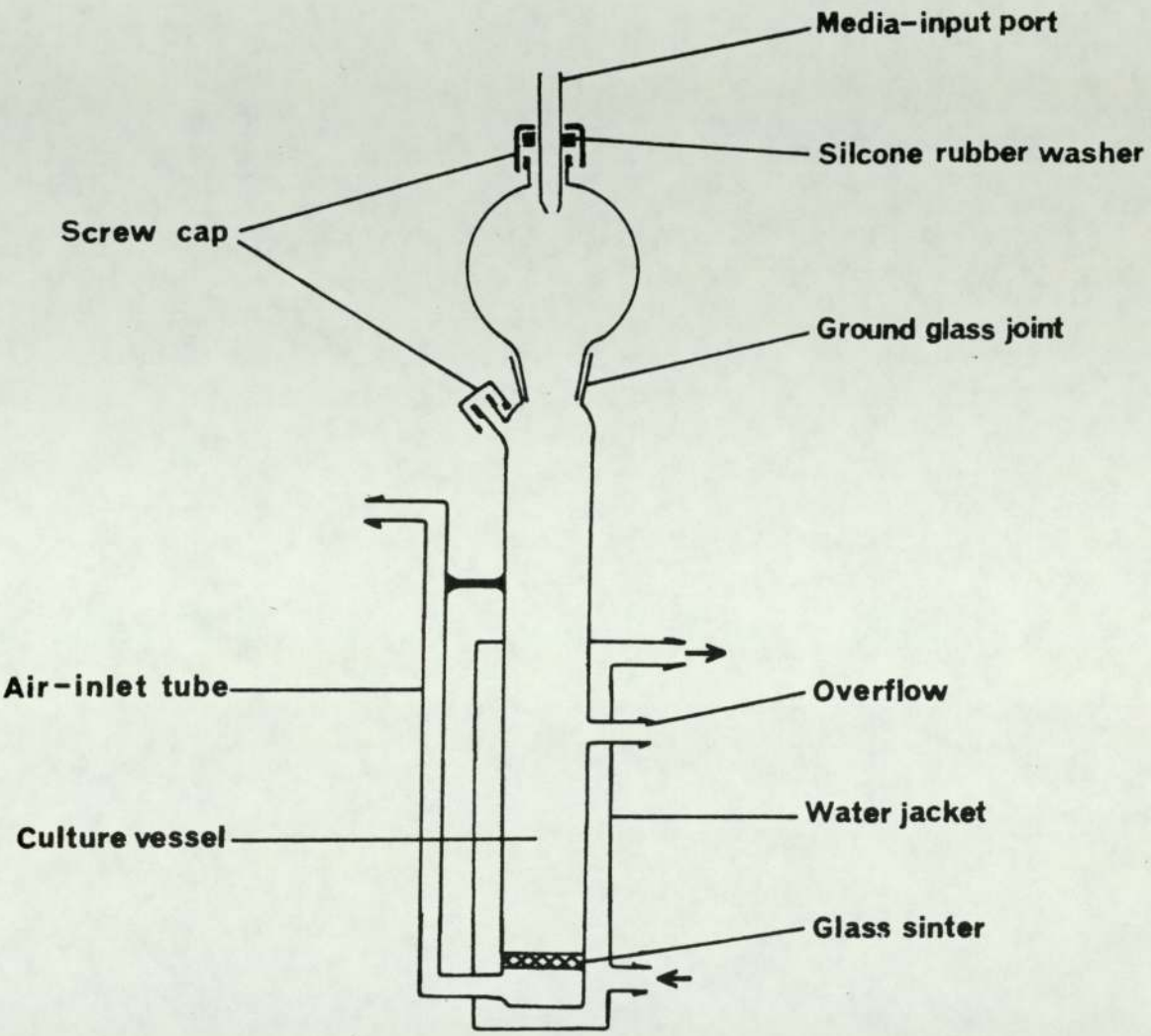


Fig. 2.2. Diagram of a 50ml glass chemostat.



2.4. Standard microbiological techniques

2.4.1. Measurement of bacterial concentration.

Opacity measurements were carried out at OD_{470} found to be the wavelength giving maximum absorbance of the culture with minimum interference from the medium.

Total counts were performed using a haemocytometer with "improved Neubauer" rulings and a chamber depth of 0.1 mm. The sample was diluted in 1% formaldehyde with the addition of Bromophenol Blue to aid discrimination. The slide was viewed under X40 magnification and the number of cells in 5 x 0.04mm^2 counted; this was repeated at least 4 times and the mean value was used to calculate the number of organisms per ml. A Coulter Counter (model ZM with channeliser C256) was also used in some experiments. The sample was diluted in isoton II (Coulter Counter) and three counts made on each sample.

Viable counts were performed using both the spread plate method (Crone 1948) and the method of Miles and Misra (1938), using nutrient agar plates. The spread plate method was preferred because the large size of the colonies formed after overnight incubation and the amount of capsular polysaccharide produced made it difficult to distinguish the colonies in the Miles and Misra drops.

Dry weights were estimated from a bacterial culture equivalent to 100 ml OD_{470} 1.0, the OD_{470} and volume of which were accurately determined. This was harvested at 20,000 rpm at 4°C for 30 min in pre-dried and weighed plastic centrifuge tubes. The pellet was dried in an oven at 60°C and kept in a desiccator over phosphorus pentoxide until a stable weight was recorded. This technique proved to be unsatisfactory as the

plastic centrifuge tubes did not readily reach a stable weight. An alternative technique was used for later experiments. Carefully measured volumes (approximately 1 ml) of a bacterial suspension of an accurately determined opacity (approximately $OD_{470}4$) were dispensed into small (10X50mm) flat bottomed glass tubes. The samples were lyophilised to dryness.

2.4.2. Determination of the minimum inhibitory concentration of antibiotics.

2.5 ml aliquots of double strength medium (SSM or nutrient broth) were dispensed into sterile test tubes. Amounts of sterile antibiotic solution and water were added to give the desired concentrations in a 5 ml volume and finally a 100 µl inoculum from an overnight culture was added. The tubes were vortexed and incubated at 37°C for 18 h. The tubes were examined for growth and the MIC defined as a range of concentrations from the lowest inhibiting to the greatest permitting growth.

2.5. Atomic absorption spectroscopy for the determination of iron.

A Perkin-Elmer manually loaded spectrophotometer (model 560) with deuterium background corrector was used. A 20 µl 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. A calibration curve was constructed using a standard iron solution (Spectrosol grade BDH). Using this set up and the conditions in Table 2.2. it was possible to measure accurately the iron concentration in the complete SSM and in all the separate stock solutions, except the phosphate buffer. The atomisation temperature of the phosphate was close to that of iron and the interference from the phosphate was too great to

Table 2.2. Program details for the spectrophotometric measurement of iron.

	Temperature (°C)	Time to reach temperature (sec)	Time temperature maintained (sec)
Step 1	140	10	30
Step 2	1200	10	30
Step 3	2000	0	10*
Step 4	2200	1	5

* This uses the max power facility. The chart recorder and peak analyser are switched on at this point.

be compensated for by the background corrector, negative peaks were observed. This interference was overcome by use of L'vov tubes and platforms (L'vov 1977), and by adjusting the atomisation temperatures and times (Table 2.2.). The L'vov tubes which are Pyro coated reduce carryover from one sample to the next and the platforms, by holding the drop away from the chamber wall facilitate more gentle and even heating and reduce splattering.

2.6. Electrophoretic analysis of the components of the outer membrane.

2.6.1. Preparation of outer membranes.

Where possible a sample of bacteria equivalent to 100 ml of OD_{470}1.0 was used. The cells were harvested (12,000 rpm for 10 min) and resuspended to approximately OD_{470}10. The suspension was sonicated on ice; 5 x 30 sec bursts with 30 sec. cooling periods. The inner membrane portion was selectively solubilised by incubation with 2% sodium-lauryl sarcosinate (Sigma Chemicals Co.) for 30 min at 20°C according to the method of Filip *et al* (1973). Any remaining whole cell debris was removed by centrifugation (7000 rpm for 10 min). The outer membrane

fraction was separated by centrifugation (20,000 rpm for 40 min) and the pellet resuspended in 500 µl water. The preparation was stored frozen (-20°C) until required.

2.6.2. Isolation of non covalent peptidoglycan associated proteins.

Membranes were prepared as above. The sample was then extracted in SDS (2% SDS, 10% Glycerol and 10mM Tris HCl buffer pH 7.8 in 0.1 M NaCl) for 30 min at 30°C to solubilise non peptidoglycan associated proteins (Mizuno and Kageyama 1979). The insoluble fraction was collected by centrifugation at 20000 rpm for 40 mins. The extraction was repeated twice and the peptidoglycan protein complex obtained was washed once in distilled water before being resuspended and frozen.

2.6.3. Polyacrylamide gel electrophoresis.

Two types of gel apparatus were used depending on the application, a large slab gel apparatus (in house), with 1 mm or 1.5 mm spacers, and a mini gel system (Mini Protean II. Bio-Rad) using 0.5 mm spacers.

Electrophoresis was carried out according to the methods described by Lugtenberg *et al* (1975) and Laemmli (1970). Polyacrylamide gels of different percentages were made from stock solutions (Table 2.3). As the quality of the SDS was found to influence the electrophoretic mobility of some proteins (Anwar, Lambert and Brown 1983) specially purified SDS (BDH) was used. Both electrodes contained buffer at pH 8.3 (28.8g glycine, 6g TRIS/HCl, 20 ml 10% SDS in 2L).

2.6.4. Polyacrylamide gel electrophoresis for analysis of proteins.

The OM preparation was denatured by mixing with an equal volume of sample buffer (5 ml 0.5M TRIS-HCl pH6.8, 10 ml 10% SDS, 5 ml glycerol, 0.5 ml mercaptoethanol, 10 ml distilled water and bromophenol blue (as

Table 2.3. Shows the composition of gels used. The quantities given are for large slab gels, smaller amounts were made for mini gel systems.

	Volume of solution used (ml)					
	Lugtenberg System				Laemmli System	
Stock Solution	Stacking Gel	10% Gel	12% Gel	14% Gel	Stacking Gel	Running Gel
Stock I	-	12.5	16.0	18.7	-	-
Stock II	5.0	-	-	-	5	25
1.5M Tris-HCl pH 8.8	-	18.5	18.5	18.5	-	15
0.5M Tris-HCl pH 6.8	7.5	-	-	-	7.5	-
Distilled water	16.0	26.0	22.5	19.8	18.5	19.8
SDS 10% (w/v)	0.3	1.5	1.5	1.5	-	-
APS 10% (w/v)	0.1	0.2	0.2	0.2	0.1	0.1
TEMED	0.08	0.14	0.14	0.14	0.01	0.01

BIS is N,N-methylene bisacrylamide

SDS is sodium dodecyle sulphate

APS is ammonium persulphate

TEMED is NNN'N'-tetramethylethalene diamine

Stock I contains 44% (w/v) acrylamide/0.8% (w/v) BIS

Stock II contains 30% (w/v) acrylamide/0.8% (w/v) BIS

a tracking dye) and heating to 100°C for 10 min. When using the large slab gel system, 20-50 µl samples were loaded into each well and electrophoresis carried out at room temperature at a constant current of 40mA until the tracking dye had moved 10cm from the interface of the stacking and running gels. 5-20 µl samples were loaded into wells on the mini gel system and a voltage of 200v was applied. The dye front was run to the end of the mini gels. The gel was fixed and stained in 0.1% Coomassie brilliant blue (BDH) in 50% ethanol/25% acetic acid, and destained in 50% ethanol/25% acetic acid.

2.6.5. Polyacrylamide gel electrophoresis for analysis of lipopolysaccharide.

Aliquots of whole cell suspension equivalent to 200 µl of $OD_{470}40$ were placed in small glass test tubes. The volume was adjusted to 200 µl and the sample heated to 100°C for 10 min. 90 µl of fresh sample buffer (5 ml 0.5 M TRIS-HCL pH 6.8, 10 ml 10% SDS 1 ml mercaptoethanol 5 ml glycerol and Bromophenol blue) was added and the sample lysed by heating to 100°C for 10 min. For protein digestion 80 µl of a 2.5 mg ml^{-1} solution of proteinase K (from *Tritirachium album* type XI Sigma) was added and the sample was incubated at 60°C for 60 min (Hitchcock and Brown 1983). Samples of extracted lipopolysaccharide were simply treated with sample buffer (as above). The large slab gel system with 1 mm spacers, made according to the Laemmli system, was used for lipopolysaccharide analysis because the samples needed to be run over a long distance to ensure separation of the high molecular weight lipopolysaccharide into bands. 5-30 µl samples were loaded into each well and electrophoresis was carried out at room temperature at 22 mA until the dye front had run 16cm from the stacking gel/running gel interface. The gel was fixed overnight in 45% ethanol/5% acetic acid.

2.6.6. Silver stain for lipopolysaccharide.

This was performed according to the method of Tsai and Frasch (1982). Double distilled water and a scrupulously clean glass dish were used and the gel was handled with washed gloves at all times because the stain is very sensitive. The fixer (see above) was replaced with the oxidising solution (0.7% periodic acid/40% ethanol/5% acetic acid) and agitated gently at room temperature for 45 min. The gel was then washed every 15 min for 1 h with large volumes of water. The staining solution was freshly prepared, 5 ml of a 20% silver nitrate solution was slowly added to a solution, containing 28 ml 0.1 M sodium hydroxide and 2 ml ammonium (approximately 40%), avoiding precipitation, 115 ml of distilled water was added. The gel was agitated in the staining solution for 45 min and washed every 15 min in a small volume of water for 45 min. It was then developed in a solution containing 10 mg citric acid and 0.1 ml formaldehyde in 200 ml of water. The developing was stopped and the gel preserved in the fixer (see above). Photographs were taken as soon as possible as the stain deteriorates.

2.7. Production of hyperimmune antiserum.

2.7.1. Preparation of inoculum.

The bacteria were grown in SSM for 18 h to approximately OD_{470} 4. Formaldehyde was added to give a concentration of 1% and the culture was incubated at room temperature (21°C) for 2 h. The bacteria were pelleted (10,000 rpm for 15 min) and washed twice in normal saline. The bacteria were then resuspended in normal saline to OD_{470}1 (approximately 10^{9} cells per ml) and dispensed into suitable aliquots for injection.

2.7.2. Inoculation schedules.

Rabbit. Male half lop rabbits (2.5-3.5 kg) were used, 1 ml of the bacterial suspension was injected subcutaneously twice weekly for at least 2 months. 10 ml samples of blood were taken from the marginal ear vein periodically and tested for antibodies in a double diffusion assay (Sect. 2.8.4.). The animal was sacrificed and blood collected by cardiac puncture, the serum was separated and stored frozen.

Rat. Wistar outbred male rats (250-300 g) were used, 0.5 ml of the bacterial suspension was injected subcutaneously twice weekly for at least 2 weeks. Blood was taken from the tail vein to assess the level of antibodies. All other details were as for rabbits.

2.8. Immunological analysis of bacterial antigens.

2.8.1. Preparation of agarose gels.

A 1% solution of agarose (LKB) was made in Tris-barbiturate buffer pH 8.6 (a stock solution containing 22.4 g diethylebarbituric acid, 44.3 g Tris, 0.533 g calcium lactate and 0.65 g sodium azide in 1L of distilled water was stored at 4°C and diluted 1:5 before use). Vigorous boiling was required to ensure that the agarose dissolved completely. The volume of agarose gel solution required for each gel was calculated according to the formula, length of gel X width of the gel X 0.132 ml. The correct volume of agarose solution was dispensed on to the hydrophilic side of a piece of Gel Bond (Bio Rad) supported on a horizontal table to ensure an even thickness of gel. When the gel was set wells were punched with either a 4 mm or 5 mm diameter cork borer giving wells of 15 µl and 25 µl volumes.

2.8.2. Preparation of samples.

Whole cell suspensions with an OD_{470}10 were sonicated for 5 min on ice for periods of 30 secs with 30 sec cooling periods. Supernatant samples were concentrated, where necessary, by lyophilisation before being loaded on agarose gels. Bromophenol blue was added where necessary as a tracking dye.

2.8.3. Washing and staining agarose gels.

The gel was placed on a glass plate, covered with a double layer of filter paper (Watman chromatography paper) and several thicknesses of paper towel. A glass plate and a weight (1 Kg) were placed on top and the gel was pressed for 10 min, it was then soaked in 0.1 M NaCl for 10 min. The washing and pressing process was repeated three more times, the final wash being in distilled water. The gel was then dried to a thin film using a hair dryer or an oven at 60°C. The gel was then immersed in stain (1 g Coomassie Brilliant Blue/90 ml ethanol/20 ml acetic acid/90 ml water) for 10 min and repeatedly washed in destain (90 ml ethanol/20 ml acetic acid/90 ml water) until the background was no longer blue.

The washing process removes soluble protein from the gel, unreacted antibodies or other proteins from the serum or the sample, leaving only the insoluble antibody antigen complex. This precipitate is stained nonspecifically by the coomassie blue which detects the antibody part even if the antigen is non proteinaceous.

2.8.4. Double diffusion (Ouchterlony) technique.

Wells were placed 8 mm apart, centre to centre, usually in a triplet or as 6 wells arranged around a central well. After filling the wells, the

plate was placed in a humidity chamber and left for diffusion overnight (18 h) at 4°C. The gel was then washed and stained.

2.8.5. Quantitative rocket immunoelectrophoresis.

This technique was first described by Laurell (1965). The agarose gel solution was cooled to 55°C (to avoid denaturation of the antibodies), and mixed with hyperimmune antiserum to give the desired dilution (1/10-1/100), before being poured as described above. 15 µl wells were cut at 10 mm intervals 15 mm from the bottom edge of the gel. To minimise diffusion of the samples the gel was placed on the cooling plate of the flat bed electrophoresis tank (LKB), and the wicks (Watman Chromatography paper) were positioned, before loading. Tris-barbiturate buffer (Sect. 2.8.1.) was used at both electrodes and to soak the wicks. Electrophoresis was carried out at 500v overnight at 10°C. The area of the rockets was estimated by photocopying the gel, after washing and staining, and cutting out the rockets and weighing them. A calibration was performed by loading a range of volumes of a preparation of the pure antigen onto the gel (Sect. 2.10.).

When the voltage is applied the antigen starts to migrate through the gel; it is initially in excess, so the antigen-antibody complex is soluble and will migrate towards the anode. During electrophoresis the number of immunocomplexes increases and eventually the equivalence point, where the precipitate is insoluble, is reached. The area of the rocket is proportional to the concentration of the antigen. The electrophoresis is performed at pH8.6 where the electrophoretic mobility of the antibodies is low.

2.8.6. Fused rocket immunoelectrophoresis.

A 25 mm strip of antibody-free agarose gel solution was poured along the bottom edge of the piece of Gel Bond. Wells were cut at 8 mm intervals, centre to centre 10 mm from the bottom edge. Samples were loaded and allowed to diffuse for 30 min at room temperature. Antibody containing gel was prepared and poured onto the remaining Gel Bond and electrophoresis was performed (Sect. 2.8.5.).

When polyvalent antiserum is used it may detect more than one antigen particularly in a whole cell sample. By allowing an initial diffusion stage a line of identity can be identified between the purified antigen and one of the precipitant bands in the sample. The rockets to be measured can thus be identified.

2.8.7. Crossed immunoelectrophoresis.

An antibody-free gel was poured and a single 25 µl well was cut in the bottom left hand corner into which the sample was loaded. Electrophoresis was carried out in the first dimension at 500v until the dye had migrated to within 5 mm of the edge of the gel. The gel was then scored 2.5 mm from the bottom edge of the gel and the upper portion was removed. Antibody-containing gel was then cast onto the empty part of the Gel-Bond (Sect. 2.8.5.) and the gel orientated on the flat bed one quarter turn clockwise relative to the first dimension. Electrophoresis in the second dimension is carried out at 100v overnight.

CIE's of whole cells result in a fairly complex pattern of precipitation bands. It is possible to identify some of these by putting a purified antigen into either an intermediate gel (between the antibody-free and the antibody-containing gels) or into a well punched 1cm from the first well along the line of migration in the first dimension. The

precipitation band formed by the purified antigen will in the first case lift out the peak formed by the homologous antigen and in the second case fuse with it.

2.9. Siderophore assays.

2.9.1. Chromatography to identify enterochelin.

The supernatant was collected from an overnight batch culture, and acidified to between pH2 and pH3. The supernatant was then extracted with 1/3 it's volume of ethyl acetate by vigorous shaking. This was allowed to equilibrate overnight at 20°C. The ethyl acetate extract was then back extracted with an equal volume of 0.1 M pH7 phosphate buffer. The ethyl acetate was reduced to dryness in a rotary evaporator at room temperature and the residue was dissolved in a small volume of methanol. Enterochelin extracted from *Escherichia coli*, kindly donated by I.G.Young (1976), was used as a standard. The extracts were analysed by paper or cellulose thin layer chromatography using the formic acid solvent system (5% w/v ammonium formate, 0.5% formic acid) described by Perry and San Clemente (1979).

2.9.2. Detection of phenolate compounds.

The presence of phenolate compounds was detected using a reagent freshly prepared by mixing equal volumes of 0.1 M $FeCl_2$ in 0.1 M HCl and 0.1 M $KFe_3(CN)_6$. A blue colour indicated the presence of phenolate compounds.

2.9.3. Detection of siderophores.

The presence of siderophores was detected using the method described by Schwyn and Neilands (1987). The reagent was made by mixing 1.5 ml of 1 mM $FeCl_3$ in 10 mM HCl with 7.5 ml 2 mM aqueous Chrome Azurol S (CAS)

(Sigma). This mixture was added to 6 ml of 10 mM hexadecyltrimethylammonium bromide (HDTMA) (Sigma) in a 100 ml volumetric flask. 4.307 g of anhydrous piperazine was dissolved in less than 50 ml of water and 6.25 ml of 12 M HCl was added. The piperazine solution was rinsed into the volumetric flask and the volume made up to 100 ml. The reagent was stored in a brown bottle. CAS is an indicator which is blue when complexed with iron. The addition of any ligand capable of binding the iron held by the CAS, eg. a siderophore, causes the release of the free dye which is a pink colour. A pink spot on the chromatogram therefore indicates the presence of a siderophore

2.9.4. Quantitative assay of enterochelin.

The method of Arnow (1937) was used to quantitate enterochelin and related phenolates. The assay was calibrated using 3,4 dihydroxy phenylalanine as a standard phenolate. A 1 ml aliquot of supernatant or standard phenolate solution was measured into a test tube, this was then acidified with 1 ml 0.5 M HCl. 1 ml of the nitrate/molybdate reagent (10 g sodium nitrate and 10 g sodium molybdate in 100 ml water) and then 1 ml of 1 M NaOH added and the solution mixed vigorously. A red colour indicated the presence of enterochelin and related phenolates. The absorbance of the solution was measured at 515 nm.

2.10. Preparative methods.

2.10.1. Purification of lipopolysaccharide by phenol extraction.

A 3l culture was grown in SSM for 18 h and the bacteria harvested (10,000 rpm for 15 min). The lipopolysaccharide was extracted according to the method of Westphal and Jann (1965). The pellet was washed once in normal saline before being resuspended in 30 ml of 30 mM Tris-HCl (pH8). The suspension was sonicated on ice (10X60 sec bursts with 30 sec

cooling periods), before being incubated with DNAase, RNAase and lysosyme for 2 h at 37°C to digest DNA, RNA and peptidoglycan. The mixture was then incubated overnight at 37°C with EDTA and pronase (*Sacchromyces griseus*). Pronase digests protein and EDTA chelates magnesium ions from the lipopolysaccharide destroying the integrity of the membrane. The mixture was heated to 85°C and mixed with 50 g phenol at 85°C, this was stirred for 30 min. The mixture was centrifuged (10,000 rpm for 30 min) and the aqueous layer carefully removed. 50 ml of distilled water was mixed with the phenol layer and the extraction repeated twice more. The aqueous extracts were pooled and dialysed for 48 h against tap water. $MgCl_2$ to a concentration of 50 mM was added before centrifugation at 40,000 rpm for 4 h. Magnesium ions aid the formation of lipopolysaccharide micells which are pelleted by centrifugation. The pellet was washed twice in distilled water before being lyophilised.

Lipopolysaccharide was also extracted from small samples of cells (2 ml of OD_{470} 2) using the method of Inzana (1983). The bacteria were pelleted (10,000 rpm for 5 min) and washed once in phosphate buffered saline supplemented with magnesium and calcium (0.144 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4). The pellet was resuspended in 300 µl of water and mixed in a small flat bottomed glass tube with an equal volume of hot 90% phenol. This was mixed vigorously at 65-70°C for 15 min. The suspension was cooled on ice and transferred to a microfuge tube (Eppendorf) and centrifuged for 15 min (Eppendorf centrifuge). The supernatant was transferred to a glass centrifuge tube and the phenol phase reextracted with 300 µl of water. The aqueous phases were pooled and NaCl added to 0.5 M. 10 volumes of absolute alcohol were added and the tube kept at -20°C for 6 h, this was then centrifuged (15,000 rpm

for 10 min) to precipitate the lipopolysaccharide. The precipitate was resuspended in 100 µl of water and the precipitation procedure repeated in a microfuge tube. The final pellet was resuspended in 50 µl of water.

2.10.2. Purification of capsular polysaccharide by acetone precipitation.

Cultures were grown in SSM for 18 h. The organisms were pelleted (20000 rpm for 60 min) and the supernatant centrifuged at 38000 rpm for 4 h to spin out lipopolysaccharide, the capsular polysaccharide was isolated by the method of Sandford and Conrad (1966). The supernatant was mixed with 3 volumes of cold acetone (-20°C) and stirred vigorously with a glass rod. The precipitated capsular polysaccharide was collected by filtration, and dried under vacuum over phosphorous pentoxide. It was then washed first with acetone and then with ether and lyophilised.

2.11. Measurement of permeability

In principal this technique involves measuring the rate at which a β-lactam antibiotic crosses the outer membrane by determining the difference between the rates of breakdown of a chromogenic cephalosporin Nitrocefin (Glaxo, Greenford, Essex) by the periplasmic β-lactamases in whole and disrupted cells (Zimmermann and Rosselet 1977). A 10 ml sample was taken from the chemostat culture vessel, the bacteria were harvested (70000 rpm for 15 min at room temperature) and resuspended to OD_{470} 1.0 in magnesium/phosphate buffer (5 mM magnesium sulphate, 10 mM sodium and di-sodium phosphate) and then maintained at 37°C. 0.2 ml of the suspension and 0.1 ml of a 0.5 mg ml^{-1} solution of Nitrocefin were mixed in a 0.3 ml glass cuvette (path length 1 mm) and the absorbance at 540 nm recorded over a period of 20 min. This was repeated three times and the initial rate of hydrolysis of Nitrocefin in intact cells

determined (V_i). The procedure was repeated using 0.2 ml of whole cell suspension sonicated to below OD_{470} 0.1 to determine the rate of the reaction unimpeded by the outer membrane. The rate of hydrolysis of a range of concentrations of Nitrocefin (25 to 125 $\mu g\ ml^{-1}$) by the disrupted whole cell suspension was measured to give a family of curves from which a Lineweaver-Burk plot was drawn. The values of K_m (Michaelis-Menten parameter), V_{max} (the maximum rate of hydrolysis of Nitrocefin) were calculated from the ordinate and abscissa intecepts respectively and S_e (the substrate concentration inside the cell) was calculated from the equation:

$$S_e = \frac{V_i \ X \ K_m}{V_{max} - V_i}$$

The ratio of the concentration inside the cell to the concentration outside the cell (S_e/So) is taken as a measure of the permeability of the outer membrane (Zimmermann and Rosselet 1977).

2.12. Appendix.

Addresses of suppliers and manufacturers:

BDH Ltd., Broom Rd., Poole, Dorset BH12 4NN

Beckman Ltd., High Wycombe, Buckinghamshire.

Bio-Rad Laboratories Ltd. Caxton Way, Watford Business Park, Watford,

Buchi Laboratoriums, Technic AG, CH-9230 Flawl, Switzerland.

Hertfordshire WD1 8RP

Cecil Instruments Ltd., Milton Industrial Estate, Cambridge CB4 4AZ

Coulter Counter, Northwell Dr. Luton Bedfordshire.

Eppendorf, Gerätebau, Nethelera and Hinz Gamh, West Germany

FSA Ltd (Fisons)., Loughborough, Leicestershire.

Gilson, Anachem Ltd., Luton, Bedfordshire.

Grant Instruments (Cam.) Ltd., Barrington, Cambridge CB2 2QZ

LKB Instruments Ltd., 232 Addington Rd., Selsdon, South Croyden, Surrey

Modulyo, Edwards High Vacuum Ltd., Crawley, Sussex.

Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire.

Platon Ltd. Wella Rd., Basingstoke, Hampshire

Oertling Ltd., Orpington ,Kent

Sartorius, 34 Gottingen, West Germany

Sigma Chemical Company Ltd., Fancy Rd., Poole, Dorset BH17 7NH

Watson Marlow, Falmouth, Cornwall

Watman Filter Paper, supplied by Fisons

Wild, Microscopic Instruments Ltd., Oxford, Oxfordshire.

3. The effect of growth rate and iron deprivation on enterochelin production and the outer membrane protein profile of *K. pneumoniae*.

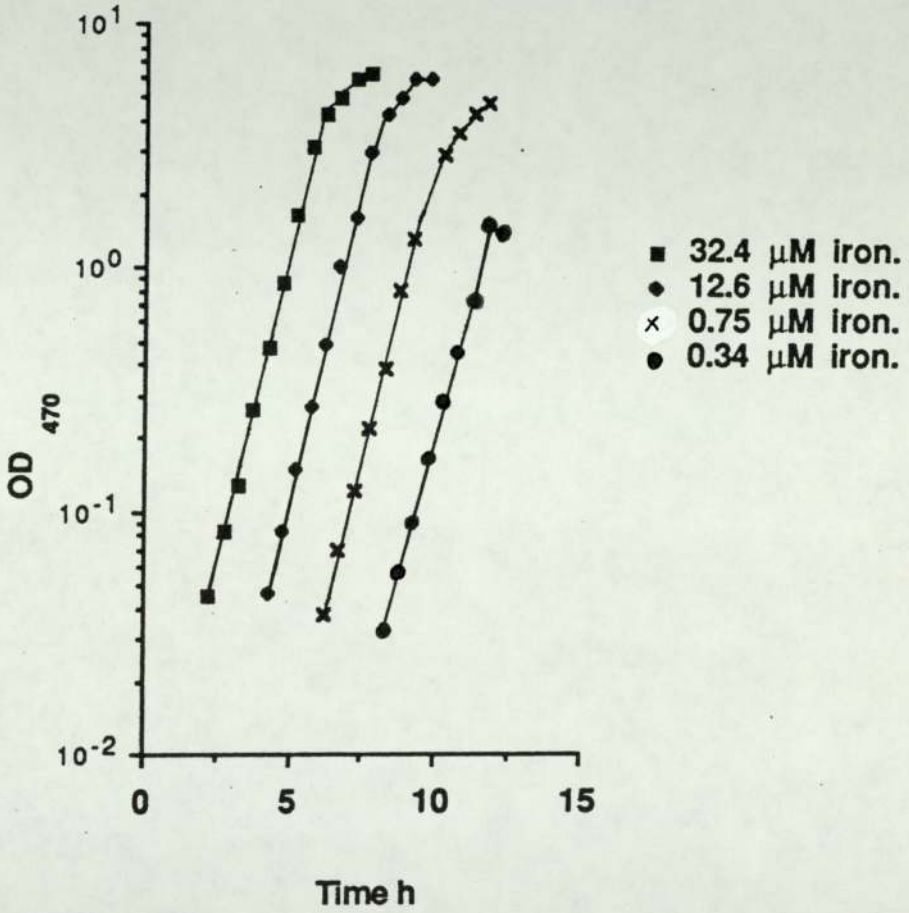
3.1. Iron requirement of *K. pneumoniae* in batch culture.

K. pneumoniae was grown in a SSM containing sufficient of each essential nutrient, except iron, theoretically to facilitate growth to OD_{470} 10 (Klemperer *et al* 1979). Fig. 3.1. shows the effect of various concentrations of iron on the growth kinetics of *K. pneumoniae* in batch culture. The iron concentration does not effect the growth rate: doubling time (t_d) 35 min $\pm$ 8% for these curves. The effect of iron concentration was seen on the point of entry into stationary phase growth. There is no difference in the curves for concentrations above 12.4 μM, which represent untreated media, with and without added iron. The effect of reduced iron concentration on the growth kinetics in batch culture was seen at iron concentrations below 1 μM. At concentrations of 0.34 μM the cultures entered stationary phase at least two doubling times before the cultures grown in complete SSM. In iron depleted batch culture the IRMP's and enterochelin production are detected 2 h into the growth curve (Williams *et al* 1984). The bacteria show characteristics of iron limitation before there is any effect on growth kinetics.

3.2. Siderophore production by *K. pneumoniae*

Strains of *K. pneumoniae* are known to produce two siderophores, enterochelin and aerobactin (Nassif and Sansonetti 1986, Perry and San Clemente 1979, Williams *et al* in press). An ethyl acetate extract of the supernatant from an overnight culture of DL1 in iron depleted SSM was subjected to paper chromatography and detected using a reagent specific for phenolates and one which detects siderophores (Sects. 2.9.2. and 2.9.3.). The phenolate reagent detected five spots with R_f values which

Fig 3.1.Effect of the concentration of iron in the medium on the growth kinetics of *K.pneumoniae.*



The curves are superimposable, they have been offset for clarity.

Table 3.1 R_f Values for enterochelin and related phenolates.

Compound	R_f Value		
	Literature[1]	Supernatant	Standard[2]
Dihydroxybenzoic acid	0.74	0.85	0.837
Dihydroxybenzoyleserine	0.66	0.624	0.631
DBS dimer	0.56	0.551	0.597
DBS linear trimer	0.35	0.419	0.418
Enterochelin	0.19	0.198	0.211

1 Perry and San Clement (1979)
2 *Escherichia coli* enterochelin Young (1976)

approximate well with those from the literature for: dihydroxybenzoic acid, 2,3-dihydroxy-N-benzoyl serine (DBS), a dimer of DBS, a linear trimer and a cyclic trimer, enterochelin (Table 3.1.). The siderophore reagent comprises a dye which is blue when iron is bound to it; a strong chelator, such as a siderophore, is capable of removing the iron causing a colour change to orange. This reagent detected all but the dihydroxybenzoic acid spot, which presumably has no iron chelating properties. No similar spots were detected with supernatant from iron replete cultures. DL1 produced iron chelating compounds with R_f values characteristic of enterochelin and its related phenolates. The same supernatant samples were assayed for phenolates using the Arnow assay (1937). Substantial amounts of the phenolate compounds were detected in the iron depleted culture only. It seems reasonable to assume that the phenolate compounds measured by the Arnow assay are enterochelin and related DBS compounds. The assay was used throughout to quantify enterochelin.

Aerobactin has not been detected in the supernatant from this strain grown in this SSM (Williams *et al* 1984), although it can be detected after growth in a Tris-buffered succinate medium (Williams *et al* in press). It was not assayed for in this study.

3.3. Continuous culture of *K. pneumoniae*.

In batch culture only the starting conditions can be defined accurately. The concentrations of the nutrients are altered during the growth cycle and bacterial metabolic products also alter the composition of the medium. The culture conditions in a chemostat can be more closely defined in terms both of the growth rate and of nutrient availability. It is theoretically possible with *K. pneumoniae* in this SSM (t_d 0.6 h) to achieve growth rates of up to D=1.2 h^{-1} but in practice the chemostat is stable only up to D=0.8 h^{-1}. Fig. 3.2. shows the changes in optical density over a range of growth rates up to the theoretical maximum. The decrease in optical density is in part accounted for by the different light scattering properties of the larger cells characteristic of higher growth rates (Fig.3.3.) but is mainly a consequence of the tendency to wash out as the maximum growth rate is approached. The relationship between dry weight and optical density did not vary much over the range of growth rates used in these experiments but the standard deviation at the fastest growth rate indicates that these chemostats are less stable (Fig. 3.4.). The iron limited chemostats were deemed to be truly iron limited because the optical density increased by more than 2.5 times to the theoretical maximum for the medium of OD_{470}10 when iron was added to the culture to give the concentration for full SSM. This also demonstrated that the oxygenation provided in the chemostat was far in

Fig. 3.2. Change in optical density over a range of growth rates in iron limited chemostat culture.

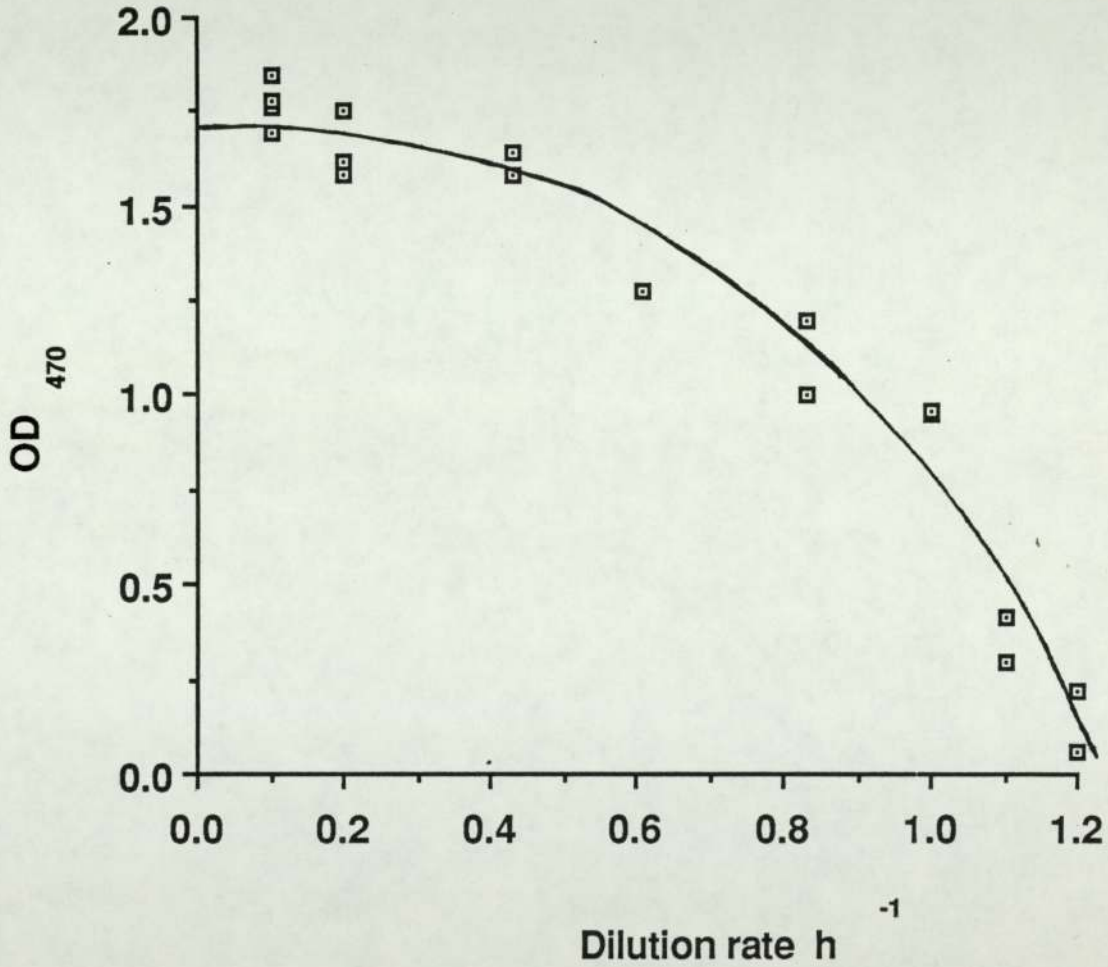


Fig 3.3. Average size of *K.pneumoniae* at different growth rates in iron limited chemostat culture.

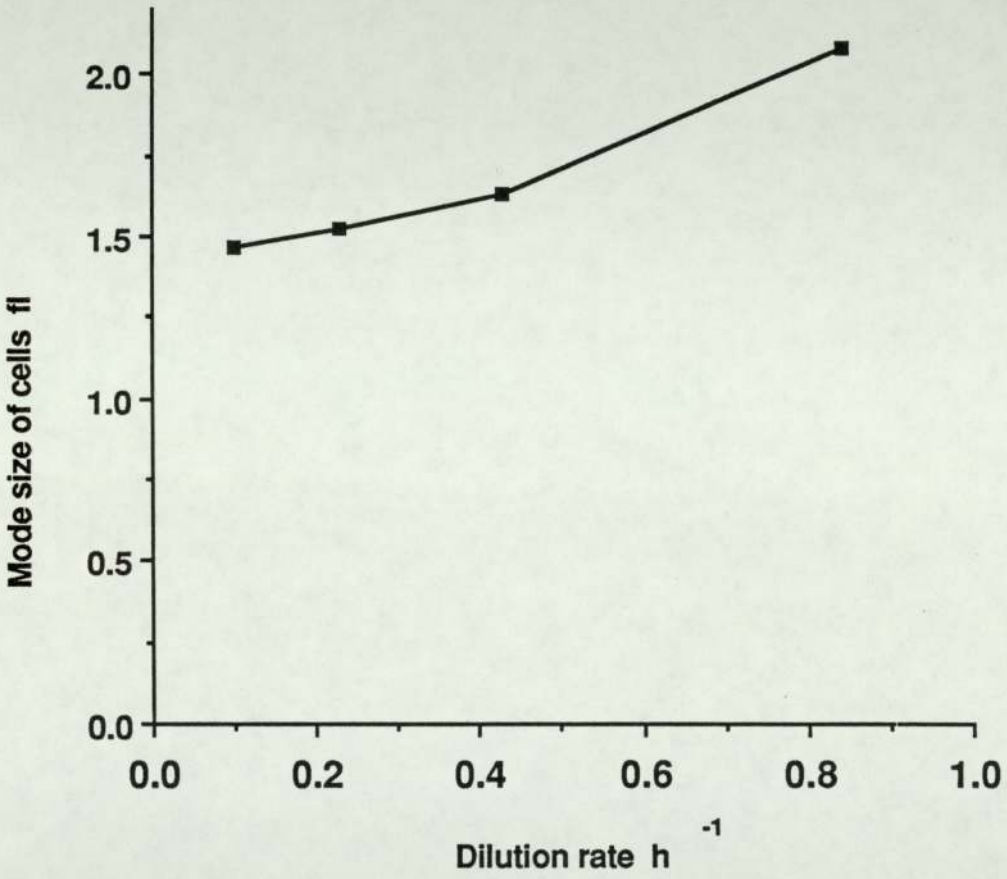
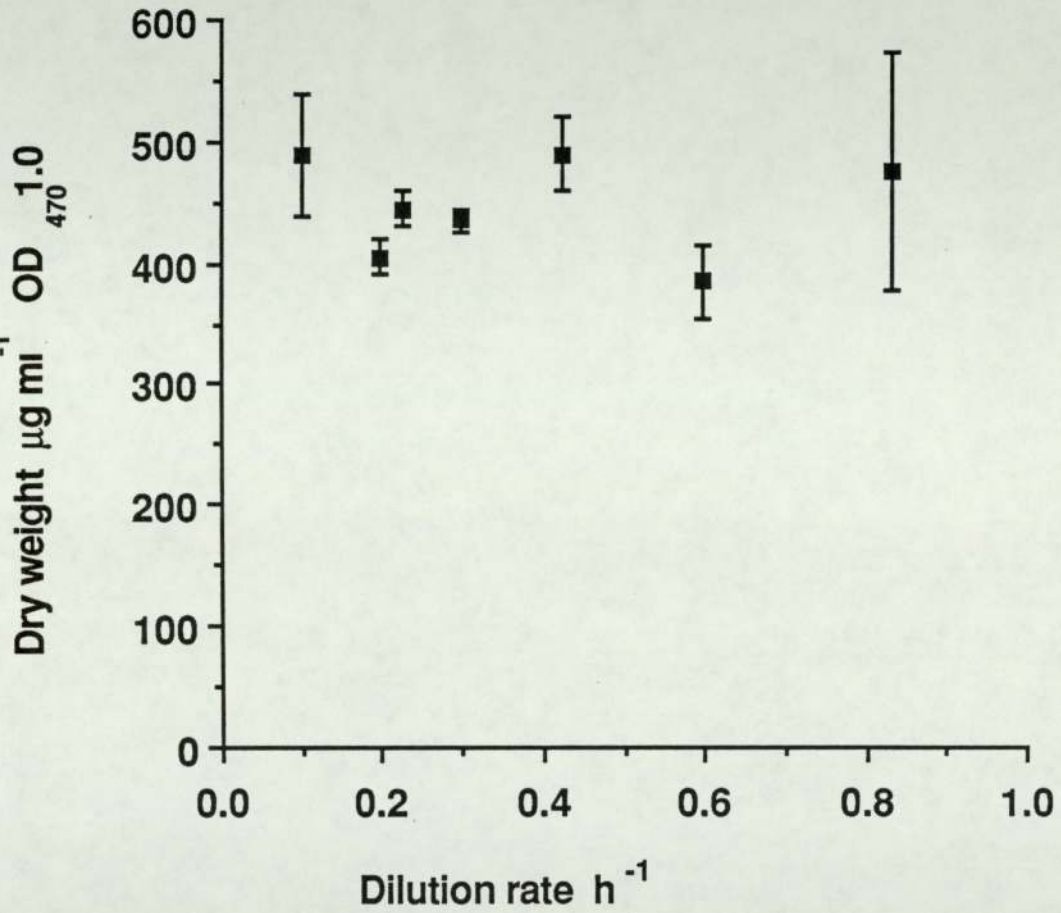


Fig 3.4. Effect of growth rate on the dry weight of *K.pneumoniae* in iron limited chemostat culture.



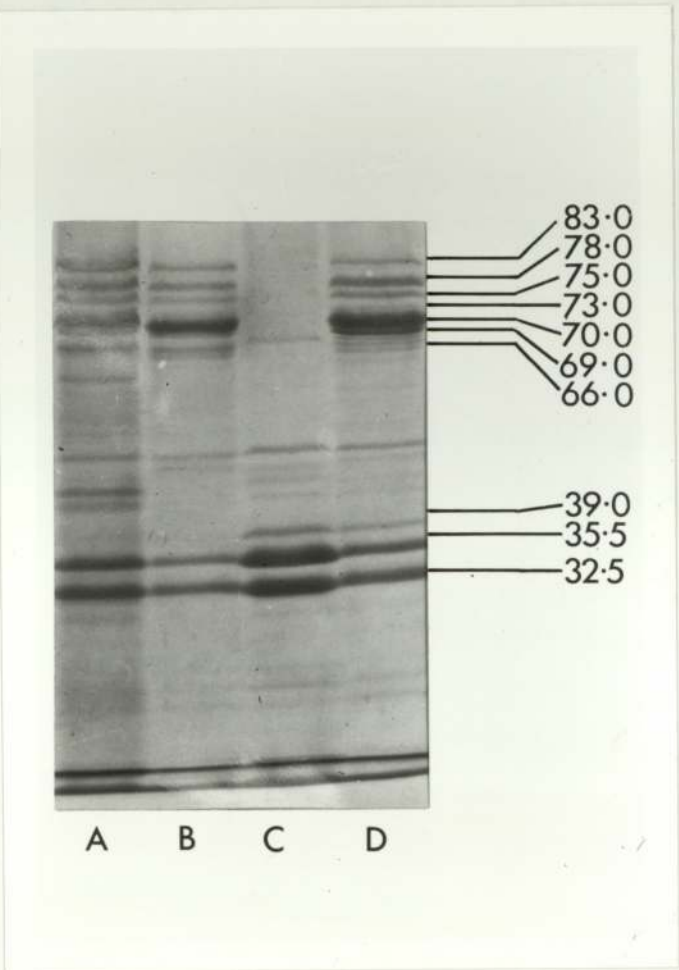
excess of the cultures requirements and that another nutrient, probably glucose, was limiting in these circumstances.

3.4. The effect of iron deprivation on enterochelin production and the outer membrane protein profile of *K. pneumoniae* in batch and iron limited chemostat culture.

The outer membrane protein profile of *K. pneumoniae* grown to stationary phase in complete SSM shows three major protein bands at 32.5, 35.5 and 39 kDa (Fig. 3.5, lane C). Two of these proteins, 35.5 and 39 kDa, were found to be peptidoglycan associated when extracted in 2% SDS at 35°C (Fig. 3.6, lane A). Lugtenberg *et al* (1977) demonstrated two similar peptidoglycan associate proteins with molecular weights of 35 and 38 kDa in *K. aerogenes*. It is proposed that these proteins are functionally related to the OmpF and OmpC porin proteins of *E. coli* with which they cross-react immunologically (Hofstra and Dankert 1979). In iron depleted batch culture seven high molecular weight proteins are seen (Fig. 3.5, lane D). These IRMP's play various roles in sequestration of iron (Sect. 1.4.5.)

The outer membrane protein profile of *K. pneumoniae* grown in iron limited chemostat culture is closely related to the iron depleted batch culture profile (Fig. 3.5, lanes A and B). The IRMP's were fully expressed at both growth rates. The 39 kDa peptidoglycan associated protein was absent at both growth rates in iron limited chemostat cultured cells. Culture conditions such as osmolarity and nutrient limitation are known to alter the expression of the OmpF and OmpC porin proteins of *E. coli* (Lugtenberg *et al* 1976, van Alphen and Lugtenberg 1977). The reduction or absence of one porin resulting from culture conditions or from mutation has been shown in some cases to be

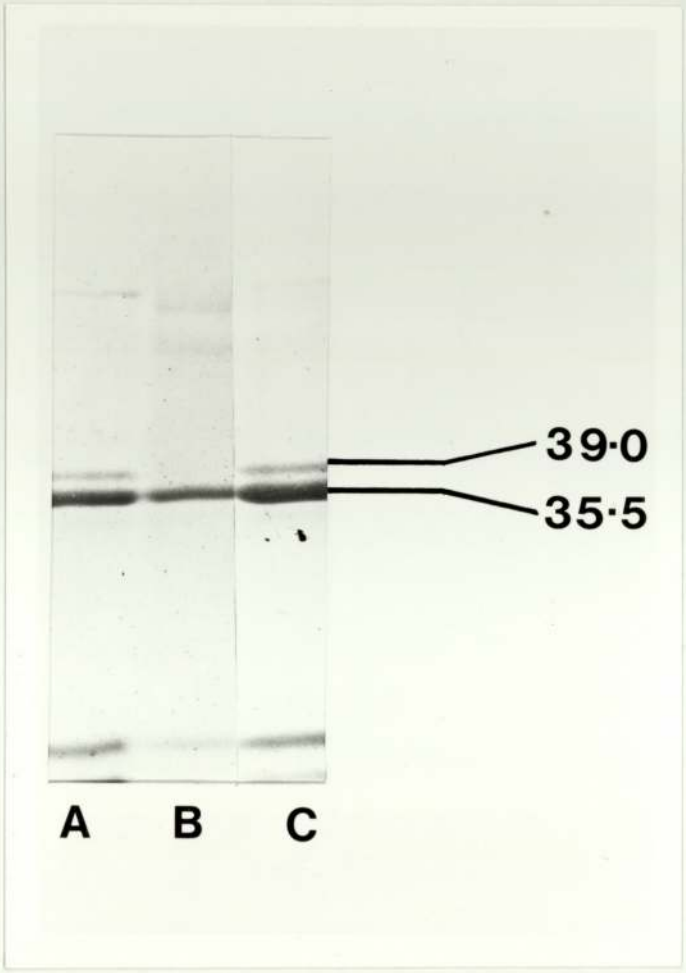
Fig. 3.5. SDS-PAGE of the outer membrane proteins of *K. pneumoniae* grown in batch and in iron limited chemostat culture.



Lane A Slow growth rate (D=0.1 h^{-1}), iron limited chemostat culture.
Lane B Fast growth rate (D=0.4 h^{-1}), iron limited chemostat culture.
Lane C Iron replete batch culture.
Lane D Iron depleted batch culture.

Apparent molecular weights are given in kDa.

Fig. 3.6. SDS-PAGE of the peptidoglycan-associated outer membrane proteins of *K. pneumoniae*.



Lane A Iron depleted batch culture.
Lane B Carbon limited low iron chemostat culture D=0.1 h^{-1}.
Lane C Carbon limited low iron chemostat culture D=0.4 h^{-1}.

Apparent molecular weights are given in kDa.

Table 3.2 Enterochelin in the supernatant of iron deprived cultures of *K. pneumoniae*.

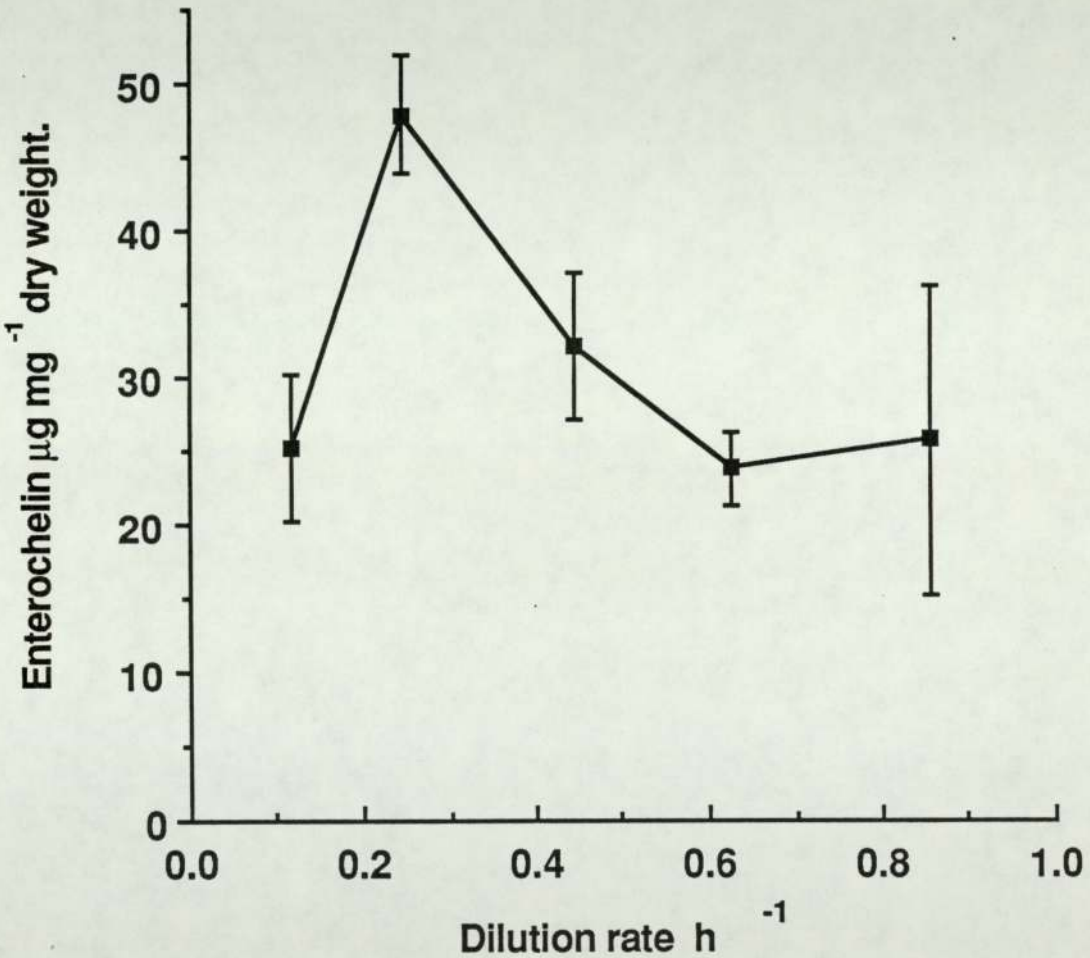
Enterochelin in supernatant, μg mg^{-1} of cell dry weight in:						
Batch culture stationary phase		Chemostat culture[1] dilution rates (D h^{-1})				
Full SSM		Fe- SSM[1] D=0.1	D=0.23	D=0.43	D=0.61	D=0.84
ND	18.19 ± 2	24.4 ± 5	47.0 ± 4	31.2 ± 5	23.0 ± 3	24.9 ± 12

1 These cultures contained 0.5 μM iron
ND None detected

compensated by an increase in the expression of the other porin (Lugtenberg and van Alphen 1983). There is however no compensatory increase in the 35.5 kDa band in the outer membrane protein profile of the cells deficient in the 39 kDa protein. There was no difference between the outer membrane protein profiles of chemostat cells grown at dilution rates of D=0.1 h^{-1} and D=0.4 h^{-1} (t_d=6.9 h and t_d=1.7 h).

Enterochelin was detected in the supernatant of *K. pneumoniae* grown in iron limited SSM both in batch and chemostat culture (Table 3.2.). At the slowest growth rate, D=0.1 h^{-1}, the enterochelin mediated iron uptake system was only partly derepressed, at faster growth rates, D=0.2 h^{-1}, the system was completely derepressed and a maximum level of enterochelin was detected. At growth rates above D=0.2 h^{-1} the amount of enterochelin detected in the supernatant fell (Table 3.2. Fig. 3.7.). The iron requirement of *K. pneumoniae* at slow growth rates may be relatively low and a basal level of enterochelin may provide sufficient

Fig. 3.7. Effect of growth rate on the amount of enterochelin detected in the supernatant of *K.pneumoniae* in iron limited chemostat cultures.

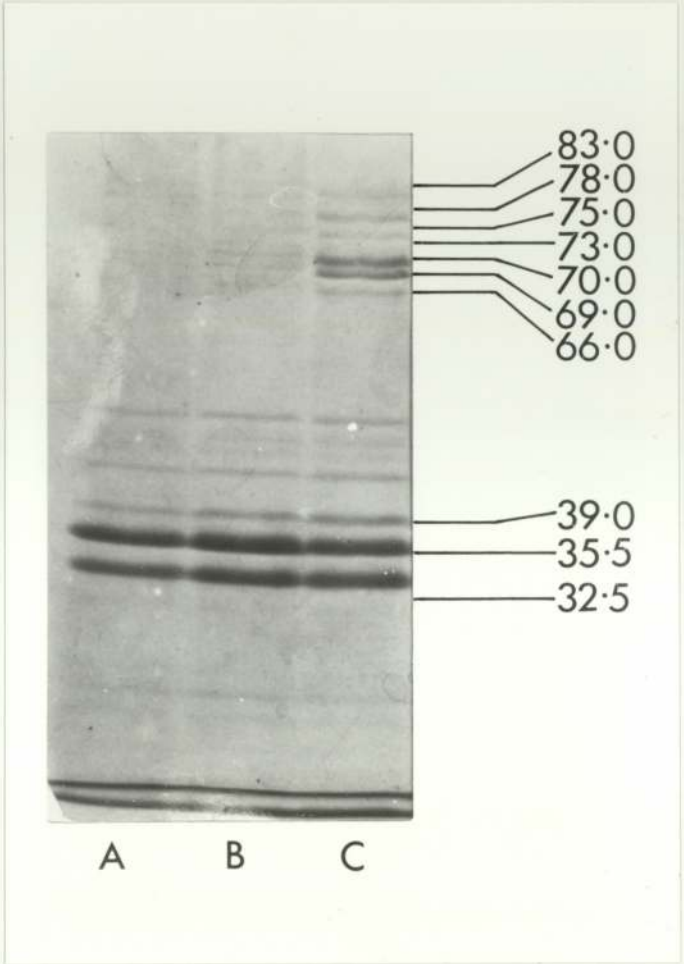


iron for the cells' requirements. At faster growth rates the greater requirement for iron was reflected in a complete derepression of the enterochelin mediated iron uptake system. The fall in the level of enterochelin as the growth rate approaches the maximum, and the organism is placed under greater metabolic stress, may indicate that the previous high level was in excess of the cells' requirements. Kadurugamuwa *et al* (1985a) showed that production of enterochelin, by *K. pneumoniae*, was reduced by half, in the presence of sub MIC concentrations of β-lactam antibiotics, without any effect on the growth rate. This supports the conclusion that *K. pneumoniae* produce enterochelin in excess of their requirements. The observed reduction in the supernatant enterochelin concentration at growth rates above D=0.2 h^{-1} may reflect an increase in the rate of uptake of the ferric enterochelin complex resulting in lower detectable levels in the supernatant.

3.5. The effect of iron concentration on enterochelin production and outer membrane protein profile of *K. pneumoniae* in the carbon limited chemostat.

The outer membrane protein profile of *K. pneumoniae* grown in carbon depleted batch culture showed the same three major outer membrane protein's seen in the full SSM (Fig. 3.8.); the IRMP's were only fully expressed when the iron level was reduced to 0.5 μM (Fig. 3.8, lane C). When grown in carbon limited chemostat culture a 49 kDa protein, which may be a doublet, was induced (Fig. 3.9.); this protein was not peptidoglycan associated (Fig. 3.6, lanes B and C). A similar protein at 48 kDa has been described (Sterkenberg *et al* 1984) which was present in comparable amounts whether the carbon source was glucose, glycerol or maltose. This carbon limitation specific protein may be involved in the uptake of carbon containing compounds, and it may be related to the MalB

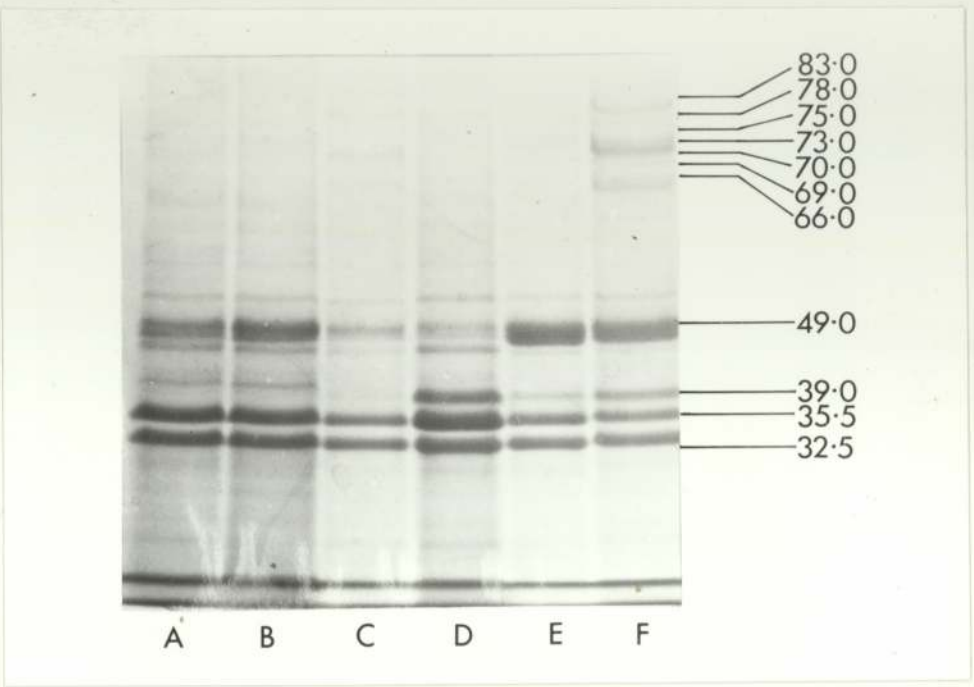
Fig. 3.8. SDS-PAGE of the outer membrane proteins of *K. pneumoniae* grown in carbon depleted batch culture with different concentrations of iron



Lane A Carbon depleted batch culture with 20 µM iron.
Lane B Carbon depleted batch culture with 1.5 µM iron.
Lane C Carbon depleted batch culture with 0.5 µM iron.

Apparent molecular weights are given in kDa.

Fig.3.9. SDS-PAGE of the outer membrane proteins of *K. pneumoniae* grown in carbon limited chemostat cultures with varying iron concentrations.



Lane A Slow growth rate (D=0.1 h^{-1}), 20 μM iron.
Lane B Slow growth rate (D=0.1 h^{-1}), 1.5 μM iron.
Lane C Slow growth rate (D-0.1 h^{-1}), 0.5 μM iron.
Lane D Fast growth rate (D=0.4 h^{-1}), 20 μM iron.
Lane E Fast growth rate (D=0.4 h^{-1}), 1.5 μM iron.
Lane F Fast growth rate (D=0.4 h^{-1}), 0.5 μM iron.

Apparent molecular weights are given in kDa.

There is some evidence that these fast growing carbon limited cells which express the 39 kDa protein are more permeable than the slow growing cells (Table 3.3). In each set of growth conditions the higher Se/So value for cells growing at D=0.4 h^{-1} indicates increased permeability.

Table 3.4 Growth parameters of *K. pneumoniae* in carbon limited chemostat culture.

Dilution Rate h^{-1}	SSM iron concentration µM	OD_{470}	Dry weight $\mu g ml^{-1}$	Total count $x10^{8} ml^{-1}$
0.1	20	0.96 ± 0.01	363 ± 53	11.18 ± 1.9
	0.5	0.87 ± 0.03	360 ± 3	12.75 ± 1.0
0.4	20	0.95 ± 0.01	382 ± 55	11.89 ± 1.6
	0.5	0.78 ± 0.01	284 ± 24	11.39 ± 0.5

The use of carbon limited chemostat cultures permitted the investigation of the effect of different iron concentrations in the medium on cells growing at different growth rates. The growth rate was controlled by glucose limitation and the iron level varied. It is not clear whether the cultures in which the iron concentration is 0.5 µM are truly glucose limited. There was a small reduction of optical density of both iron deprived cultures, a drop in dry weight only in the fast growing, iron deprived culture and no significant change in the total number of organisms per ml of culture between the iron replete and iron deprived cultures (Table 3.4.). It is important to bear these facts in mind when analysing the effect of the concentration of iron in these cultures.

Like the batch cultures the IRMP's were not induced in cultures with iron concentrations of 1.5µM and above (Fig. 3.9, lanes A,B,D and E),

but were only fully induced at a concentration of 0.5 μM in the fast growing cultures (Fig. 3.9, lanes C and F). Enterochelin was however detected in the supernatant of both iron deprived cultures; the amount produced at the slow growth rate was less than half that at the fast growth rate (Table 3.5). It is possible that the residual level of expression of the IRMP's, which is seen in all cells, is sufficient to take up enough ferric enterochelin to satisfy the cells iron requirements which are likely to be lower in slow growing than in fast growing cells (Sect. 3.4.). The production of enterochelin and it's receptor protein are generally considered to be coordinately controlled (Fleming *et al* 1983, Klebba *et al* 1982, McIntosh and Earhart 1977). In glucose limited chemostat cultures at a slow growth rate ($D=0.1\ h^{-1}$) this coordinate control does not appear to operate.

Table 3.5 Influence of medium iron concentration on enterochelin production by *K. pneumoniae* in carbon limited chemostat culture.

SSM iron concentration μM	Enterochelin in supernatant μg mg^{-1} of cell dry weight in:		
	Batch culture stationary phase	Chemostat culture	
		Slow $D=0.1\ h^{-1}$	Fast $D=0.4\ h^{-1}$
20	ND	ND	ND
0.5	14.1 ± 6.5	11.8 ± 2.1	29.6 ± 0.9

ND Not detectable

3.6. Conclusion.

It is possible that *K. pneumoniae* adopts a slow growing relatively metabolically inactive form as a survival strategy *in vivo*. In this state the cells' requirement for iron would not be great; this is reflected by the data in the iron limited chemostat by the low level of enterochelin production by slow growing cells in both iron and carbon limited chemostat cultures. This is emphasised further in the carbon limited cultures by the failure to induce the IRMP's, sufficient iron for the cells' requirements being acquired through the residual IRMP's expressed in the outer membrane at all times. At faster growth rates the uptake of iron, which is vital for many metabolic processes, becomes much more important. This is reflected by an increase in the amount of enterochelin produced in both iron and carbon limited chemostat cultures to a level which may be in excess of the cells' requirements and by derepression of the IRMP's in the carbon limited chemostat. It is not possible to increase further the production of enterochelin as the cells' need for iron increases still further but it may be possible to increase the efficiency of the ferric enterochelin uptake system. If uptake of ferric enterochelin is controlled by the cytoplasmic iron concentration, cells with a high metabolic activity would use up the iron pool resulting in increased iron uptake.

It appears that the presence or absence of porin proteins may contribute to changes in permeability of the outer membrane associated with different growth conditions but that it is not the only factor in control of permeability, in *P. aeruginosa* for instance many of the pores appear to be closed (Angus *et al* 1982, Nicas and Hancock 1983).

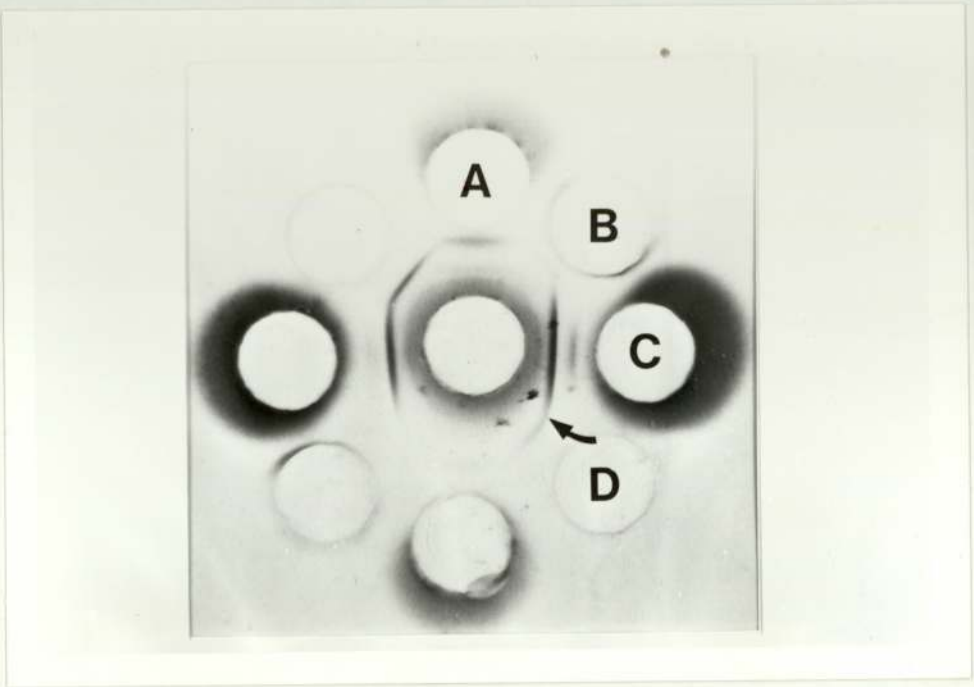
4. Analysis of the extracellular polysaccharides of *K. pneumoniae*

4.1. Measurement of capsular polysaccharide

Antiserum raised in rabbits against whole cells of *K. pneumoniae* DL1 was tested using double diffusion immunoprecipitation in agarose gels against whole cell sonicates of DL1 and 5055 (a strain which has the same lipopolysaccharide serotype but a different capsular polysaccharide type). Samples of extracted lipopolysaccharide and capsular polysaccharide from DL1 were included to help identify the precipitin bands (Fig. 4.1.). One precipitin band was formed between each of the wells containing the extracted polysaccharides (B and D) and the well containing the antiserum indicating the relative purity of the extracts. Two antigens were recognised by the serum in whole cell sonicates of DL1 (C), the bolder of these precipitin bands fuses with the band produced in response to capsular polysaccharide indicating that they are the same antigen. Lipopolysaccharide is much more weakly detected by the antiserum and the pattern of fusion is not clear, it seems likely that the other band detected in DL1 and the outer band detected in 5055 are lipopolysaccharide. There is a weak reaction between the anti DL1 antiserum and another antigen expressed by 5055. This could be a partial cross reaction with the type K2 capsule or another common antigen. The major antigen detected by anti DL1 antiserum raised in both rats (not shown) and in rabbits is the capsular polysaccharide.

The same antiserum used in crossed immunoelectrophoresis detected only one major antigen in whole cell sonicates of DL1 (Fig. 4.2, A). This antigen was identified by including an intermediate gel containing extracted capsular polysaccharide (Fig. 4.2, B). The horizontal precipitin band formed as a result of the reaction of the antiserum with

Fig. 4.1. Double diffusion immunoprecipitation showing antigens detected by rabbit anti DL1 antiserum.



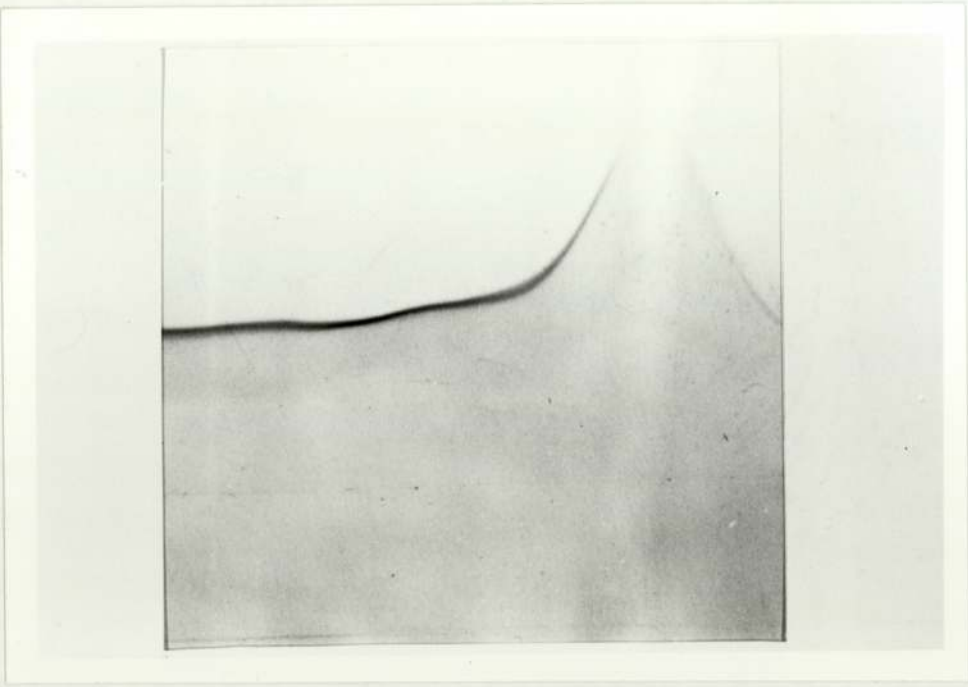
Central well contains rabbit anti DL1 antiserum.
Well A contains whole cell sonicate of *K. pneumoniae* 5055.
Well B contains extracted lipopolysaccharide.
Well C contains whole cell sonicate of *K. pneumoniae* DL1.
Well D contains extracted capsular polysaccharide.

The arrow indicates the fusion between the precipitant bands formed in response to capsule and whole cells of DL1.

Fig. 4.2. Crossed immunoelectrophoresis of sonicated whole cells of DL1 into rabbit anti DL1 antiserum.

Fig. 4.2.A. Intermediate gel contains 1% agarose.

Fig. 4.2.B. Intermediate gel contains 1 mg ml^{-1} capsular polysaccharide in 1% agarose.



The single peak in Fig. 4.2.A. fuses with and is lifted by the horizontal band produced by the capsular polysaccharide in the intermediate gel.

the capsular polysaccharide in the intermediate gel fused with and lifted the peak detected in the whole cell sample. This indicated once again that the antiserum detected capsular polysaccharide as the primary antigen and could be used in rocket immunoelectrophoresis to quantify the capsular polysaccharide.

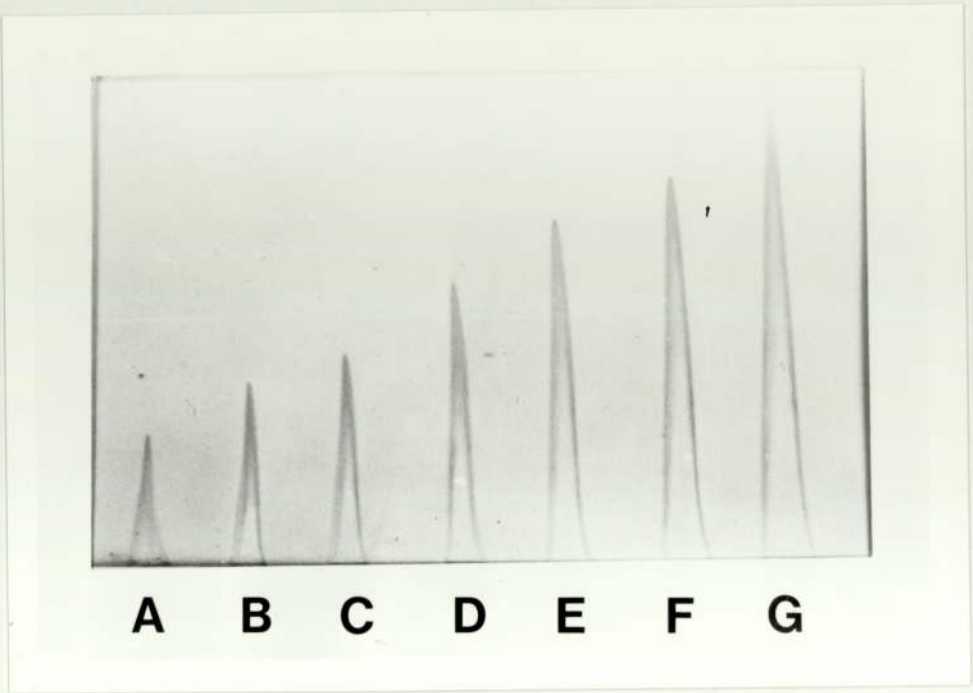
A calibration curve was constructed using a range of loadings of extracted capsular polysaccharide for each gel (Fig. 4.3.). These always resulted in an acceptable straight line but varied from one experiment to the other as a result of differences such as the thickness of the gel and the running conditions.

4.2. PAGE analysis of lipopolysaccharide

It proved to be difficult to separate the lipopolysaccharide of DL1 to show a clear ladder pattern. Many variations of the Lugtenberg system (1975) were tried with different percentages of acrylamide with and without urea and both proteinase K digestion (Hitchcock and Brown 1983) and lipopolysaccharide extraction by the micromethod of Inzana (1983) were used to prepare samples. The Laemmli gel system (1970) as used by Hitchcock and Brown (1983) gave the best separation of the high molecular weight lipopolysaccharide. This system uses a higher acrylamide to bis ratio than the Lugtenberg system which results in a smaller pore size in the gel; this may enhance separation.

The basis by which silver stain detects lipopolysaccharide is not well understood. It has been suggested that the periodate oxidising reagent reacts with cis-glycols in the polysaccharide side chains (Kropinski *et al* 1985). The lipopolysaccharide from DL1 is of the O1 serotype which consists of 1,3, galactose. The only cis glycol group on galactose is

Fig. 4.3. Rocket immunoelectrophoresis of a range of loadings of capsular polysaccharide into agarose gel containing anti DL1 antiserum.



Well A contains 0.5 µg extracted capsular polysaccharide.
Well B contains 1.0 µg extracted capsular polysaccharide.
Well C contains 1.5 µg extracted capsular polysaccharide.
Well D contains 2.0 µg extracted capsular polysaccharide.
Well E contains 3.0 µg extracted capsular polysaccharide.
Well F contains 4.0 µg extracted capsular polysaccharide.

There is a linear relationship between the amount of capsular polysaccharide loaded and the peak height.

lost when the monosaccharides are linked through the 1 and 3 positions. Kropinski *et al* (1986) postulate that it is the lipid A portion of the lipopolysaccharide molecule which is important in the silver stain reaction. It is possible that the lipid A moiety is important in the reaction but that, particularly in the high molecular weight lipopolysaccharide, the polysaccharide side chains may also contribute to the overall effect. In the case of DL1 the 1,3 galactose side chains will not enhance staining and could cause steric interference with the oxidation process, making it difficult to stain the high molecular weight lipopolysaccharide.

Care should be taken when interpreting silver stained lipopolysaccharide gels in the light of the variation which can result from small alterations in concentration and loading of the sample and in the precise details of the silver stain method.

4.3. The effect of growth rate on the capsular polysaccharides of *K. pneumoniae* in iron limited chemostat culture.

Samples from the overflow from the chemostats were collected into ice; the bacteria were pelleted by spinning at 20,000 rpm for 30 min. The polysaccharide detected in the supernatant was designated as cell free polysaccharide and the polysaccharide detected after sonic disruption of the pellet as cell associated polysaccharide. The amount of cell associated polysaccharide detected was greatest in slow growing ($D=0.1\ h^{-1}$) *K. pneumoniae*; only a quarter of this amount was detected at the fastest growth rate ($D=0.61\ h^{-1}$) (Fig. 4.4). The amount of cell free polysaccharide detected in the supernatant also decreased in relation to increased growth rate (Fig. 4.5). The two types of polysaccharide are manifestations of the same phenomenon (Domenico *et al* 1985). The cell

Fig 4.4. Cell associated capsular polysaccharide produced by *K.pneumoniae* at different growth rates in iron limited chemostat cultures.

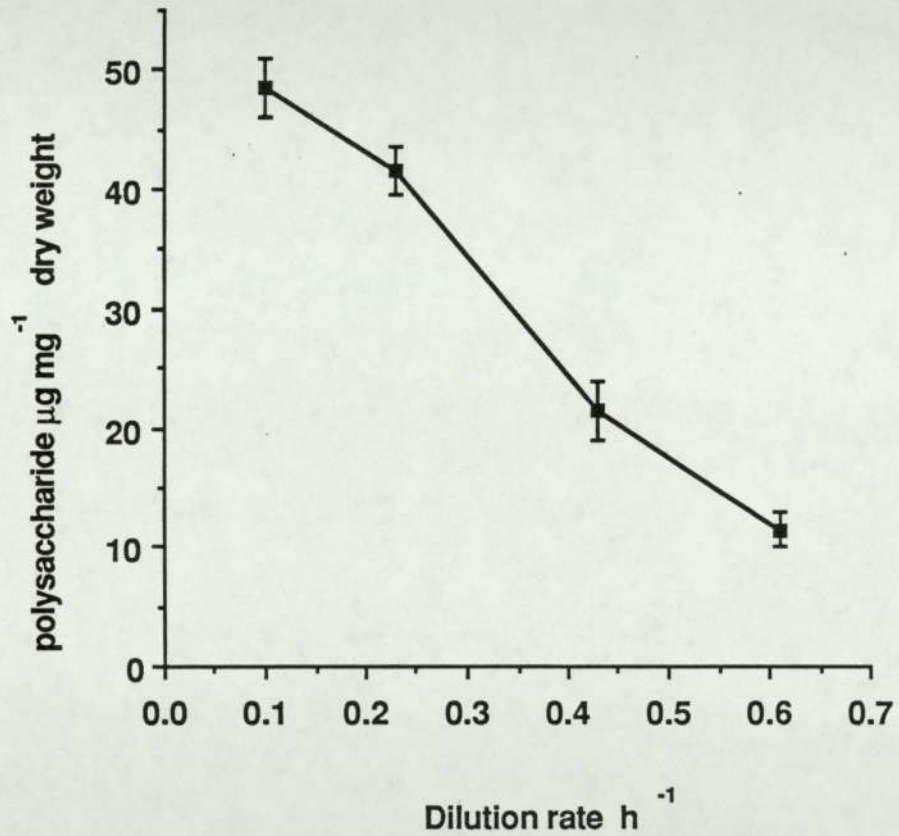
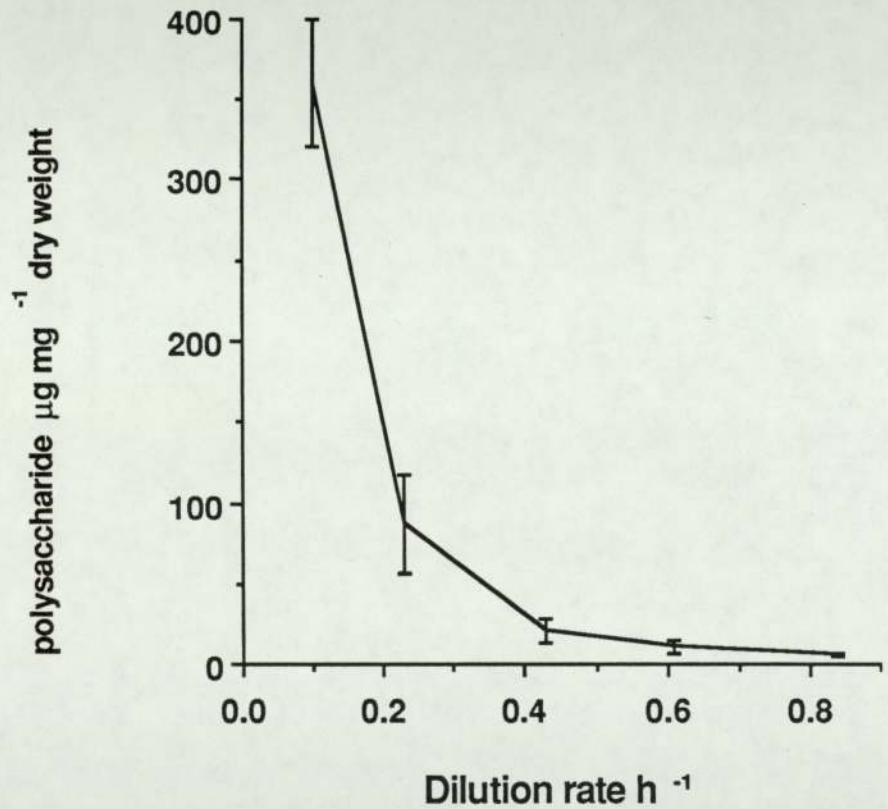


Fig 4.5. Cell free capsular polysaccharid produced by cultures of *K. pneumoniae* at different growth rates in iron limited chemostat culture.



associated polysaccharide comprises the capsule and the cell free polysaccharide being molecules of the same polysaccharide which are either not anchored or which lose their anchorage in the envelope (Sect. 1.4.6). It would be reasonable to expect that these two forms of capsular polysaccharide would show the same trend in relation to growth rate.

The total amount of capsular polysaccharide detected at the slowest growth rate amounts to 40% of the dry weight of the cell. This figure may be an over estimate if the extracted capsular polysaccharide used as a standard contains non antigenic contaminants, never-the-less it reflects the importance of the capsular polysaccharide which makes up a large part of the cells' output. The high levels of polysaccharide may be a response of slow growing cells to the *in vivo* environment. Capsular polysaccharide plays an important role in resistance to host defence mechanisms (Kim *et al* 1986, Williams *et al* 1983) which would be of particular importance to cells adapted for persistence *in vivo*.

The surprisingly large quantities of cell free polysaccharide produced (seven times more than the amount of cell associated polysaccharide at $D=0.1\ h^{-1}$) suggests that it serves a function and is not simply the result of deterioration of the capsule. Capsular polysaccharide is detected in the blood of animals infected with *K. pneumoniae* (Pollak 1976); it may play a role in "mopping up" opsonic antibodies preventing the opsonisation of the encapsulated bacteria. Anti capsular antibodies are the major component of the immunological response to *Klebsiella* (Sect. 4.1., Fukutome *et al* 1980) and these antibodies are protective (Cryz *et al* 1986b, Robert *et al* 1986) so any interference with their effectiveness would enhance survival of the bacteria. It has also been

suggested that cell free polysaccharide has a variety of negative effects on maturation and function of phagocytic cells (Kato *et al* 1976, Yokochi *et al* 1977) which may be of importance. Bacteria with a faster growth rate would be able to persist despite the host's defence mechanisms; production of polysaccharide would be of less importance.

The production of the three types of exopolysaccharide: peptidoglycan, lipopolysaccharide and capsular polysaccharide are dependent on the presence of the same carrier lipid (Norval and Sutherland 1969). Sutherland (1977) proposes a system of priorities for the polymer synthesis with peptidoglycan being the first priority, followed by lipopolysaccharide and finally capsular polysaccharide. It is possible that at slow growth rates there is more isoprenoid lipid available allowing synthesis of greater amounts of capsular polysaccharide. Previous studies comparing the relative amounts of extracellular and cell associated polysaccharide have found that the ratio of one to the other remains constant (Domenico *et al* 1985, Ehrenworth and Baer 1956); these studies used batch cultures in complex media. Duguid and Wilkinson (1953) however showed that the proportion of total polysaccharide which was cell associated in cultures grown at 35°C was only half that at 15°C. It may be true that a strain of *Klebsiella* growing in a defined set of growth conditions always produces the same proportions of cell free to cell associated polysaccharide but this ratio may be varied under a different conditions and is not necessarily representative of the *in vivo* case.

4.4. The effect of growth rate on the lipopolysaccharide of *K. pneumoniae*

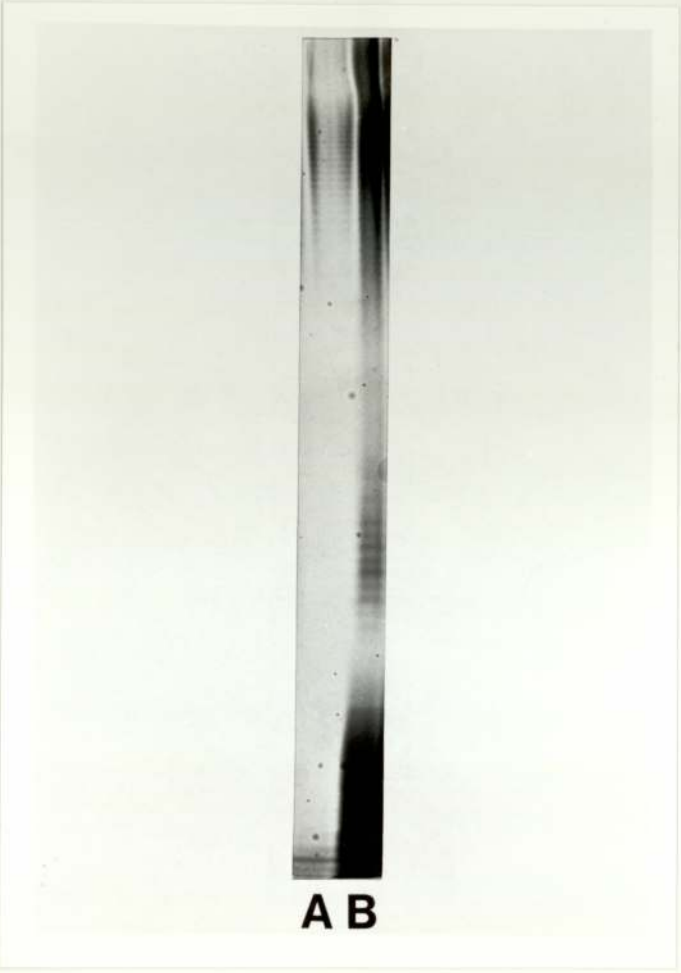
The lipopolysaccharide ladder pattern of *K. pneumoniae* showed a large amount of rough lipopolysaccharide and fine banding in the high molecular weight range. The ladder pattern is in fact continuous between these two regions as demonstrated by overloading the gel (Fig. 4.6.). This pattern suggests that there is a higher proportion of the very high molecular weight lipopolysaccharide than of the medium chain length in the outer membrane.

There is very little difference in the lipopolysaccharide ladder patterns of cells grown at different rates in the iron limited chemostat (Fig. 4.7). There is relatively less rough lipopolysaccharide in cells grown at D=0.1 h^{-1} than at higher growth rates which may indicate that there are fewer lipid A molecules without side chains at this growth rate. The polysaccharide side chains of lipopolysaccharide may play a similarly protective role to that of the capsular polysaccharide. The lipopolysaccharide and in particular the O antigen side chain has been shown to play an important role in resistance of *K. pneumoniae* to the action of complement (Tomás *et al* 1986, Williams *et al* 1983) by preventing access of complement components to their targets. It would be important then at slow growth rates that as great a proportion as possible of the lipid A molecules possessed protective O polysaccharide side chains.

4.5. Conclusion

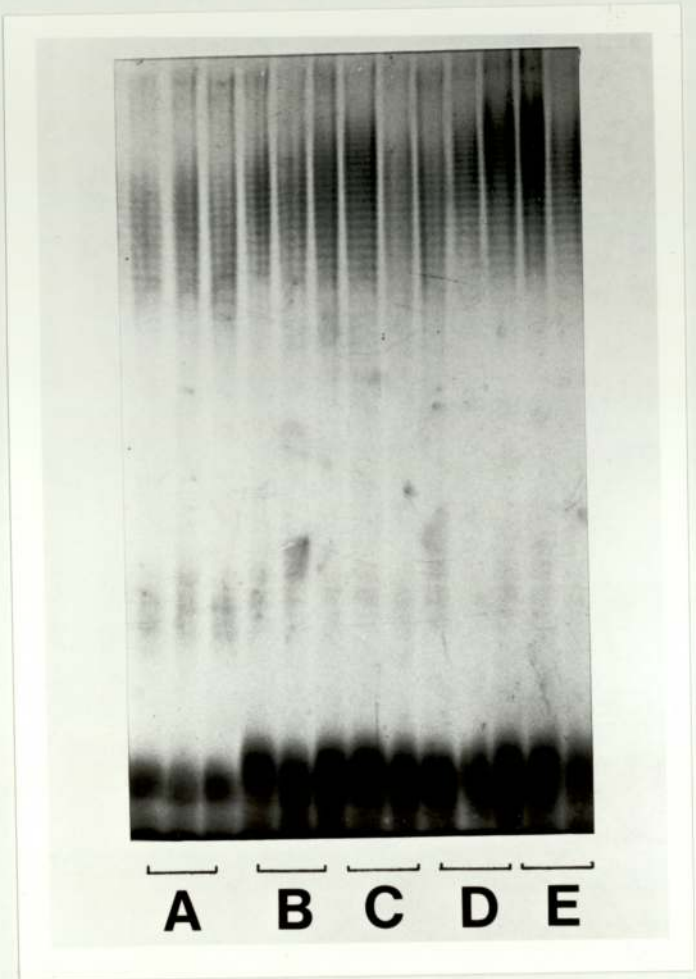
Bacteria adopt many different strategies for survival in the host environment. With respect to growth rate there are two possible strategies; grow slowly and divert resources to expensive survival

Fig.4.6. PAGE of lipopolysaccharide of *K. pneumoniae* grown in batch culture.



Lane A lipopolysaccharide extracted from 50 µl OD_{470} 40.
Lane B lipopolysaccharide extracted from 200 µl OD_{470} 40.

Fig 4.7. PAGE of lipopolysaccharide profile of *K. pneumoniae* at different growth rates in iron limited chemostat cultures.



Lanes A D=0.1 h^{-1}.
Lanes B D=0.23 h^{-1}
Lanes C D=0.43 h^{-1}
Lanes D D=0.61 h^{-1}
Lanes E D=0.84 h^{-1}

mechanisms or grow fast enough to out-strip the host's attempts at eradication. Cells adopting the latter course will divert resources into areas which are essential for growth and multiplication and neglect factors which are important only in cells growing slowly enough to be cleared by the hosts defence mechanisms. The fast growing chemostat cells conform to this strategy, producing only the minimum amount of capsular polysaccharide, the lipopolysaccharide on the other hand which occupies a higher position in the cells priorities remains relatively unchanged. Slow growing cells on the other hand must be protected from the host defence mechanisms if they are to survive. A thick polysaccharide capsule and particularly growth as a biofilm have been shown to mask surface antigens, protect from the effects of serum and phagocytes and to hinder the penetration of antibiotics. It also seems likely that extracellular polysaccharide is important in interactions with the immune system and that the O polysaccharide side chain of the lipopolysaccharide is important in protection against complement activity. These strategies are all characteristic of the slow growing chemostat cells.

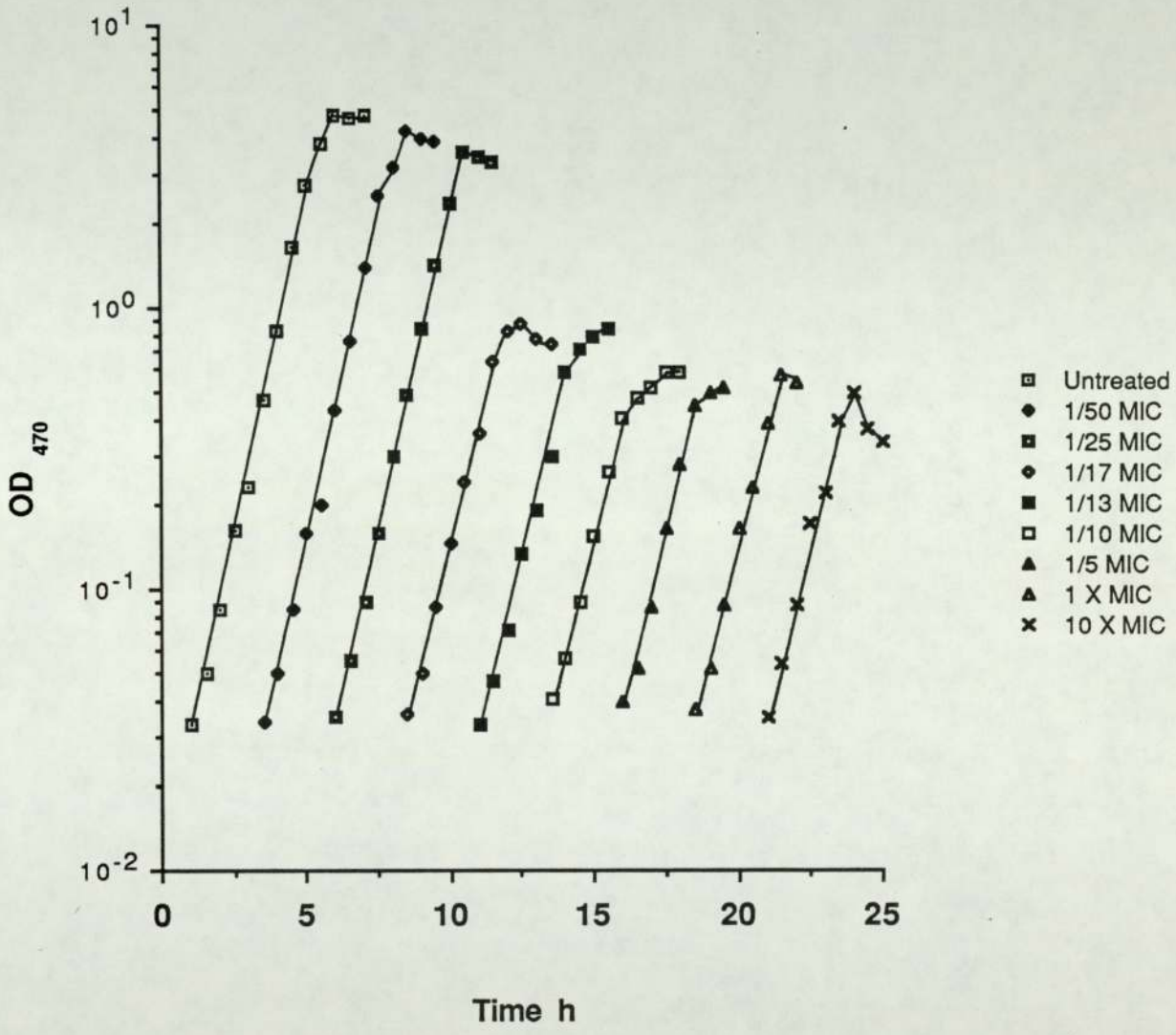
mechanisms or grow fast enough to out-strip the host's attempts at eradication. Cells adopting the latter course will divert resources into areas which are essential for growth and multiplication and neglect factors which are important only in cells growing slowly enough to be cleared by the hosts defence mechanisms. The fast growing chemostat cells conform to this strategy, producing only the minimum amount of capsular polysaccharide, the lipopolysaccharide on the other hand which occupies a higher position in the cells priorities remains relatively unchanged. Slow growing cells on the other hand must be protected from the host defence mechanisms if they are to survive. A thick polysaccharide capsule and particularly growth as a biofilm have been shown to mask surface antigens, protect from the effects of serum and phagocytes and to hinder the penetration of antibiotics. It also seems likely that extracellular polysaccharide is important in interactions with the immune system and that the O polysaccharide side chain of the lipopolysaccharide is important in protection against complement activity. These strategies are all characteristic of the slow growing chemostat cells.

5. The effect of sub-MIC's of two cephalosporin antibiotics on iron limited chemostat cultures of *K. pneumoniae*.

5.1. Preliminary work in batch culture.

Two β-lactam antibiotics were chosen for this study which had shown interesting effects on *K. pneumoniae* in previous studies in thislaboratory (Kadurugamuwa *et al* 1985a,b); these were Ceftriaxone (Roche) and Cefotaxime (Roussel). The MIC for both Ceftriaxone and Cefotaxime in SSM was found to be 0.25 μg ml^{-1}, the iron content of the media did not affect this value. The effect of a range of concentrations of each of the drugs on the growth kinetics of *K. pneumoniae* in batch culture was examined; the effects of the two antibiotics was very similar. Fig. 5.1 shows the growth curves for Ceftriaxone. There was no effect of the antibiotic on the growth rate in batch culture, the mean t_d for Fig. 5.1 was 1 doubling every 42 min ± 5%. A range of concentrations of Ceftriaxone from 1/10 to 10X the MIC caused early onset of stationary phase and clumping of the bacteria. Concentrations of between 1/10 and 1/25 MIC allowed growth of up to one more doubling before onset of stationary phase and less clumping was observed. Concentrations of 1/25 and 1/50 MIC had little effect on the growth curves, which were barely distinguishable from the growth curves for untreated calls. Samples of the culture were taken from 180 min after the start of the experiment and preserved in 4% formaldehyde, these were examined for morphological changes. Double cells were seen in all the samples examined from antibiotic treated cultures; later in the growth curves elongated forms of about four or more cell lengths were seen especially at the higher concentrations (estimations of cell length were made relative to a single log phase cell). The sub-MIC concentrations, defined as a concentration not effecting the growth kinetics but causing

Fig 5.1 The effect of Ceftriaxone on the growth kinetics of *K.pneumoniae.*



The curves are superimposable, they have been offset for clarity.

morphological changes, were taken to be 1/50 and 1/25 MIC for both Ceftriaxone and Cefotaxime in batch culture.

5.2. Introduction to chemostat experiments.

Effects of sub-MIC's of β-lactam antibiotics on the production of enterochelin and on the formation of a capsule in batch cultures of *K. pneumoniae* have previously been reported (Kadurugamuwa *et al* 1985a). It was decided to examine these effects in the more closely defined conditions of the chemostat and to examine how they were influenced by growth rate. The effects of sub-MIC's of β-lactam antibiotics in batch culture are seen early in the growth curve so it was decided to examine chemostat grown cells over the first seven generations after addition of the antibiotic. Iron limited chemostat cultures were chosen as iron limitation is one of the important factors *in vivo*. Chemostats were allowed to stabilise at the required growth rate for 5 volume changes; antibiotic was added to the culture vessel and to the media pot to give a concentration of 1/5 or 1/50 MIC and the chemostats run for a further 5 volume changes with frequent sampling into ice from the overflow.

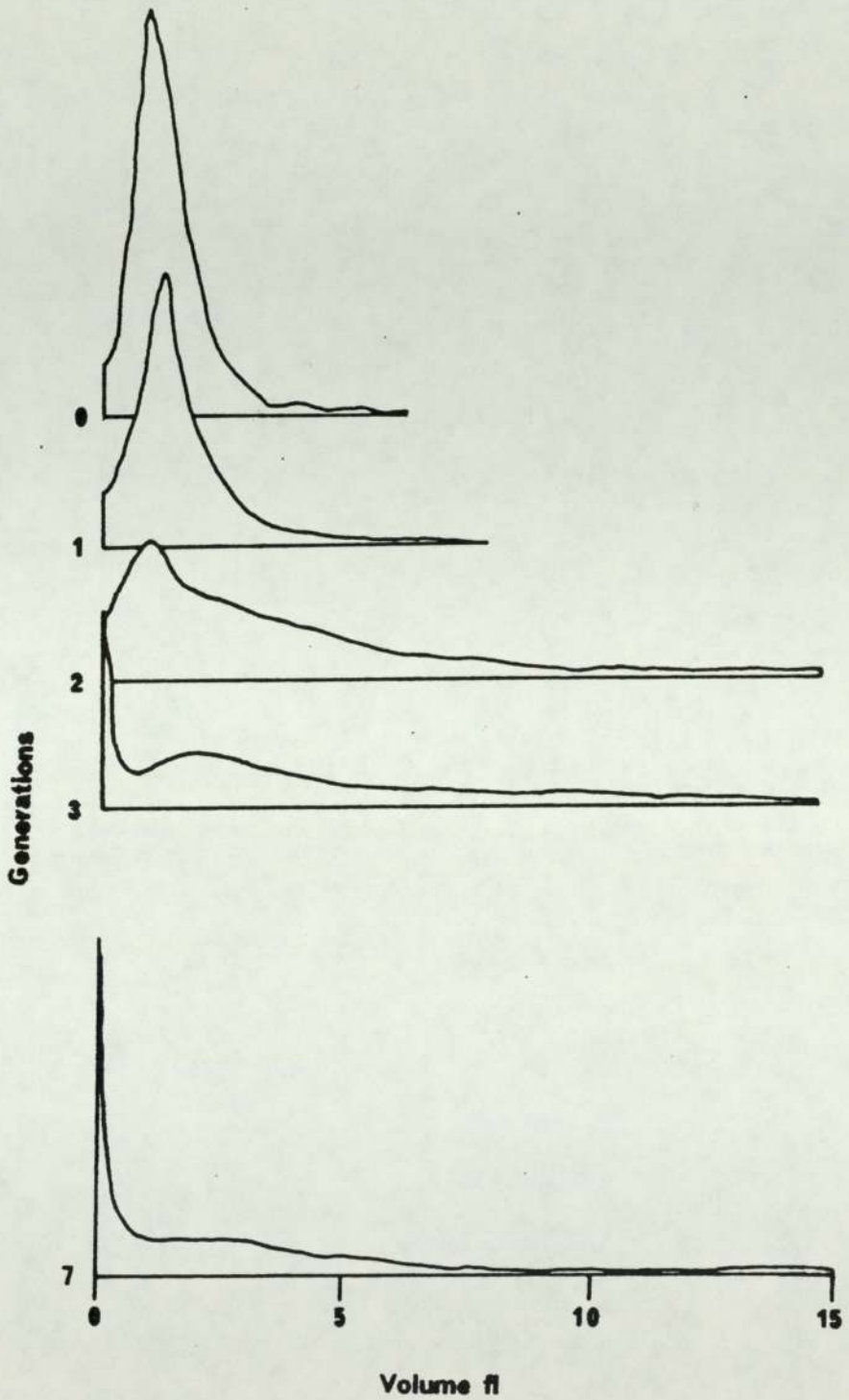
All points on the graphs represent an average value for a sample collected over 0.4 of a volume change (0.58 generations). It was not possible to measure the dry weight of the cells at each time point from a 20 ml sample; the estimation of cell associated polysaccharide was performed on a weighed sample of freeze dried cells to give the figure in terms of dry weight. The enterochelin level is expressed in terms of ml^{-1} of OD_{470} 1.0. This is somewhat unsatisfactory as the optical density of the culture will fall as the cell size increases in response to the β-lactam antibiotic. It has been shown that the effect of MIC

concentrations of β-lactam antibiotics on Gram negative bacteria in chemostat culture is directly proportional to generation time (Cozens *et al* 1986b, Tuomanen *et al* 1986). The data in this study is expressed in terms of generation time making it possible to compare the effects at different growth rates.

5.3. Morphological effects of sub-MIC's of β-lactam antibiotics on *K. pneumoniae* in iron limited chemostat culture.

The distribution of cell sizes for *K. pneumoniae* grown under iron limitation in chemostat culture is very narrow over the range of growth rates stud ied, with the vast majority of cells at a given growth rate being of similar size. Fig. 5.2. time 0 shows the cell size distribution for *K. pneumoniae* in iron limited chemostat culture at $D=0.2\ h^{-1}$. Fig. 5.3; an indian ink stain of the same cells shows their relatively uniform morphology. Addition to the culture of 1/5 MIC of Cefotaxime resulted in the appearance of elongated cell forms. The cell size distribution curves in Fig. 5.2 became broader and flatter indicating a broader range of cell sizes. The peak, indicating the size of the majority of the cells shifted to the right and many more very elongated cells were detected (Fig. 5.4.). After three generations a large number of particles of less than one cell in length were detected. These were small rounded cells with a complete capsule (Fig. 5.5) but it is not clear wether they are functioning cells with a full genetic and metabolic complement. The smearing process in the preparation of indian ink stained slides effectively separated the bacteria into different size categories; Figs. 5.4 and 5.5. are fields from the same slide. No judgement about the proportion of cells of different sizes can be made from these preparations. The data for the morphological effects of Cefotaxime on chemostat cultures with a dilution rate of $D=0.2\ h^{-1}$ was

Fig. 5.2 The effect of 1/5 MIC of Ceftriaxone on the volume of *K. pneumoniae* grown in iron limited chemostat culture at D=0.1 h^{-1}.



The curves represent the size distributions obtained from the Coulter Counter at various time intervals after the start of the antibiotic treatment. For each curve the horizontal axis represents the volume of the bacteria and the verticle axis the number of bacteria of a given size.

Fig 5.3 Indian ink stain of *K. pneumoniae* grown in iron limited chemostat culture at D=0.2 h^{-1}. The capsule can be seen as a bright area around the cells.

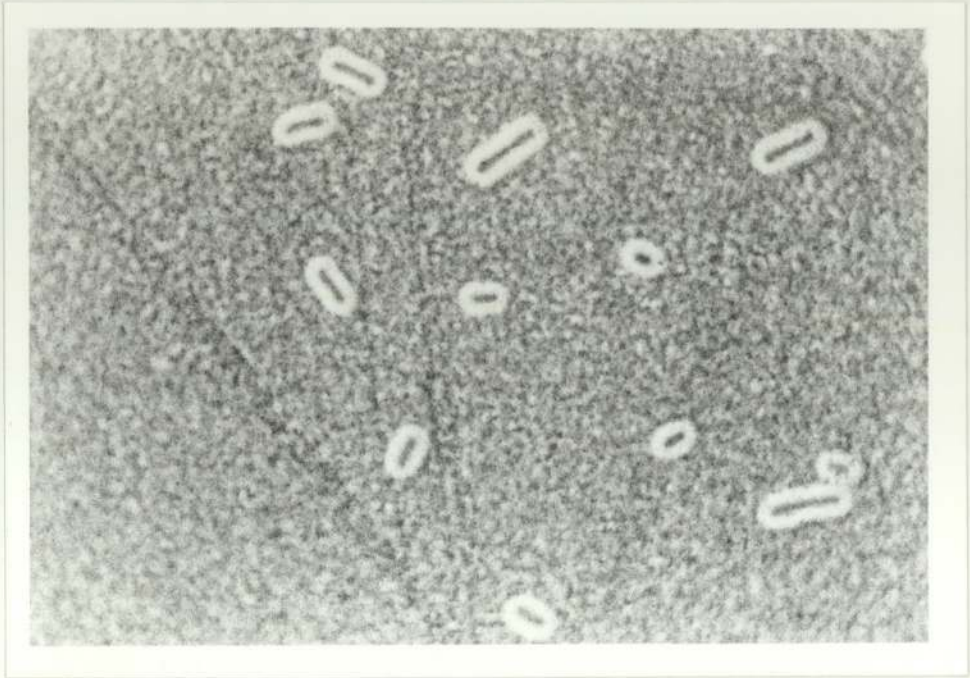


Fig 5.4 Indian ink stain of *K. pneumoniae* grown in iron limited chemostat culture at $D=0.2\ h^{-1}$ in the presence of 1/5 MIC of Ceftriaxone. The photograph shows a single, greatly elongated cell.

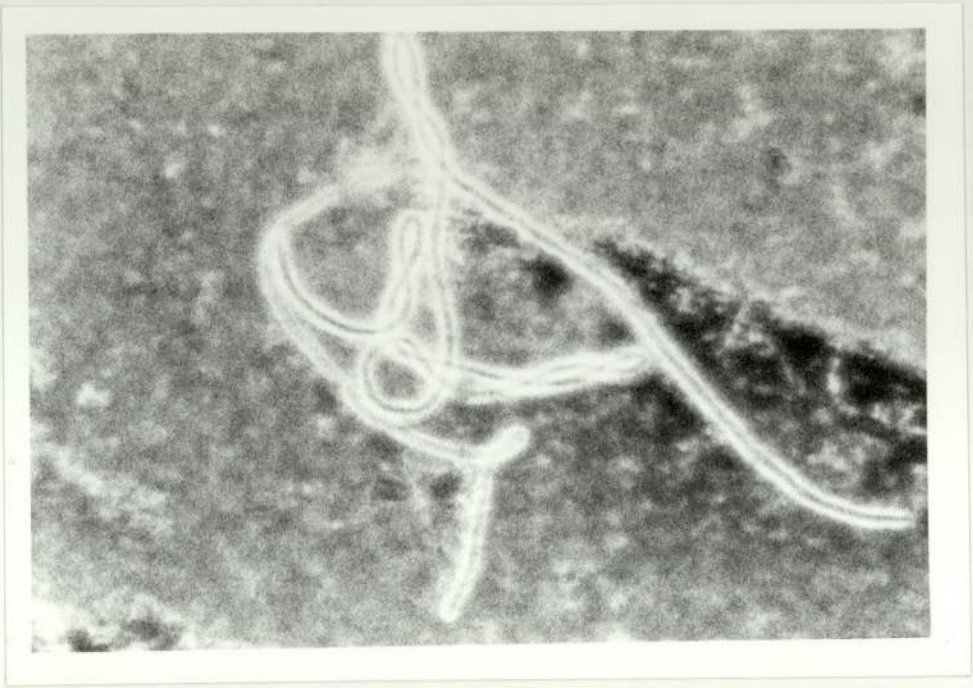
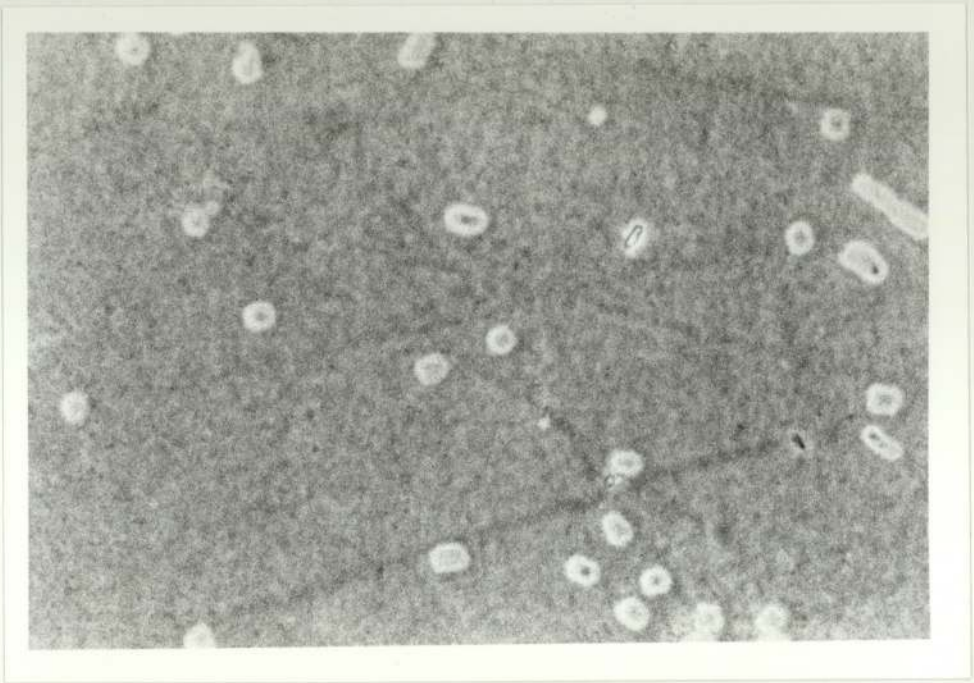


Fig 5.5 Indian ink stain of *K. pneumoniae* grown in iron limited chemostat culture at D=0.2 h^{-1} in the presence of 1/5 MIC of Ceftriaxone. The photograph shows many cells smaller than those seen in the untreated culture (Fig 5.3.).



selected as being representative of the effects of both antibiotics on organisms grown at dilution rates from D=0.1 h^{-1} to D=0.4 h^{-1}. There was no difference in the morphological response of *K. pneumoniae* to sub-MIC's of these β-lactam antibiotics at different growth rates related to generation times; in terms of absolute time the response would be twice as quick for every doubling of growth rate.

Treatment of the chemostat cultures with 1/50 MIC of Ceftriaxone and Cefotaxime had very little effect on the morphology of the bacteria. The bacteria were examined microscopically using phase contrast and indian ink staining; elongated forms representing four and eight cell lengths were observed but not in sufficient numbers to show up on the size distribution curves. These effects are in contrast to batch culture where there is a distinct morphological response to 1/50 MIC.

5.4. The effect of sub-MIC's of β-lactam antibiotics on enterochelin production by iron limited chemostat cultures of *K. pneumoniae*.

There was no major effect on the amount of enterochelin detected in the supernatant of iron limited chemostat cultures of *K. pneumoniae* treated with 1/50 MIC of either Ceftriaxone (Fig. 5.6.A) or Cefotaxime (Fig. 5.6.B) The fluctuations in the level detected may have been a result of the unstabilising effect of the antibiotics on the steady state in the chemostat (Dean and Rogers 1967). 1/5 MIC of both antibiotics did effect the amount of enterochelin detected Fig. 5.7.

Fig. 5.7.A shows the effect of 1/5 MIC of Ceftriaxone. There was little effect on the slowest growing culture (D=0.1 h^{-1}). At this growth rate there is only a basal level of enterochelin production and the acquisition of iron may be relatively unimportant among the cells'

Fig.5.6.A. The effect of 1/50 MIC of Ceftriaxone on the amount of enterochelin in the supernatant of iron limited chemostat cultures of *K.pneumoniae.*

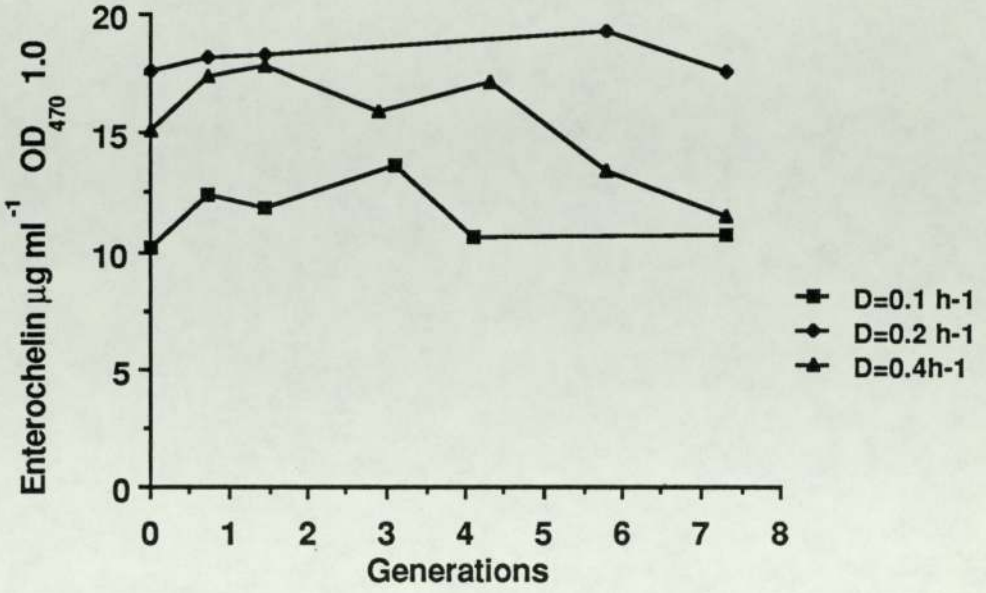


Fig 5.6.B The effect of 1/50 MIC of Cefotaxime on the amount of enterochelin in the supernatant of iron limited chemostat cultures of *K.pneumoniae.*

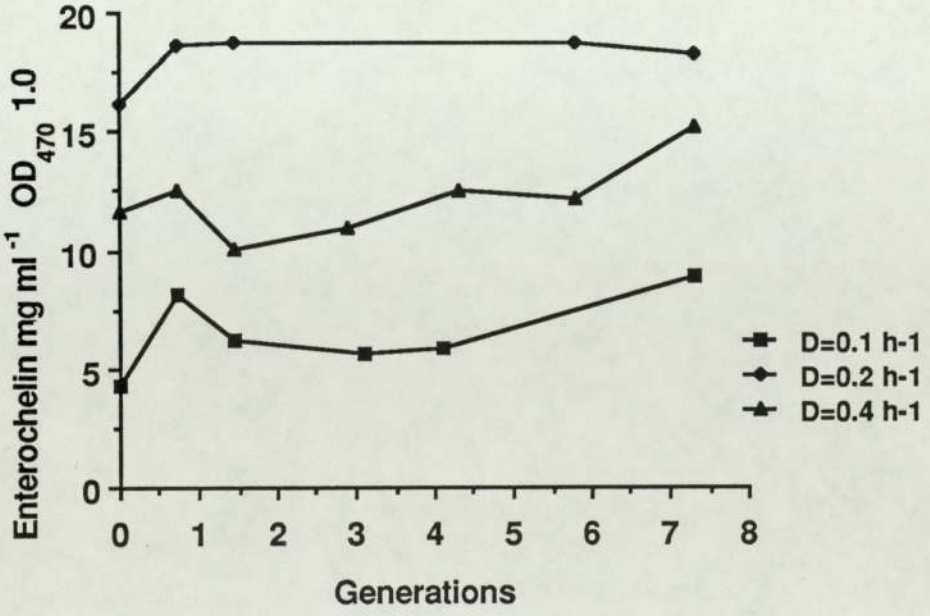


Fig. 5.7.A. The effect of 1/5 MIC of Ceftriaxone on the amount of enterochelin in the supernatant of iron limited chemostat cultures of *K. pneumoniae.*

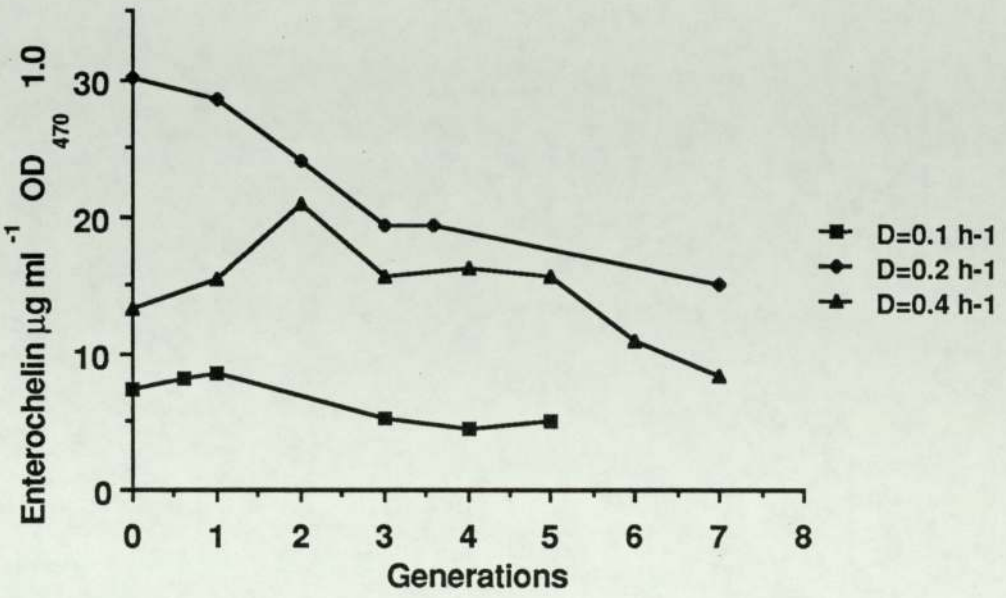
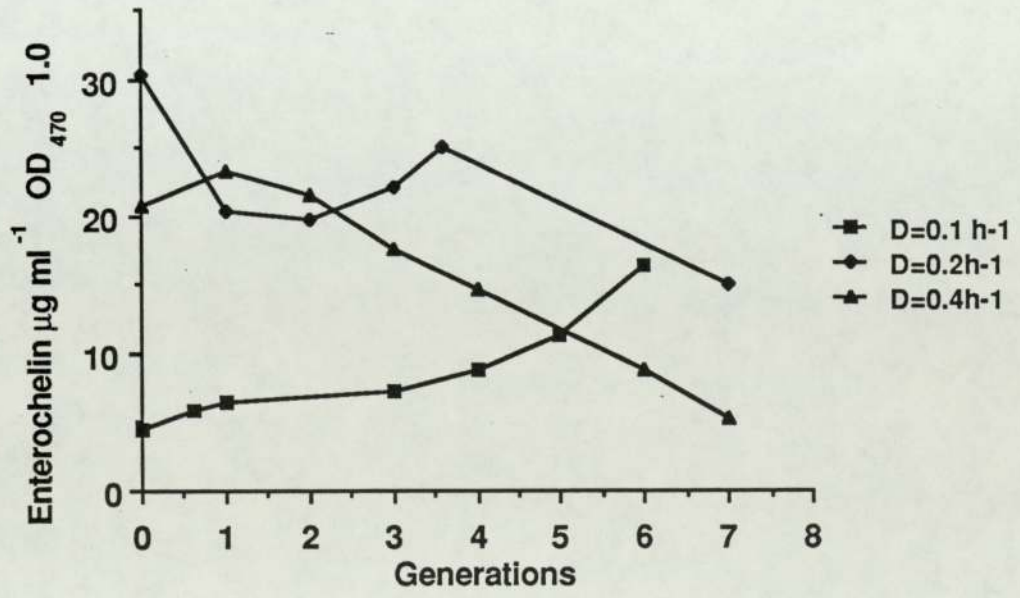


Fig 5.7.B. The effect of 1/5 MIC of Cefotaxime on the amount of enterochelin in the supernatant of iron limited chemostat cultures of *K. pneumoniae.*



metabolic priorities (Sect. 3.). At D=0.2 h^{-1} enterochelin production is maximal in untreated cells, this level fell rapidly on addition of 1/5 MIC of Ceftriaxone. The lower level of enterochelin detected in the supernatant of untreated cultures with a dilution rate of D=0.4 h^{-1} is a result of greater efficiency of uptake of enterochelin rather than decreased production (Sect. 3.4). Treatment with Ceftriaxone caused an initial increase in the detected level of enterochelin followed by a decrease. This could indicate that there was an effect of the antibiotic at this growth rate on the efficiency of uptake of the ferric enterochelin complex and that the effect on enterochelin production was seen only later on.

The effect of Cefotaxime was less clear cut but followed the same general trends (Fig. 5.7.B). There was an increase in the amount of enterochelin detected in the supernatant at D=0.1 h^{-1} which may indicate that under metabolic stress the bacteria were able to increase enterochelin production from the basal level characteristic of this growth rate. The amount of enterochelin detected in the supernatant of the D=0.2 h^{-1} chemostat does not show the same clear downward trend seen in Fig. 5.7.A.

Work on batch cultures of *K. pneumoniae* has shown that sub-MIC's of cephalosporins cause a decrease in the amount of enterochelin detected in the culture supernatant (Kadurugamuwa *et al* 1985a), this effect is seen in chemostat culture but is complicated by the underlying influence of growth rate on detectable enterochelin. The effect is seen only at growth rates where production of enterochelin is maximal. If the initial increase at D=0.4 h^{-1} indicates a decrease in the rate of uptake of the

ferric enterochelin complex, this may be masking a great effect on enterochelin production.

5.5. The effect of sub-MIC's of β-lactam antibiotics on the extracellular polysaccharides of *K. pneumoniae* in iron limited chemostat cultures.

The amount of cell associated capsular polysaccharide detected in samples of cells treated with 1/50 MIC of these cephalosporins showed wide fluctuations but no clear trends (Fig. 5.8 A and B); there appeared to be a downward trend in the cells treated with Ceftriaxone but this was not seen in the Cefotaxime treated cells. The effect of 1/5 MIC was much clearer. There was no effect on the amount of capsule associated with cells growing at D=0.4 h^{-1}, but there was a general decrease over the course of the experiment in the amount of capsular polysaccharide detected at the slower growth rates (Fig. 5.9 A and B). A reduction of the degree of encapsulation of *K. pneumoniae* treated with sub-MIC concentrations of β-lactam antibiotics has previously been noted for batch cultures (Kadurugamuwa *et al* 1985a,b) this effect is seen in chemostat cultures at slow growth rates where the capsule is large, but not at the faster growth rates where the amount of capsular polysaccharide is already reduced. It is possible that capsular polysaccharide synthesis is strictly controlled to give a minimum size of capsule, in response to environmental conditions involving changes in growth rate this can be increased but an external stress such as that imposed by sub-MIC treatment can modify this response.

Electronmicrographs of *K. pneumoniae* grown in iron limited chemostat cultures at D=0.2 h^{-1} show the effect of the cephalosporins on the structure of the capsule. The glutaraldehyde/osmium tetroxide double

Fig. 5.8.A. The effect of 1/50 MIC of Ceftriaxone on the production of capsular polysaccharide by *K. pneumoniae* grown in iron limited chemostat culture.

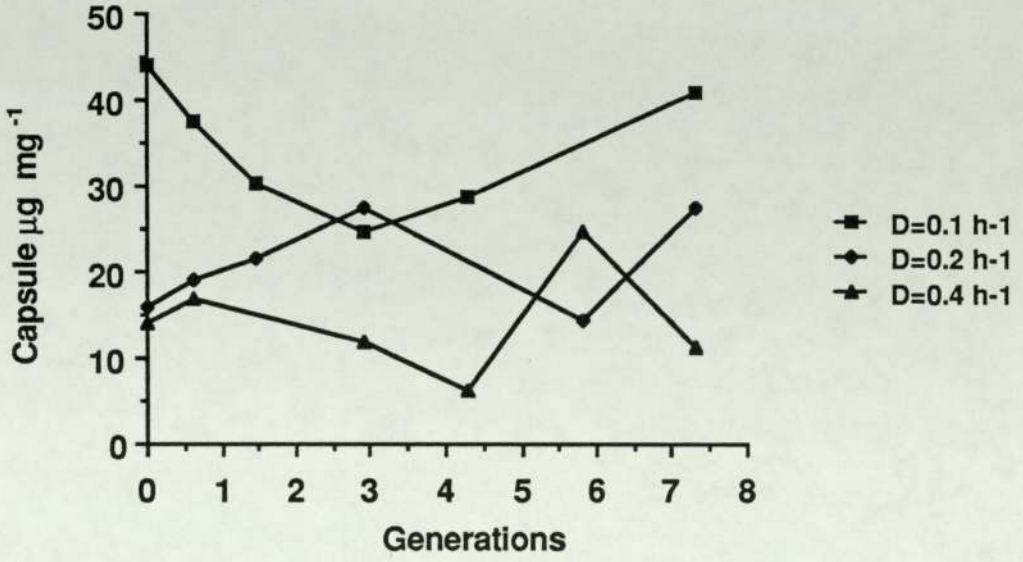


Fig. 5.8.B. The effect of 1/50 MIC of Cefotaxime on the production of capsular polysaccharide by *K. pneumoniae* grown in iron limited chemostat culture.

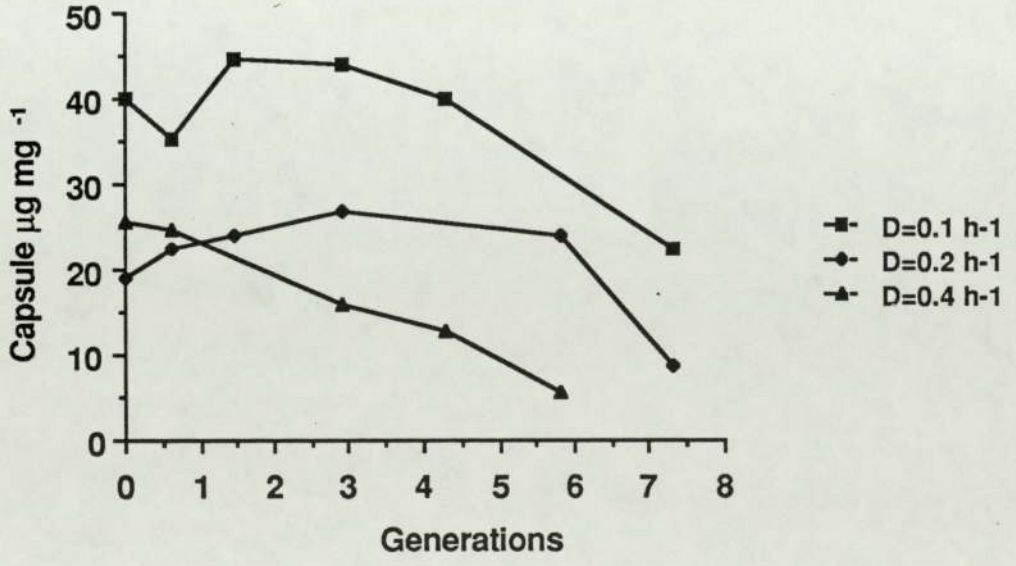


Fig 5.9. A. The effect of 1/5 MIC of Ceftriaxone on the production of capsular polysaccharide by *K. pneumoniae* grown in iron limited chemostat culture.

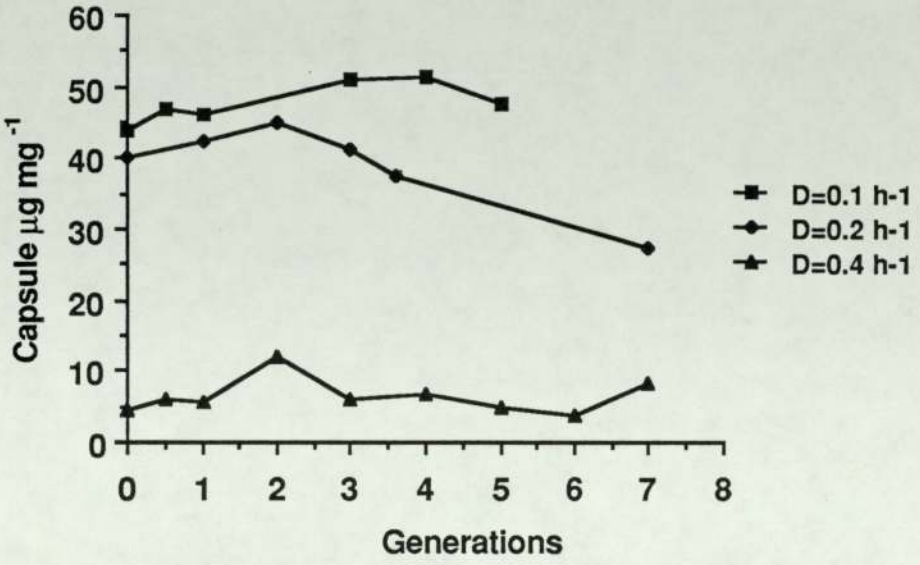
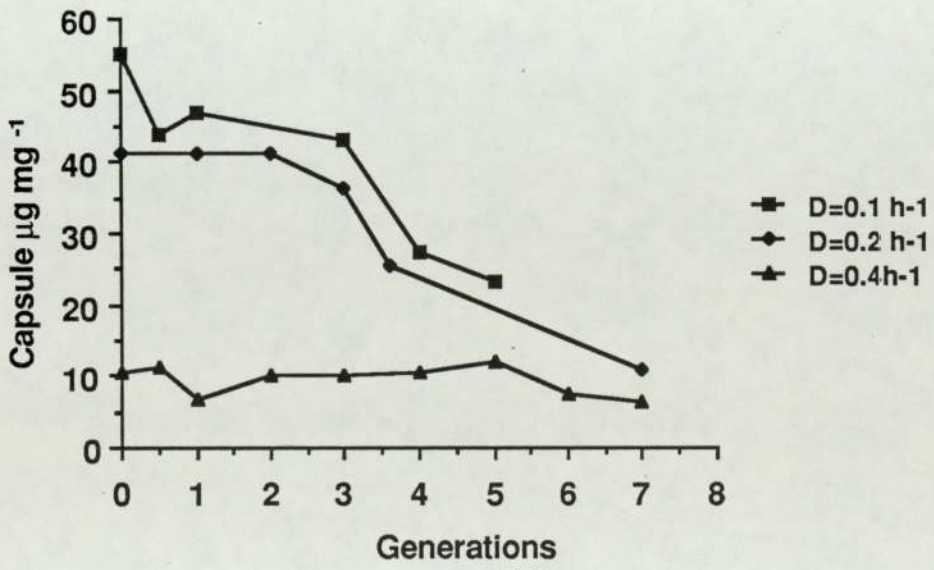


Fig 5.9.B. The effect of 1/5 MIC of Cefotaxime on the production of capsular polysaccharide by *K. pneumoniae* grown in iron limited chemostat culture.



fixation technique has been shown to be successful in preserving the fine structure of the capsular material of *K. pneumoniae* (Schmid *et al* 1981). A comparison of the capsule sizes of the untreated cells stained with indian ink (Fig. 5.3.) and electronmicrographs of the same cells (Fig. 5.10.) shows that there is a 30% reduction in the thickness of the capsule in the electronmicrographs but that the capsule is still well preserved. Preparation of indian ink slides involves suspension in a "watery film" containing the suspended ink particles; this causes some expansion of the capsule (Duguid and Wilkinson 1953). This may have led to some over estimation of the dehydration resulting from the treatment of the bacteria for electron microscopy.

The capsule of untreated chemostat grown *K. pneumoniae* shows up as a discreet filamentous layer under the electron microscope (Fig. 5.10.). This capsule structure for *K. pneumoniae* has previously been demonstrated (Schmid *et al* 1981) and is different from the capsule of *Escherichia coli* which has an overall granular matrix with thin fibres extending radially through the capsular material (Bayer *et al* 1985). Treatment with a sub-MIC concentration of Ceftriaxone resulted in a more sparse distribution of filaments (Fig. 5.11) and eventually left the cells "bald" (Fig. 5.12.). The capsule filaments in the intermediate sample (Fig. 5.11.) exhibited a greater variety of lengths, than did those in the untreated cells; some of them being very short and portions of the cell envelope were left unprotected by capsule. Prolonged exposure to the antibiotics resulted in complete loss of the filamentous structure of the capsule. The condensed material, outside the envelope in Fig. 5.12 may represent the remains of the capsule or may be the lipopolysaccharide. It is important when interpreting electronmicrographs to bear in mind the possibility of artefacts

Fig 5.10 Transmission electronmicrograph of *K. pneumoniae* grown in iron limited chemostat culture at $D=0.2\ h^{-1}$. The capsule can be seen as a filamentous region extending outwards from the cell.

Fig 5.11 Transmission electronmicrograph of *K. pneumoniae* grown in iron limited chemostat culture at D=0.2 h^{-1} in the presence of 1/5 MIC of Ceftriaxone for 3.6 generations. The filaments of capsular material are more sparsely distributed over the surface of the cell.

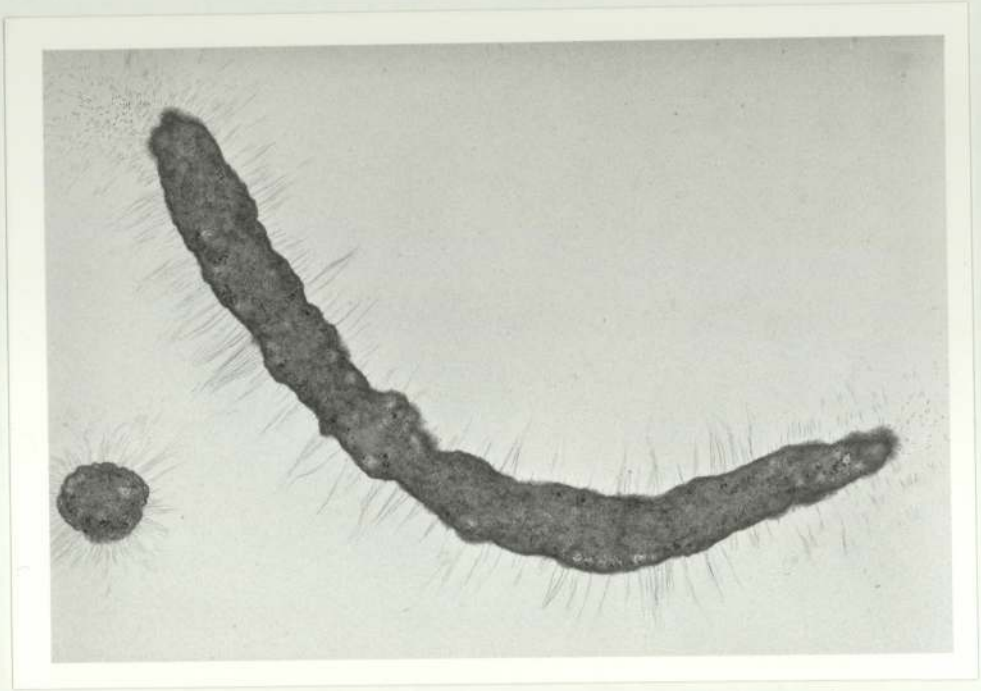
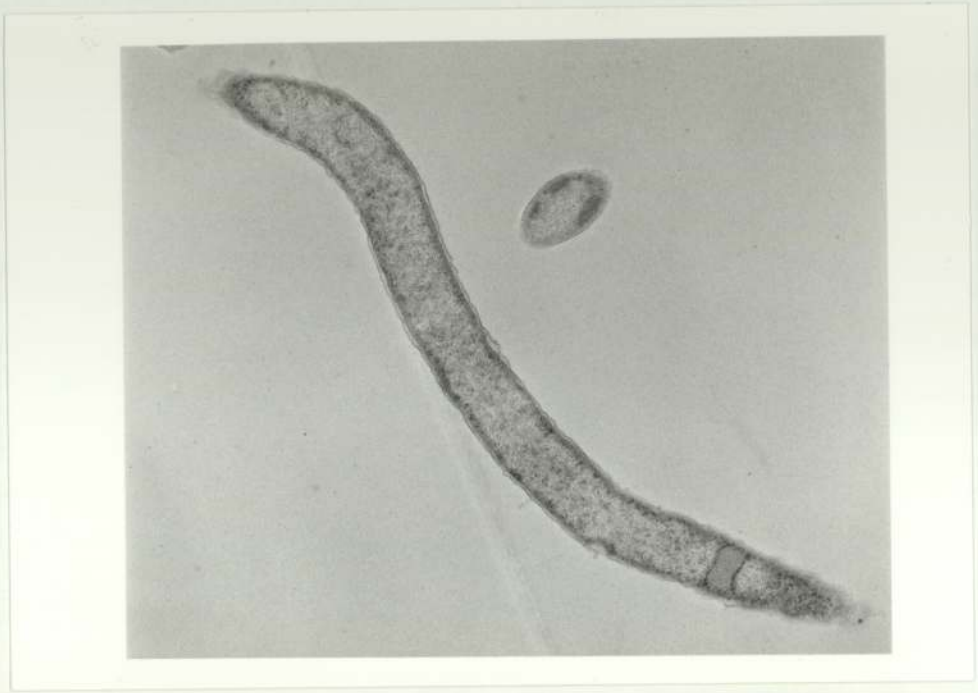


Fig 5.12 Transmission electronmicrograph of *K. pneumoniae* grown in iron limited chemostat culture at D=0.2 h^{-1} in the presence of 1/5 MIC of Ceftriaxone for 7 generations. The capsule is no longer visible.



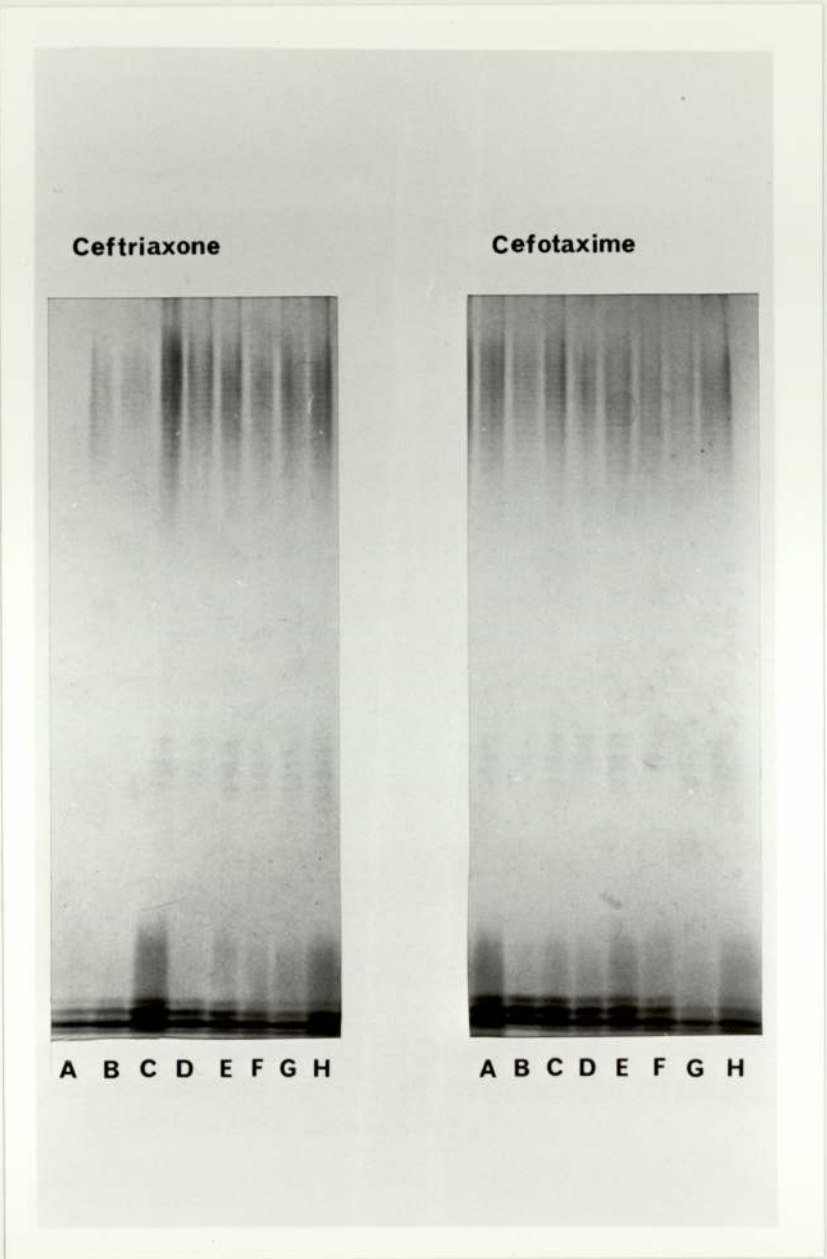
introduced by the fixing and staining processes, but the correlation with the quantitative data from rocket immunoelectrophoresis strengthens this interpretation of the data.

The effect of the higher sub-MIC concentrations of Ceftriaxone and Cefotaxime is to reduce the amount of cell associated capsular polysaccharide in slow growing cells. This reduction takes the form both of shorter filament lengths and of an overall reduction in the number of filaments. It would be interesting to know whether the amount of cell free polysaccharide decreased proportionally, indicating that the effect was on the synthesis of the polysaccharide, or whether there was a proportional increase in the amount of cell free polysaccharide indicating a failure of attachment.

Analysis of the lipopolysaccharide ladder pattern at various time intervals after treatment with the antibiotics showed no variation from that seen in untreated cells (Fig. 5.13. A and B). This observation was not affected by growth rate or by the antibiotic used. This indicates that there was no influence of the antibiotics on the relative proportions of long and short polysaccharide side chains in the lipopolysaccharide. It is possible that the total amount of lipopolysaccharide was altered by the treatment but not sufficiently to be detected by PAGE.

It has previously been shown that treatment of *K. pneumoniae* with sub-MIC's of various antibiotics in batch culture decreases the capsule sufficiently to expose previously masked cell envelope components (Kadurugamuwa *et al* 1985b, Williams 1987). It is possible that this may be of some significance *in vivo* where bacteria are slow growing and

Fig 5.13 The effect of 1/5 MIC of β-lactam antibiotics on the lipopolysaccharide ladder pattern of *K. pneumoniae* grown in iron limited chemostat culture at D=0.4 h^{-1}.



Lane A 0 generations
Lane B 1 generations
Lane C 2 generations
Lane D 3 generations
Lane E 4 generations
Lane F 5 generations
Lane G 6 generations
Lane H 7 generations

where encapsulation is of particular importance as a mechanism of resistance to host defences. It is possible that the patchy encapsulation seen in Fig. 5.11. would allow penetration both of opsonic antibodies and of complement components to the outer membrane more readily than the continuous capsule in Fig. 5.10.

Conclusion.

These experiments with sub-MIC's of cephalosporin antibiotics in iron limited chemostat culture have only been performed once and should be regarded as preliminary results. It is obvious from the data presented above that the effect of a given concentration of antibiotic in chemostat culture is not the same as the effect in batch culture. The effect of 1/50 MIC of both cephalosporins in batch culture conformed to the definition of a sub-MIC; growth rate was not affected but distinct morphological changes were observed in early log phase in batch culture. The same concentration of antibiotic in chemostat culture had no effect on the morphology of the cells over a period of seven generations, but did cause fluctuations in the levels of the two parameters measured: capsular polysaccharide and enterochelin. These concentrations of antibiotic were affecting the chemostat and it is possible that over a longer time course the chemostat would reach a new steady state and that the cells would exhibit a phenotype characteristic of sub-MIC treated bacteria. A concentration of 1/5 MIC did have a marked effect both on cell morphology and on the other parameters measured. This concentration does affect the growth kinetics in batch culture causing early onset of stationary phase and is probably too high a concentration to allow the chemostat to reach a new steady-state.

Sterkenberg (1984a) showed that the minimum bactericidal concentration of Penicillin G for chemostat cultures of *K. aerogenes* was dependent on the amount of cells in the culture. He found that lowering the concentration of the limiting nutrient, and consequently decreasing the biomass of the culture, resulted in a decrease in the MBC of Penicillin G for the culture. In the batch culture experiments the starting optical density of the cultures was in the region of OD_{470} 0.02, the optical density of the chemostat cultures was about 100 times greater. The rapid effects of the antibiotics seen in batch culture may be a result of the relatively greater drug to bacteria ratio in the early stages of the culture. It may require many more generations for the same effect to be seen in the chemostat where the ratio of drug to bacteria is much less. It seems likely however that the chemostat conditions are more closely related to the conditions *in vivo* where antibiotic regimes are designed to maintain a constant antibiotic concentration over the period of treatment.

The data describing the effects of sub-MIC concentrations of antibiotics has been expressed in terms of generations rather than absolute time because it makes for more meaningful comparisons between data from different growth rates. In translating this data to the *in vivo* situation account should be taken of the relation to absolute time. Any effect that happens at the same rate, expressed in terms of generations, in two populations of cells growing at different growth rates in fact happens faster, in terms of absolute time, at the faster growth rate. For example the rate of reduction of capsule by Cefotaxime is approximately the same at both $D=0.1\ h^{-1}$ and $D=0.2\ h^{-1}$ in terms of generation time (Fig. 5.7 B), but expressed in terms of absolute time the effect on the faster growing cells is more rapid. In general, where

there is an effect of sub-MIC treatment it occurs at the same rate in cells growing at different growth rates when expressed in terms of generation time. Translated into absolute time these effects are slower in slow growing cells, demonstrating that slow growing cells are more resistant to the effects of sub-MIC's as well as the effects of MIC's of β-lactams (Cozens *et al* 1986b, Sterkenberg 1984a).

A concentration of 1/5 MIC of these two cephalosporins affected the expression of two important virulence factors for *K. pneumoniae*. The effect was not as straight forward as that seen in batch culture as it was complicated by the underlying effects of growth rate on both enterochelin (Sect. 3.) and capsule (Sect. 4.). Even at this relatively high concentration there was no effect on the lipopolysaccharide that could be detected with the techniques used. The effect of the antibiotics on the expression of these virulence factors was seen at the growth rates were they are of most importance to the bacteria: at fast growth rates for enterochelin and at slow growth rates for capsular polysaccharide production. In both cases these effects may be of importance in the *in vivo* situation. In the initial stages of infection the bacteria may be growing rapidly (Morris-Hooke 1985, Rubin *et al* 1985) and in these circumstances the acquisition of iron, a nutrient essential to many of the cells' metabolic processes (Neilands 1974), is of great importance. In the early stages of a course of antibiotics sub-MIC concentrations may well prevail, while these concentrations are not bactericidal there may be sufficient effect on the cell, such as the effect on the enterochelin iron uptake system to retard the course of the infection. Bacteria in a chronic infection are probably slow growing (Eudy and Burrous 1973, Cozens *et al* 1986a) and adopt a biofilm form of growth which may protect them from the hosts immune system (Costerton

and Marrie 1983). This mode of growth may also reduce their vulnerability to antibiotic agents (Nickel *et al* 1985a,b) resulting in them being exposed to sub-MIC concentrations. The effect of sub-MIC's of ß-lactams in decreasing the amount of capsular material resulting in a more sparsely covered cell envelope would facilitate the actions of the host immune system an enhance clearance of the bacteria.

6. Discussion

The importance of the influence of the environment on the phenotype of bacteria is becoming recognised in fields such as design of vaccines (Griffiths and Bullen 1987), and antibiotics (Brown and Williams 1985b) as well as in procedures such as sterility testing (Gilbert *et al* 1987). It is possible using the chemostat to grow bacteria under conditions of controlled growth rate and well defined nutrient availability. In this study chemostat culture was used to mimic some of the factors which are of relevance to the the *in vivo* situation.

The pattern of expression of the 39 kDa porin protein is a good example of the influence of the environment on the bacterial phenotype. This protein which was expressed in batch culture in full SSM as well as in iron and carbon limited media was not presentin iron limited chemostat culture and was only present at the fast growth rate in carbon limited chemostat culture. This pattern of expression is complex and is not related simply either to growth rate or nutrient availability. The expression of this porin was related to permeability in the carbon limited chemostats and variations in it's expression caused by these and other environmental influences could have important consequences in terms of antibiotic sensitivity.

In the case of both of the limitations used in the chemostats in this study limitation specific proteins were expressed, the role of IRMP's in iron uptake is well understood (Griffiths 1987b). The role of the 49 kDa glucose inducible protein is less clear but it is probably involved in the uptake of limitation releasing compounds (Sterkenberg *et al* 1984).

Two factors of established importance in the virulence of *K. pneumoniae* are the possession of a polysaccharide capsule (Domenico *et al* 1985) and the expression of high affinity iron uptake mechanisms (Williams *et al* in press); the expression of both of these virulence factors was shown to vary with growth rate. The results of these experiments suggest that the acquisition of iron is of relatively less importance in slow growing *K. pneumoniae* than in those growing at near maximal rates, even in iron limited cultures. This conclusion was supported by the observation that the high affinity iron uptake system was not fully expressed at the slowest growth rate iron limited or carbon limited low iron chemostats.

Encapsulation may be of particular importance to slow growing bacteria *in vivo* as a protection against host defence mechanisms (Kato *et al* 1976). There is a body of evidence that suggests that cell free as well as cell associated capsular polysaccharide is important in immobilising various prongs of the host defences (Domenico *et al* 1985). The amount of both of these forms of exopolysaccharide was shown to be greater at slow growth rates in iron limited chemostat cultures; it is likely that bacteria in chronic infections are growing slowly (Eudy and Burrous 1973, Cozens 1986a) and that in the case of *K. pneumoniae* the exopolysaccharides are of major importance in protecting the bacteria from clearance by the host defence mechanisms. There was little variation in the lipopolysaccharide over the range of growth rates studied. There is some evidence that the bacteria prioritises the synthesis of lipopolysaccharide above capsular polysaccharide (Sutherland 1977); at growth rates approaching the maximum capsular polysaccharide synthesis may be sacrificed to enable the cell to maintain an intact lipopolysaccharide layer.

In the initial stages of an infection the number of bacteria will be relatively small and the host parasite interaction weighted in favour of the host (Smith 1978). In these circumstances the ability to reproduce rapidly may be important in the establishment of an infection. The ability to acquire iron, which is important in many metabolic processes, would be of particular importance in these circumstances, hence the full expression of the high affinity iron uptake system. Metabolic resources may well be diverted away from capsule production which is of less importance to bacteria which are growing fast enough to outstrip the rate of clearance by the host. As the infection proceeds the bacterial growth rate is reduced (Eudy and Burrous 1973, Rubin *et al* 1985) and the bacteria adopts a form of growth which confers resistance to antimicrobial agents as well as to the host defence mechanisms (Costerton and Marrie 1983). Slow growing bacteria may have a lower requirement for iron as well as other nutrients but produce a large amount of capsular polysaccharide which plays a crucial role in protection against host defence mechanisms.

Antibiotics, particularly at sub-MIC concentrations, are an important facet of the environment encountered by bacteria *in vivo* (Brown and Williams 1985b, Lorian 1980). The effects of sub-MIC's have mainly been studied in batch culture where initially there is a low number of bacteria and a relatively high concentration of antibiotic, as the experiment progresses the number of bacteria increases and the concentration of antibiotic falls under the influence of for instance ß-lactamases. The chemostat made it possible to study the effect of a constant concentration of antibiotic on a population of cells which was, at least initially, stable. The concentration of a ß-lactam antibiotic which caused a morphological effect but did not effect the growth rate

was higher in chemostat than in batch culture. It has been shown that the MBC of β-lactam antibiotics for *K. aerogenes* grown in chemostat culture increases as the number of cells increases (Sterkenberg 1984a). It is likely that the sub-MIC is similarly affected and that the lower concentration required to effect batch cultures of *K. pneumoniae* is a reflection of the small number of cells present at the beginning of the experiment.

The interpretation of the effects of sub-MIC's of β-lactam antibiotics on the exopolysaccharides and on enterochelin production is complicated by the underlying effects of growth rate on these virulence factors. In general the presence of sub-MIC concentrations of these antibiotics affects each of these factors most in the circumstances where they are of most importance. Enterochelin production was not effected by sub-MIC's at slow growth rates where it is of relatively little importance to the bacteria and where production is at a basal level. Enterochelin production was however greatly reduced at faster growth rates and this reduction could have a marked effect on the bacteria's survival as the acquisition of iron is critical in these circumstances.

In circumstances where capsule production is at a basal level, in this case in rapidly growing bacteria, there was very little effect of the sub-MIC. Capsule production was however markedly reduced by sub-MIC concentrations of β-lactam antibiotics in *K. pneumoniae* growing at slow growth rates. Reduction of the capsule in these bacteria is important in enhancing the susceptibility of *K. pneumoniae* to clearance by the host defence mechanisms (Kadurugamuwa *et al* 1985b, Williams 1987). The electronmicrographs show that the reduction in the amount of capsule initially results in the cell being more sparsely covered in capsule. It

is tempting to consider that this structure would facilitate better access of antibodies and complement to the outer membrane.

This preliminary work shows that the presence of sub-MIC concentrations of β-lactam antibiotics has important synergistic effects on the clearance of bacteria by the host immune system and that individual virulence factors are effected most in circumstances where they are of most importance to the survival of the bacteria.

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