PHYSIOLOGICAL AND MOLECULAR GENETIC ANALYSIS OF CAPSULE PRODUCTION IN *KLEBSIELLA PNEUMONIAE*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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ABSTRACT

Capsular polysaccharide (CPS) production in *Klebsiella pneumoniae* K1 was investigated in batch and chemostat cultures. The CPS was analyzed by physical and chemical methods. Molecular cloning techniques have been used to characterise the capsule biosynthetic genes of *K. pneumoniae* K1.

The production of CPS by *K. pneumoniae* was enhanced under nitrogen-, phosphate-, or sulphate-limited conditions but not in carbon-limited conditions. Lower temperature and neutral pH increased the level of CPS production. The synthesis of polysaccharide was observed throughout growth with maximum rate occurring after exponential phase of growth. The rate of polysaccharide synthesis was 0.48 mg ml^{-1} h^{-1} at a specific growth rate of 0.44 h^{-1} in batch culture. The rate of polysaccharide synthesis was higher at low dilution rates in nitrogen-limited chemostat culture. Growth of *K. pneumoniae* in nitrogen-limited culture resulted in the development of nonmucoid variants. The emergence and the increase in the population of nonmucoid variants may be due to their selective advantage over capsule producing cells. The irreversible loss of the mucoid phenotype could be a mutational lesion within the capsule genes as a result of continuous selective pressure. The CPS isolated from *K. pneumoniae* K1 had an approximate molecular mass of $6x10^6$ and more than 60% of the total weight was comprised of carbohydrate.

K. pneumoniae chromosomal DNA was cloned into *E. coli* using cosmid vectors. Recombinant cosmids with a molecular size of 35-42 kb that expressed *Klebsiella* specific antigen were identified. Further subcloning and analysis of the antigenic component expressed by the cosmid clones suggested the protein nature of the antigen rather than K antigen *per se*. Southern hybridisation analysis of the cloned genes with the chromosome of *Klebsiella* and *E. coli* strains suggested their absence in *E. coli* but presence in all *Klebsiella* strains analyzed. It is possible that the antigen may be a surface protein that is expressed by *Klebsiella* strains.

Using Tn*10* or Tn*phoA*, noncapsulated mutants of *K. pneumoniae* were generated. Immunological analysis of noncapsulated mutants indicated complete loss of capsule production. Analysis of the noncapsulated mutants by Southern hybridisation also showed the insertion of transposons at different sites within the chromosome, suggesting that several genes could be involved in capsule biosynthesis. DNA fragments close to the insertion sites were cloned and partially mapped. Recombinant plasmids failed to express the K antigen in *E. coli* or complement noncapsulated strains of *Klebsiella*. This could either due to the biosynthetic gene cluster being large to be cloned in a single vector system or the requirement of additional genes which may not be closely linked.

Two regulator genes, $rcsA_{K1}$ and $rcsB_{K1}$ that positively regulate colanic acid synthesis in *E. coli* K12 were cloned from *K. pneumoniae* K1. The $rcsA_{K1}$ gene encoded a 23,000 Dal protein while the $rcsB_{K1}$ did not encode any protein in maxicell system. Multicopy $rcsA_{K1}$ gene induced colanic acid synthesis at 30 and 37°C. Multicopy $rcsB_{K1}$ gene was only involved in increasing the levels of colanic acid synthesis in *E. coli* containing multicopy $rcsA_{K1}$ gene. Analysis of the $rcsA_{K1}$ gene sequence showed a single ORF of 621 bp while $rcsB_{K1}$ contained a possible ORF of 180 bp. The two genes are oriented towards each other with their stop codons 444 bp apart. Both genes were homologous to the previously cloned *rcsA* and *rcsB* genes from *K. pneumoniae* K21b. The identity of these genes with other *Klebsiella rcs* genes showed that the genes are highly conserved in this genus. The $RcsA_{K1}$ protein also shared extensive homology with those of *E. coli* and *Erwinia* RcsA proteins. The $rcsB_{K1}$ gene and its predicted amino acid sequences did not show any homology with other regulatory genes and their protein sequences. Both $rcsA_{K1}$ and $rcsB_{K1}$ genes could be part of a set of a regulatory system that participates in the regulation of capsule biosynthesis in *Klebsiella*.

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Abbreviations

Ap^r	ampicillin resistance
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degree centigrade
cfu	colony forming units
cm^r	chloramphenicol resistance
cm^{-3} min^{-1}	cubic centimetre per minute
cos	containing the cohesive end of lambda
CPS	capsular polysaccharide
D	dilution rate
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
ddATP	dideoxyadenosine-5'-triphosphate
ddCTP	dideoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
ddGTP	dideoxyguanosine-5'-triphosphate
ddTTP	dideoxythymidine-5'-triphosphate
dTTP	deoxythymidine-5'-triphosphate
DNA	Deoxyribonucleic acid
Dal	dalton
DMSO	1,4-dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminotetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
GDP	guanosine-5'-diphosphate
h	hour
kb	kilobase
KDO	2-keto-3-deoxyoctonate
kg	kilogram

Km^r	kanamycin resistance
λ	lambda
LPS	lipopolysaccharide
mg	milligram
mg ml^{-1} h^{-1}	milligram per millilitre per hour
min	minute
ml	millilitre
MSM	minimal salts medium
Nal^r	nalidixic acid resistance
nm	nanometre
OD	optical density
OPD	orthophenyldiamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PPG	polypropylene glycol
rcs	regulator of capsule synthesis
rmp	regulator of mucoid phenotype
RNase	ribonuclease
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
Sm^r	streptomycin resistance
SPPI	*B. subtilis* phage I
Tc^r	tetracycline resistance
TAE	tris acetate EDTA
TEMED	N,N,N,N-tetramethylethylene-diamine
TBE	tris borate EDTA
TBS	tris buffered saline
μCi	microCurie
μg	microgram
μl	microlitre
$^v/_v$	Volume/volume
$^w/_v$	weight/volume

Table of contents

CHAPTER I

GENERAL INTRODUCTION

1.1. Introduction

A wide range of bacteria from clinical and environmental habitats produce complex and diverse exopolysaccharides. Our understanding of bacterial polysaccharides is considerable because of sophisticated analysis at the molecular level. However, there are still major gaps in knowledge regarding the structural organisation, diversity and regulation of exopolysaccharide biosynthesis. This also presents a particular unresolved problem in the pathogenesis of encapsulated bacteria, including *Klebsiella.*

1.2. General features of *Klebsiella*

Klebsiella is a genus of capsulated, non-motile, and non-spore-forming bacilli which belong to the family *Enterobacteriaceae. Klebsiella,* named after Edwin Klebs (1834-1913), produce typical large greyish-white, glossy dome-shaped colonies on solid media. Members of this genus are active in fermenting a wide variety of carbohydrates.

Four different techniques are routinely used for typing of *Klebsiella* species. These include serological, biochemical, bacteriocin, and bacteriophage typing. Of all the methods, serological typing is by far the most widely used for characterisation of *Klebsiella.* Serological classification of *Klebsiella* to at least 77 chemically distinct serotypes is primarily based on capsular (K) antigens (Orskov and Orskov, 1978; 1984). The somatic antigens (O antigens) of *Klebsiella* are the O-specific polysaccharides of lipopolysaccharides. Although 8 different O antigens have been reported, the O1 serotype is the commonest of all. Because the chemical composition

of the O antigens of *Klebsiella* strains is quite simple, and the O1 antigen is common to many capsular serotypes, differentiation on a chemical basis is not reliable (Orskov and Orskov, 1978).

Although the subdivisions of the genus *Klebsiella* are still disputed, the three species, *K. pneumoniae* (*sensu lato*), *K. ozaenae*, and *K. rhinoscleromatis* described by Orskov (1974) are widely used. In the United Kingdom, seven species are widely recognised based on the classification of Cowan (1974). The seven species characterised include *K. pneumoniae* (*sensu stricto*), *K. edwardsii*, *K. oxytoca*, *K. aerogenes*, *K. ozaenae*, *K. atlantae*, and *K. rhinoscleromatis*.

1.3. Role of *Klebsiella* in infections

Klebsiella can be isolated from many parts of the environment: natural waters; soil; air; and human and animal sources. Most of the strains are saprophytes. *K. pneumoniae* was first recognised as a pathogen by Freidlander in 1882 from a pneumonic patient. Before the introduction of chemotherapeutic agents, *Klebsiella* was not considered as an important cause of disease. However, after the second half of this century infections due to *Klebsiella* have increased steadily. Since then *Klebsiella* has emerged as a leading cause of morbidity and mortality in hospital-acquired infections.

The incidence of *Klebsiella* infections among other Gram-negative bacillary infections in hospitalised patients has been reported to be as high as 16% (Cooke *et al*, 1979). Epidemiological surveillance in both the UK and USA showed that 7% of all hospital acquired infections were caused by *K. pneumoniae*. It is now known as the second most common opportunistic pathogen after *Escherichia coli* causing Gram-negative infections (Williams and Tomas, 1990). The reasons for this high incidence

are not well understood. However, the extensive use of antibiotics and the increased use of surgical and invasive procedures in hospitals could play an important role (Casewell, 1982).

Klebsiella infects any age group and is capable of invading any part of the body. Elderly patients (Steihauer *et al*, 1966), patients immunocompromised by burns or cytotoxic therapy (Umsawasdi *et al*, 1973) and neonates (Morgan *et al*, 1984) have shown to be high risk groups for *Klebsiella* infections.

Common infections with *Klebsiella* include those of the urinary tract, pneumonia, bacteraemia, meningitis, endotoxic shock, osteomyelitis, peritonitis, and necrotising enterocolitis (Edmonson and Sanford, 1967; Hill *et al*, 1974; Montgomerie and Ota, 1980). Respiratory infection is the most devastating and difficult to control and the mortality rate can exceed 50% (Ma *et al*, 1983; Jarvis *et al*, 1985).

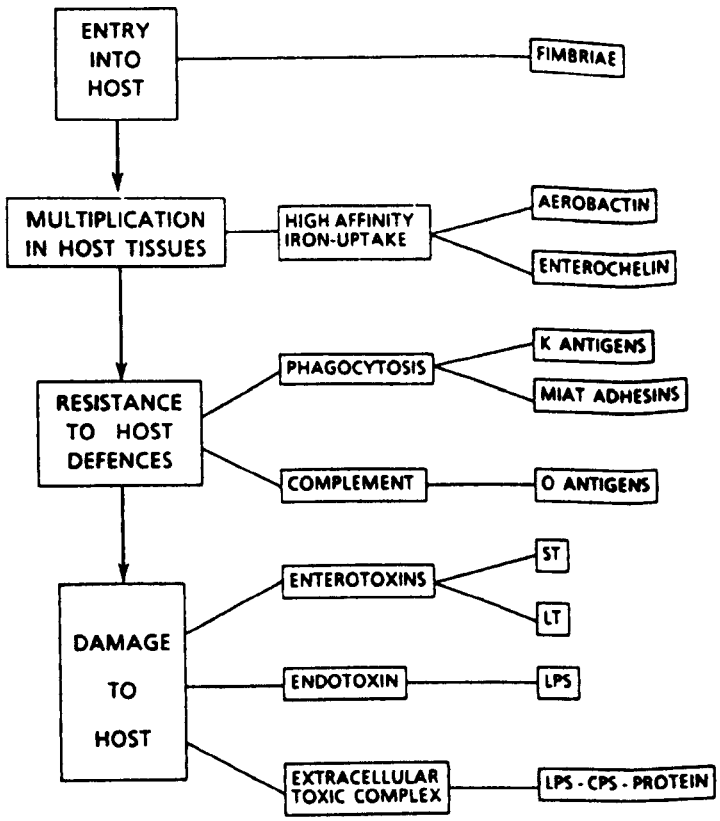
The majority of life-threatening diseases caused by *Klebsiella* are hospital-acquired (Montgomerie, 1979; Jarvis *et al*, 1985; dela Torre *et al*, 1985). Hospital staff and the environment can be reservoirs for *Klebsiella*. Studies have shown that hospital personnel play a critical role in the transmission of *Klebsiella* to patients (Casewell and Phillips, 1981). Although apparently healthy subjects can harbour *Klebsiella*, the carriage rate is much higher in hospital patients (Mengistu and Gedebou, 1986). Increase in colonisation by *Klebsiella* may predispose patients to infection.

Colonisation and the increase in the carriage rate is thought to be multifactorial, with the use of antibiotics playing a crucial role. Antibiotics may suppress the growth of the normal microflora and exert a selective pressure in favour of an opportunistic pathogen. This may subsequently lead to an outbreak of infection with resistant *Klebsiella* strains (Casewell and Phillips, 1981; Courtney *et al*, 1980).

Seroepidemiological studies have shown that most of the known capsular serotypes can be isolated from clinical specimens. However, some serotypes are more frequently encountered than others and show a preference for certain anatomical sites (Casewell and Talsania, 1979; Cryz *et al*, 1986; Simoons-Smit *et al*, 1987). In spite of the presence of several capsular serotypes, only a limited number of serotypes are responsible for the majority of *Klebsiella* infections.

Strains which belong to O1:K1 and O1:K2 serotypes have been associated with enhanced virulence as compared with other serotypes in experimental infections (Mizuta *et al*, 1983; Cryz *et al*, 1986). The lower number serotypes (serotypes 1-6) appear to be frequently associated with respiratory infections. Serotypes 9, 10, and 24 are frequently isolated from urine while serotype 8 is commonly isolated from the skin. These serotypes may possess an intrinsic factor, probably due to the type of capsule or some unidentified factor, which enables them to colonise readily and subsequently cause disease (Casewell and Talsmania, 1979; Riser and Noone, 1981). At present, it is not well understood how far endemic or epidemic strains of a known serotype show a predilection for invasion of particular body sites.

Differences in the clinical course of infection, the high prevalence of certain serotypes, the predominance of some serotypes in particular body sites, and the virulence of some serotypes in experimental animals have been attributed to virulence determinants. These determinants include capsular polysaccharide, lipopolysaccharide, surface appendages, and protein components (Fig. 1.1).



Key:

CPS Capsular polysaccharide
LPS Lipopolysaccharide
LT Heat-labile enterotoxin
ST Heat-stable enterotoxin
MIAT Mannose-inhibitable adhesins/coliphage T3-T7 receptors

Figure 1.1. Role of virulence determinants in the pathogenicity of *Klebsiella pneumoniae* (Williams and Tomas 1990).

1.3.1. Capsules

All living cells universally express carbohydrates on their surfaces which are involved in intercellular interactions and in reaction of the cell with different molecules. The extracellular polysaccharide produced by many bacteria is not indispensable for cell growth. It does, however influence the way that the bacterial cell recognises and interacts with its environment.

Capsular polysaccharides have two major properties; their highly hydrated state and the fact that they impart a negative charge to the cell surfaces. Anionic polysaccharide may exert an effect on the movement of molecules. They may regulate the traffic of charged molecules by selective binding of cations (Dudman, 1977). The capsule may bind heavy metals, including toxic metals and may act as a barrier to their entry into the cell. The hydrated polysaccharide has the capacity to be retained at the surface of the cell protecting the bacteria from desiccation (Dudman, 1977). The capsule of *Klebsiella* is highly hydrated and greater than 90% is water. Because of its hydrophilic nature it confers a negative charge on the surface of the cell (Sutherland, 1977).

Many pathogenic bacteria such as *Salmonella typhi*, *Neisseria meningitidis*, *Escherichia coli*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and almost all clinical isolates of *Klebsiella* strains are invariably capsulated when screened directly in established infections or in primary cultures. Capsules have long been associated with virulence properties of these pathogens. The capsule may contribute to the resistance of the pathogen to host immune defence mechanisms in various ways.

1.3.1.1. Impairment of phagocytosis

Surface tension of bacterial surfaces is a crucial property. Adhesion and phagocytosis occur as a result of contact between the bacterium and the phagocytic cell. The surface charge of some bacteria can inhibit contact between the phagocyte and the bacterial cell (Stendahl 1983). The hydrophilic properties of the capsule reduce surface tension at the interface between the phagocytic cell and the bacterium due to net electrostatic charge. The capsule of *Klebsiella* K21b which confers a strong negative charge on the surface of the cell in aqueous two-phase polymer systems has been shown to provide resistance to uptake by polymorphonuclear cells (Allen *et al*, 1987). Although colanic acid of *E. coli* confers a negative charge on the surface of the bacterial cell it does not protect it from phagocytosis, suggesting that all capsules do not act as anti-phagocytic agents.

A correlation between the surface properties of pathogenic bacteria and the degree of uptake by phagocytic cells has also been related to contact angle between the flat surface of the bacteria and drops of saline solution. Bacteria with a lower contact angle than the phagocytic cell are not easily phagocytosed (van Oss and Gillman, 1972). This phenomenon is also partly attributed to the presence of the capsule. The presence of specific antibodies to K or O antigens may increase the hydrophobicity and render the pathogens susceptible to phagocytosis (Williams *et al*, 1986).

An increase in the volume of cell-associated polysaccharide significantly increases the antiphagocytic properties of the capsule. Heavily capsulated *Klebsiella* strains are more virulent than less capsulated strains in experimentally infected of mice (Domenico *et al*, 1982). Severe metritis in horses has been reported to be due to

infections with capsulated *Klebsiella* K1 rather than noncapsulated strains (Kikuchi *et al*, 1987). However, the presence of a capsule may also facilitate phagocytosis. *Klebsiella* serotypes that contain Man-α2/3-Man or Rha-α2/3-Rha repeating units in their capsules can be easily phagocytosed by macrophages expressing a mannose/N-acetylglucosamine-specific lectin (Athamna *et al*, 1991).

Specific antibodies are necessary for effective opsonization and phagocytosis of capsulated strains (Williams, *et al*, 1983; Simoons-Smit *et al*, 1986). Anti-K2 antibody has been shown to be highly opsonic *in vitro* and can protect against *Klebsiella* infection (Trautmann *et al*, 1988; Held *et al*, 1992). The capsule of *Klebsiella* however may negate the protective effect of anti-capsular antibody and may render the bacterium less susceptible to phagocytosis. Circulating exopolysaccharides in the blood of patients infected with *K. pneumoniae* may neutralise the anti-capsular antibody before opsonisation of the pathogen can occur (Pollack, 1976). The capsule may physically detach from the bacterial cell upon antibody binding. This might be due to a change in the spatial arrangement of the capsule. Since the capsule is no longer bound to the cell surface, the bacterial cell will not be opsonised and phagocytosed.

1.3.1.2. Serum resistance

The complement system, a series of proteins (C1-C9) plays a major role in the mammalian host defense mechanisms. Complement may be activated either by the classical or alternative pathways. The classical pathway requires antibody-antigen complex formation for activation, while the alternative pathway is usually activated by substances such as bacterial polysaccharides. Both result in the activation of the

vital third component of complement, C3. The deposition of the active protein C3b on to the bacterial surface activates the C5 cascade. Lysis of bacteria occurs when the terminal complement components C5b-C9 form the membrane attack complex (MAC) on the bacterial cell membrane.

Capsulated pathogens are resistant to the direct complement activation mechanism and require immune complex formation on their surfaces to activate complement. The capsule impedes the opsonins (immunoglobulin G, or C3b) from penetrating the capsular layer and prevents complement-activating components from binding (Pluschke *et al,* 1983; Kasper, 1986). The capsules of some strains of *Klebsiella* from capsular serotypes K1, K10 and K16 not only mask the LPS molecules but also do not activate complement, and thus render resistance to complement-mediated killing (Merino *et al,* 1992).

The capsule may also inactivate the amplification loop of the alternative pathway. Sialic acid polysaccharides of *E. coli* K1, group B meningococci and group B streptococci type III have a high affinity for factor H (Brown *et al,* 1983). The interaction of capsule and factor H favours the deposition of H-C3b complexes instead of the active converting enzyme C3bBbP on the bacterial surface. The H-C3b complex blocks the amplification loop and renders the bacterium resistant to opsonophagocytosis in the absence of antibody. Some bacterial strains such as pneumococci capsular serotypes 7 and 12 do not bind factor B efficiently (Brown *et al,* 1983). The capsule may physically block access of the phagocytic cell C3b receptors to the cell wall of the pathogen and may also mask other surface components, such as outer membrane proteins, from circulating antibody.

1.3.1.3. Immunological Tolerance

The capsule of *Klebsiella* has been implicated in the induction of immunological paralysis. When rabbits were immunised with a heavily encapsulated strain of *Klebsiella* K5, little anti-capsular antibody was produced, while inoculation of the same strain that possessed a much smaller capsule resulted in a high level of antibody production (Orskov, 1956). This suggested that induction of immuno-tolerance was dose-dependent. Injection of large quantities of capsular polysaccharide (CPS) decreased the amount of detectable homologous antibodies, while small quantities of CPS induced high levels of protective antibodies (Batshon *et al*, 1963).

The persistence of antigen in the blood is thought to be due to the release of undigested polysaccharide from the macrophages. The polysaccharide will bind antibody, and be phagocytosed as an antigen-antibody complex. The antibody is eventually degraded within the macrophage while the polysaccharide remains intact and is excreted back into the circulation and the whole process repeated.

1.3.1.4. Other functions of capsules

Capsules may also play other important roles. In some bacteria, the capsule may promote adherence to the surfaces of living things or inanimate objects and the formation of microcolonies and biofilms (Costerton *et al*, 1987). Although the capsule of *Klebsiella* is not primarily involved in adherence to host cell surfaces, it might be involved in enhancing biofilm formation on mucosal surfaces at the later stages of colonisation (Williams and Tomas, 1990). The capsule might also enhance the survival of the bacterial cell by preventing loss of water. The survival of capsulated strains of *Klebsiella* strains on the skin for longer period as compared to noncapsulated strains

has been documented (Hart *et al*, 1981). Although the capsule may serve as the surface receptor for certain specific bacteriophages (Whitfield and Lam, 1986), it may protect the bacteria from viral infection by adsorption and penetration by phages that have receptors other than surface polysaccharide.

The capsule may also interfere with the development and activation of macrophages. Using *in vitro* cultivation techniques, Yokochi *et al* (1977 and 1979) demonstrated that the capsule of *Klebsiella* could be responsible in blocking the generation of macrophages from precursor cells. Such an effect was not due to the toxicity associated with preparation of the antigen. The phenomenon can occur in patients with large quantities of circulating capsular antigens which may lead to the persistence and further invasion of the body by the pathogen.

1.3.2. Lipopolysaccharide

LPS is a major component of the outer membrane and is unique to Gram-negative bacteria. The O antigen LPS plays a crucial role in protecting pathogenic *Klebsiella* from complement-mediated serum killing (Williams *et al*, 1983; Tomas *et al*, 1986; Ciurana and Tomas, 1987). Some *Klebsiella* serotypes where their lipopolysaccharides are exposed at the surface (Tomas *et al*, 1988; 1991) have been shown to be resistant to complement-mediated killing (Merino *et al*, 1992). Such resistance was not mediated by capsule since isogenic noncapsulated mutants ($O1^+:K^-$) were also resistance to serum killing.

Klebsiella serotype strains expressing high molecular weight LPS antigens are more resistant to the bactericidal action of complement than strains producing low molecular weight O antigen repeating units (McCallum *et al*, 1989). The O LPS

antigen can activate the complement, however, the deposition of C3b on the surface of the cells may not allow the C5b-C9 (MAC) to form for the lysis of cells. This may be due to steric hinderance by the O antigen chain, or the MAC may be formed far away from the target membrane (Merino *et al*, 1992). This suggests that the length of the O-polysaccharide side chain is very important in mediating serum resistance.

1.3.3. Endotoxins and enterotoxins

The lipid A component (endotoxin) of many Gram-negative pathogens including *Klebsiella* is responsible for the toxic properties associated with LPS. The endotoxin may produce fever, inflammation, shock, blood coagulation, and may also activate macrophages (Prescott *et al*, 1990). *Klebsiella* has also been reported to produce heat-stable (ST) and heat-labile (LT) enterotoxins (Kleipstein *et al*, 1983; Betley *et al*, 1986).

1.3.4. Extracellular toxic complex

In some *Klebsiella* strains the endotoxic properties and virulence have also been associated with the presence of an extracellular toxic complex (ETC). ETC composed of CPS (56-79%), LPS (14-33%), and protein (4-10%) could be responsible for extensive pulmonary damage in experimental animals (Straus *et al*, 1985; Straus, 1987). When inoculated intraperitonially, the purified ETC was capable of producing severe pulmonary infection.

1.3.5. Fimbriae

The ability of a pathogen to colonise and establish infection depends upon the ability of the strain to overcome mucocilliary clearance and to adhere to mucosal surfaces. Some members of *Enterobacteriaceae* commonly produce fimbriae (pili). Fimbriae are filamentous structures which may play a role in preventing the pathogen from mechanical clearance from the host. They also facilitate attachment to epithelial receptors by fimbriae-associated adhesins.

Klebsiella usually produces type 1 and/or type 3 fimbriae. Type 1 fimbrial adhesins are mannose-containing-glycoproteins and can induce mannose-sensitive haemagglutination *in vitro*. These pili have been shown to mediate the attachment of *Klebsiella* to uroepithelial cells through receptors containing mannose, and enhance virulence (Fader and Davis, 1980, 1982).

Type 3 fimbriae are characterised as mannose-resistant haemagglutins. Strains that express type 3 fimbriae agglutinate erythrocytes that are pre-treated with 0.01% tannic acid (Clegg and Gerlach, 1987). *In vitro* culture, type 1 or 3 fimbrial adhesins of *K.pneumoniae* have been shown to mediate adherence to epithelial cells of the respiratory tissues (Fader *et al*, 1988; Hornick *et al*, 1992; Tarkkanen *et al*, 1992). This suggests that fimbriae play an important role in the colonisation and invasion of respiratory tissues.

1.3.6. Mannose-inhibitable adhesins

Non-fimbrial adhesion factor commonly found in enterotoxigenic *E. coli* (Darfeuille-Michaud, *et al* 1986) is also known to be involved in the adherence of *K. pneumoniae* to intestinal tissues. The outer membrane proteins which may act as

receptors for coliphage T3 and T7 may serve as a mannose-inhibitable adherence mechanism (MIAT). The MIAT mediates adherence to the epithelial cells and may also protect the pathogen from phagocytosis and intracellular killing by polymorphonuclear cells (Pruzzo *et al*, 1980; 1982; and 1989). Since the MIAT system has only been found in noncapsulated strains, it is not yet clear how it is involved in *Klebsiella* infections as most of the clinical isolates are capsulated. An R-plasmid encoded non-fimbrial protein (29,000 Dal) has also been shown to facilitate adhesion of *Klebsiella* cells with epithelial cells (Darfeuille-Michaud *et al*, 1992).

1.3.7. Iron-sequestering mechanisms

The growth of pathogenic bacteria and subsequent invasion and destruction of tissues depends not only on evading host defence mechanisms but also on their ability to compete for essential elements such as iron. To overcome iron-limitation, enteric pathogens have developed iron-scavenging mechanisms which can remove ferric ions from host iron-binding proteins.

Iron-sequestering compounds are secreted into the surrounding medium and then actively taken up, complexed with ferric ions, through inner and outer membrane components. Substances like hydroxamate siderophore aerobactin, phenolate siderophore enterochelin and a number of repressible proteins that are produced by *K. pneumoniae*, have been shown to play this role (Williams *et al*, 1984; 1987; 1989; Tarkkanen *et al*, 1992). The virulence of some strains of K1 and K2 serotypes have also been associated with the presence of a 180 kb plasmid that encodes for the production of aerobactin and its receptor protein (Nassif and Sansonetti, 1986).

1.4. Structure and biosynthesis of capsule

1.4.1. Structure of capsule

Bacterial polysaccharides can be classified into three groups; intracellular, structural, and extracellular polysaccharides. The classification of these polymers is mainly based on their structural relationship with the cell. Intracellular polysaccharides are polyglucose polymers that occur as granules in the cytoplasm of the cell and are sources of energy and carbon. Peptidoglycan, the principal polysaccharide structural component of the Gram-negative and Gram-positive cell wall is a complex molecule composed of hexosamine and amino acids. It is a highly polymerised cross-linked structure and gives mechanical rigidity to the bacterial cell.

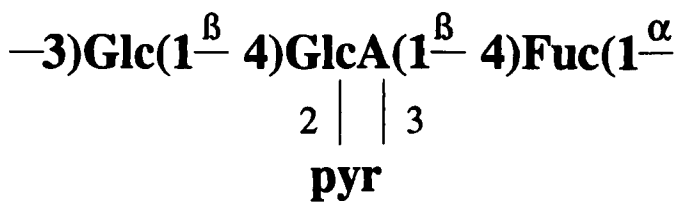
Extracellular polysaccharides located outside the cell wall are frequently termed as capsules or slime polysaccharides. The distinction between the two is arbitrary and is merely based on the nature of attachment to the cell surface. The former has a definite form and is firmly attached to the cell while the latter is loosely attached to the cell and excreted into the surrounding medium (Hammond *et al*, 1984). Both forms of polysaccharides are chemically indistinguishable from one another. Some bacteria may produce both forms while others only produce one. Some strains of *Klebsiella* are also known to produce both capsule and slime polysaccharide with identical chemical structures. Other bacteria such as *Rhizobium*, *Agrobacterium*, and *Alcaligenes* can produce more than one chemically distinct capsular polysaccharide (Sutherland, 1988).

Capsular polysaccharides of bacteria are complex and heterogeneous in nature ranging from homopolysaccharides to heteropolysaccharides. They are formed from a wide range of monosaccharides. Neutral sugars such as D-glucose, D-galactose, D-

mannose, and 6-deoxy hexoses are the most common constituents. Both amino sugars and uronic acids are also common. Many capsular polysaccharides may also contain non-sugar constituents including phosphate, formate, pyruvate, and acetyl groups (Sutherland, 1977 and 1985; Hammond *et al*, 1984).

Capsules produced by *Klebsiella* are heteropolysaccharides consisting of oligosaccharides with a degree of polymerisation ranging from 2 to 6 sugar units. The chemical composition is restricted to a few sugars namely, D-glucose, D-galactose, L-fucose, L-mannose, L-rhamnose, D-glucuronic acid, and D-galacturonic acid.

The chemical structures of most of the 77 serotypes of *Klebsiella* have been characterised. A comparative study by Sutherland (1979) showed little variation in the composition of monosaccharide units. However the arrangement of these sugar units within the oligosaccharide resulted in structural variation. The repeating unit of *Klebsiella* K1 capsule has been established by Erbing *et al* (1976). It consists of a linear trisaccharide repeat as illustrated below.



The repeat contains a 1,3-linked α-D-glucose, a 1,4-linked ß-D-glucuronic acid, and a 1,4-linked ß-L-fucose residue. The molecule also contains pyruvate ketals attached to glucuronic acid.

The sugar derivatives (mostly glucuronic acid) and non-sugar constituents attached to side chains function as determinant groups. The immunodeterminant sugar that contributes to serologic specificity can be terminal or within the polysaccharide chain. In *Klebsiella*, immunodeterminant sugars usually reside within the side chain

rather than the backbone of the polysaccharide. Because of the restricted number of sugar moieties, the structure of capsules of different serotypes may have similar regions. The degree of similarity determines whether one serotype cross-reacts with the antiserum of another serotype. Cross-reactivity of two antigens means that the two capsular antigens have the same, but not necessarily identical, antigenic determinants.

Studies on cross-reactions of more than 60 capsular, type-specific *Klebsiella* polysaccharides in 26 specific type anti-pneumococcal antisera has enabled the assignment of several structural features, such as non-reducing terminal residues of D-glucuronic acid, L-rhamnose, D- galacturonic acid, and the linkage of some monosaccharide chains (Heidelberger and Nimmich, 1976; Heidelberger *et al*, 1978).

Klebsiella capsules are also very similar to group I capsules of *E. coli* (Table 1.1). The structure of K5 and K54 antigens are structurally the same as *E. coli* K55 and K28 respectively (Altman and Dutton, 1985). In addition, the O8, O9-, and O20-specific LPS in which group I capsule are co-expressed, have the same structure as *Klebsiella* O3, O5, and O4 LPS respectively (Jansson *et al*, 1985).

The lipid-linked polysaccharides are integral constituents of the outer membrane of Gram-negative bacteria. The LPS consists of a hydrophobic moiety (lipid A) linked to the polysaccharide moiety (O-specific polysaccharide) through an oligosaccharide linkage region, the core (Jann and Jann, 1987). Lipid A which is integrated into the outer membrane bilayer consists of a fatty acid chain linked to glucosamine. The polysaccharide which is the serologically dominant part of the LPS molecule, is responsible for the diversity of O-antigenic types on the Gram-negative bacterial surface.

The O-specific polysaccharide is composed of repeating oligosaccharide units, and may also contain rare sugars. As a result of variation in the sugar composition and in the configuration of glycosidic linkage, the structure is highly diverse per unit structure. The polysaccharides that contain acid components such as hexuronic acid and glycerol phosphate are synthesised by some *E. coli* strains. Other Gram negatives produce neutral O-specific polysaccharides.

In *K. pneumoniae*, 8 structurally distinct O antigens (O1, O3, O4, O5, O7, O8, O9 and O12) have been characterised (Orskov and Orskov, 1978; Kenne and Linderberg, 1983) amongst which the O1 serotype is the most common. The O1 serotype is composed of α-D-Gal*p*-1-3-ß-D-Gal*f* repeating units. Recent chemical analysis of the O1 polysaccharide of *Klebsiella* demonstrated the presence of two distinct polysaccharides, D-galactan I and D-galactan II (Whitfield *et al*, 1991; Kol *et al*, 1991). Both are homopolymers of galactose. D-Galactan I is composed of a repeating unit of →3)-ß-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→. D-Galactan II is composed of a repeating unit of →3)-α-D-Gal*p*-(1→3)-ß-D-Gal*p*-(1→. It has a higher molecular weight, is less heterogenous among various strains than D-galactan I, and confers resistance to serum (McCallum *et al*, 1989; Whitfield *et al*, 1991). The neutral polysaccharides of *K. pneumoniae* O3 and O5 are homopolysaccharides containing 2- and 3-linked mannose residues and are identical with that of *E. coli* 09 and 08 respectively.

1.4.2. Biosynthesis of capsule

Synthesis of polysaccharides is accomplished through a series of biochemical reactions that occur in different cellular compartments. The initial stage of biosynthesis

of surface polysaccharides is generally similar , suggesting that they share a common carrier system (Tonn and Gander, 1979; Troy, 1979). Initial formation of polysaccharide starts in the cytoplasm with the synthesis of sugar residues and their activation by inter-conversion into nucleotide derivatives. Subsequent polymerisation is mediated by an inner membrane-bound transferase complex. The polymerised polysaccharide is then transported from the cytoplasmic membrane via the periplasmic space and outer membrane and deposited on the cell surface as extracellular polysaccharide (Jann and Jann, 1990).

The isoprenoid alcohol is involved in the synthesis of polysaccharide polymers consisting of oligosaccharide repeating units such as the O antigen components of LPS (Wright *et al*, 1967) and peptidoglycan (Higashi *et al*, 1967). The biosynthesis of CPS is also dependent on various intermediates, especially those containing isoprenoid alcohols (Sutherland, 1977). In spite of the similarities in the early stages of biosynthetic mechanisms of all surface polymers, some biochemical aspects of CPS including the mechanisms of post polymerisation-modification, translocation , and the mode of attachment to the cell surface remain obscure.

Little is known about the biosynthesis of *Klebsiella* and *E.coli* group I CPS. The biosynthesis of CPS studied by Troy *et al* (1971) is the most pertinent source of information to date. They reported the role of lipid intermediates in the synthesis of CPS in particulate cell envelope fractions from *K. aerogenes*. The carrier phospholipid identified as undecaprenyl phosphate ($P\text{-}C_{55}$) was identical to that involved in the biosynthesis of LPS and peptidoglycan. Based on biochemical and enzymatic analyses the sequence of biochemical reactions was then proposed:

i) UDP-gal + P-C_{55} → Gal-P-P-C_{55} + UMP;

ii) Gal-P-P-C_{55} + GDP-Man → Man-Gal-P-P-C_{55} + (UDP);

iii) Man-Gal-P-P-C_{55} + UDP-GlcUA → GlcUA-Man-Gal-P-P-C_{55}+(UDP);

iv) GlcUA-Man-Gal-P-P-C_{55} + UDP-Gal → Gal-(GlcUA)-Man-Gal-P-P-C_{55} + (UDP).

The sugar derivative is sequentially transferred to the lipid carrier to form di-, tri-, and tetrasaccharide derivatives. The tetrasaccharide linked to the lipid carrier is eventually polymerised to form the capsular polymer. The cell-free synthesised polysaccharide was identical to the native *K. aerogenes* capsular polysaccharide which was composed of galactose, mannose, and glucuronic acid. There is also evidence that the above-mentioned biochemical reactions operate in *E. coli* and other types of *Klebsiella* capsules. Indirect involvement of the oligosaccharide-lipid intermediates in the syntheses of colanic acid and group II CPS of *E. coli* has also been reported (Whitfield and Troy, 1984).

1.5. Genetic regulation of capsule biosynthesis

There is still a paucity of information about the genetic mechanisms that control the biosynthesis and diversity of bacterial capsules in general and *Klebsiella* polysaccharides in particular. With the application of molecular techniques, the genetic regulation of some polysaccharides, such as group II and related capsules has been well characterised.

Table 1.1 Classification of *E.coli* capsules (Jann and Jann, 1990).

Property	Capsule group	
	Group I	Group II
Molecular weight	>100,000 Dal	<50,000 Dal
Acidic component	Glucuronic acid Galacturonic acid Pyruvate	Glucuronic acid NeuNAc KDO, Phosphate
Expressed at <20°C	Yes	No
Co-expressed with	O8, O9,O20	Many O antigens
Lipid at the reducing end	Core-lipid A	Phosphatidic acid
Removal of lipid at pH 5-6/100°C	No	Yes
Chromosome determination at	*rfb(his), rfc (trp),*	*kps (serA)*
CMP-KDO synthetase activity elevated	No	Yes
Intergeneric relation with	*Klebsiella*	*H. influenzae, N. meningitidis*

1.5.1. Group II *Escherichia coli* capsule

E. coli capsules are generally placed into at least two groups, group I and II. The classification is based on several features: molecular weight, chemical composition, mode of expression, and genetic determination (Jann and Jann, 1987, 1990; Table 1.1). Unlike group I, group II capsules of *E. coli* are homopolysaccharides and their structures are less complex. Group II capsules of *E. coli* also share common features with other Gram-negative bacterial capsules including those of *N. meningitidis* and *H. influenzae*. The existence of a possible third group *E. coli* capsules, group I/II has also been suggested by Finke *et al* (1990). Unlike group II capsules, group I/II capsules are expressed at 18°C and do not have elevated CMP-KDO synthetase activity.

The genes encoding for group II capsules of *E. coli* K1 (Silver *et al,* 1981; Echarti *et al,* 1983), K5, K7, K12, and K92 (Roberts *et al,* 1986) have been cloned and well characterised. In *E. coli* K1 about 17 kb DNA is required for the complete expression of the capsule (Boulnois *et al,* 1987). DNA hybridisation, mutational and complementation analyses have revealed similarity in the genetic organisation of group II capsule genes.

The gene cluster known as *kps* is located near *serA* on *E. coli* linkage map (Vimr, 1991). It is divided into three functionally distinct regions. Region 2 encodes for sugar units and is flanked on each side by regions 1 and 3 which encode general functions. All the enzymes necessary for the synthesis, activation, and polymerisation of CPS are encoded within region 2 (Boulnois *et al,* 1987). Region 2 which determines the primary structure of capsule, is unique for a given K antigen and mutation within this region will result in the total loss of capsule production (Boulnois

and Jann, 1989).

Analysis of several *kps* gene clusters shows that the size of the central region (region 2) is related to the complexity of the K antigen in each strain. For example, an *E. coli* K4 capsule, a fructose-substituted chondroitin is complex in structure. Region 2 that encodes it is also large in size and shows very little homology with region 2 of other serotypes (Drake *et al*, 1990). In contrast, region 2 determinants of the K1 and K5 capsules are similar and share common restriction sites (Roberts *et al*, 1986).

Region 3 gene products are involved in the translocation of polysaccharide across the cytoplasmic membrane (Boulnois and Jann, 1989). Transposon insertions that fall within region 3 result in the deposition of synthesised K antigen polysaccharide in the cytoplasm. This region has been sequenced and seems to be conserved in all serotypes analyzed (Smith *et al*, 1990; Pavelka *et al*, 1991).

Two genes, *kpsM* and *kpsT* which encode 29,600 and 24,900 Dal proteins respectively have been characterised (Pavelka *et al*, 1991). These proteins have been proposed to act in translocation of the polysaccharide across the cytoplasmic membrane The KpsM protein is assumed to be an integral membrane protein while KpsT contains a consensus ATP-binding domain. Both belong to the family of prokaryotic and eucaryotic proteins with transport function, "traffic ATPases" or ABC (ATP-binding cassettes) transporters (Ames *et al*, 1990; Higgins e*t al*, 1990; Hyde *et al*, 1990).

Region 1 encodes for proteins that are involved in the translocation of mature polysaccharide across the outer membrane and its subsequent expression on the surface of the cell (Boulnois and Roberts, 1990). There is a high percentage of

homology between region I determinants from different *kps* gene clusters. Five polypeptides from region I of *E. coli* serotypes K1, K5, and K7 have been reported (Silver *et al*, 1984; Roberts *et al*, 1986). Mutations that occur within region I will result in the accumulation of matured polysaccharide in the periplasmic space (Boulnois *et al*, 1987).

1.5.2. *Neisseria meningitidis* capsule

The capsules of *N. meningitidis* group B share common features with those of *E. coli* K1 capsules. Both are structurally and immunologically identical but their genetic determinats share no DNA homology (Echarti *et al*, 1983). However, analysis has revealed a similar genetic organisation of capsular genes (Frosch *et al*, 1991).

The capsule polysaccharide synthesis (*cps*) genes of *N. meningitidis* group B have been characterised into five functional groups. The central region (region A) contains a biosynthetic gene. The sialyltransferase encoded by region A has similar amino acid sequence with that of the sialyltransferase encoded by the *neuS* gene of region II of *E. coli* K1 *kps*. Region B and C encode proteins that are involved in the translocation of polysaccharide from the cytoplasm to the periplasm and from the periplasm to the cell surface respectively. In addition, regions D and E have been identified as regulator genes (Frosch *et al*, 1989).

1.5.3. *Haemophilus influenzae* capsule

There are six serotypes in *H. influenzae*, type a to type f. Type b strains are responsible for over 95% of invasive disease in humans. The structure of type b capsule is similar to that of *E. coli* K100. The genes encoding for *H. influenzae* type

b capsule, *cap b* have been characterised. About 98% of the clinical isolates of type b *H. influenzae* strains contain a tandem duplication of 17 kb in their chromosome (Hoiseth *et al*, 1986; Kroll and Moxon, 1988). The tandem repeats are separated by a small DNA segment (about 1.3 kb size) known as the bridge region.

The bridge region is the critical factor in the expression of the capsule. A deletion of the DNA bridge leads to the complete loss of capsule synthesis, while a deletion of one copy of the 17 kb DNA merely reduces the level of polysaccharide synthesis (Kroll and Moxon, 1988). The loss of one of the tandem duplications with the bridge occurs by a *recA*-dependent recombination event (Kroll *et al*, 1988).

The genes encoding the capsules of *H. influenzae* have similar organisation to group II *E. coli* capsular genes. Like *E. coli kps* genes, the *cap b* genes are classified into three regions. Region 2 (central region) is involved in the biosynthesis of type specific polysaccharide, while region I and III (flanking regions) are involved in the transport of polysaccharide and are common in all serotypes (Kroll *et al*, 1989 and 1990).

Four gene products of region I of the *cap b* locus of *H. influenzae* termed BexA, BexB, BexC, and BexD (Kroll *et al*, 1990) share greater than 50% amino acid sequence homology to CtrA, CtrB, CtrC, and CtrD proteins respectively which are encoded by region C of *N. meningitidis cps* locus (Frosch *et al*, 1991). Some of the transport proteins are also homologous to *kps* gene products of region I and III of *E. coli* capsule genes. Similarity in the genetic organisation of capsule determinants among different species suggests a common origin of the genes for capsule production (Boulnois and Roberts, 1990; Frosch *et al*, 1991).

1.5.4. *Pseudomonas aeruginosa* capsule

P. aeruginosa produces an acetylated exopolysaccharide known as alginic acid. The polymer is composed of D-manuronic acid and L-glucuronic acid. Unlike other heteropolysaccharides, the molar ratio of the monosaccharides is highly variable. Alginate-producing strains are exclusively found in association with respiratory tract infections followed by cystic fibrosis. The protective alginate is considered as an important virulence determinant.

A cluster of genes that direct the biosynthesis of alginic acid has been mapped at 34 minutes of the standard genetic map of *Pseudomonas* (Deretic *et al*, 1987a). The *algD* gene that encodes GDP mannose dehydrogenase (Darzins et al, 1985; Deretic et al, 1987b), is regulated by three closely linked genes termed *algR*, *algP*, and *algQ* (Deretic and Konyecsni, 1989; Konyecsni and Deretic ,1990). The gene product of *algR* has been reported to be homologous to other gene products that control important cellular functions in response to environmental stimuli (Deretic *et al*, 1990). Studies have also shown that the overproduction of alginic acid is due to mutations in the *algST* and *muc* loci within the chromosome (MacGeorge *et al*, 1986; Flynn and Ohman, 1988).

1.5.5. Colanic acid

Many enteric bacteria such as *E. coli* K12 and *Salmonella* spp are capable of producing colanic acid. *Klebsiella* does not produce colanic acid polysaccharide but some K antigens such as K21 cross-react with antisera specific to colanic acid. Colanic acid has no role in contributing to virulence. However, it may protect the cell from desiccation in the environment. Because of its widespread nature and interesting

genetic regulation, it has been well studied.

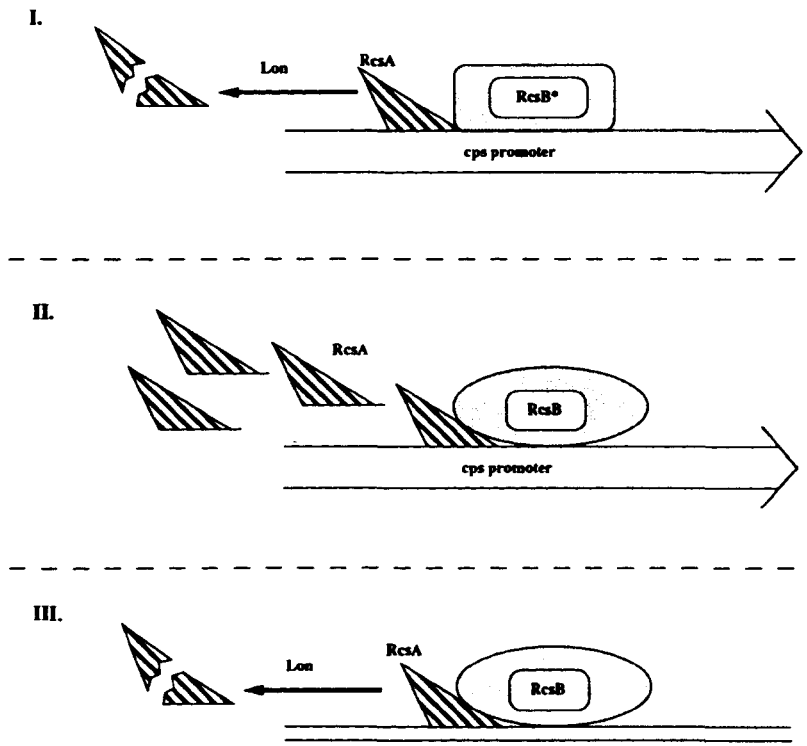
Colanic acid polysaccharide is similar to group I capsules of *E. coli*. The organisation of the *cps* genes is not well understood. In *E. coli* K12, the largest cluster of *cps* genes (*cpsA-E*) is located at 42 min adjacent to *rfb* and near *his* operon while *cpsF* is located at 89 min (Trisler and Gottesman, 1984). The *cps* cluster might include genes that are involved in the synthesis, polymerisation, transport and modification of the capsule (Gottesman and Stout, 1991). The genes are regulated at the transcriptional level by the *lon* gene and three regulator genes known as *rcsA*, *rcsB*, and *rcsC* (Trisler and Gottesman, 1984; Gottesman *et al*, 1985).

In *E. coli* K12, the *lon* gene directs the synthesis of ATP-dependent protease. Mutations at the *lon* locus result in sensitivity to UV irradiation and overproduction of colanic acid. In lon^+ cells the RcsA protein, an activator of *cps* genes is unstable and rapidly degraded by Lon protease. Overproduction of colanic acid in lon^- mutant cells is due to the increased stability of the RcsA protein (Torres-Cabassa and Gottesman, 1987). The *rcsB* and *rcsC* genes that map near to each other at 48 minutes also regulate the biosynthesis of colanic acid. The former acts as a positive regulator while the latter acts as a negative regulator (Brill *et al*, 1988). The two gene products constitute a two component sensor-regulator system in which RcsC acts as a sensor of environmental stimuli and RcsB acts as an effector to induce polysaccharide synthesis (Stout and Gottesman, 1990).

In Gram-negative bacteria, a family of regulatory proteins has been characterised. Regulator proteins that control responses to environmental stimuli have similar protein sequences. The family of two-component regulatory system consists of a sensor protein that detects the environmental stimulus and transmits a signal to

an effector (regulator) component that elicits a response. This usually occurs at the transcriptional level. There are membrane proteins that share homology in their C-terminal domains. The effector proteins, which are cytoplasmic have similar amino acid sequences in their C-terminal domains (Ronson *et al*, 1987; Henner *et al*, 1988). The members of the regulatory proteins regulate various cellular functions including, oxygen limitation, chemotaxis, osmolarity, nitrogen, and phosphate limitations. The *E. coli* RcsB and RcsC proteins share homologous sequences with other two-component regulator proteins in their respective domains. RcsC has been shown to be a transmembrane protein with N-terminal and C-terminal domains. The N-terminal 298 amino acids domain is periplasmic and the C-terminal 614 amino acids domain is cytoplasmic (Stout and Gottesman, 1990).

Three possible alternative mechanisms for the activation of colanic acid polysaccharide synthesis have been proposed by Gottesman and Stout (1991; Fig 1.2). Environmental signals may activate RcsC protein to an active protein kinase. The active kinase will then phosphorylate RcsB protein. The activated RcsB protein may then stimulate high levels of capsule synthesis in the absence of RcsA protein. The second alternative system is the presence of abundant RcsA protein in *lon* mutant cells or presence of *rcsA* in a multicopy plasmid will induce high levels of capsule expression. This pathway does not require RcsC-dependent RcsB activation. The third alternative system occurs in the absence of high levels of RcsA or activated RcsB protein and may be common in wild type *E. coli K12* strains.



cps transcription	Form of RcsB	Accumulation of RcsA
High	Activated	Low
High	Inactivated	High
Low	Inactivated	Low

Figure 1.2. Model for the regulation of *cps* expression in *E. coli*. Three possible routes for *cps* activation (Gottesman and Stout, 1991). I. In cells in which RcsC is actively phosphorylating RcsB, most RcsB is phosphorylated. The activated RcsB induces a high level of *cps* expression. II. In *lon* mutant cells, non-phosphorylated RcsB may be competent to induce a high level of *cps* expression in the presence of abundant RcsA. III. In *lon*$^+$ cells the RcsA is limiting and the RcsB is not activated by phosphorylation, *cps* is not transcribed.

1.5.6. *E coli* group I and *Klebsiella* capsules

E. coli group I capsules are heteropolysaccharides and are complex in structure. Some of the capsules which belong in this group have many similarities with *Klebsiella* capsules. Little has been studied on the genetics of this group. There are two subgroups of *E. coli* group I capsular antigens, amino acid containing repeating units and amino acid free repeating units. The latter is very similar to the capsular antigens of *Klebsiella* (Jann and Jann, 1990).

E. coli group I and *Klebsiella* capsules are genetically determined at the *his*-linked *rfb* gene cluster (Schmidt *et al*, 1977; Laakso *et al*, 1988). According to the report of Schmidt *et al* (1977), a second *trp*-linked gene might be required for the complete expression of capsular antigen. It has also been suggested that a *trp*-linked gene may encode a K-specific polymerase which may be analogous to the *rfc* gene that encodes O antigen polymerase in *Salmonella* and some *E. coli* strains (Orskov *et al*, 1977). However other studies have shown that the transfer of *his*-linked genes into auxotrophic and noncapsulated *E. coli* K12 were enough for complete expression of group I capsules (Laakso *et al*, 1988). They suggested that either the *trp*-linked gene(s) might be located closer to *his* rather than previously reported or not required.

Klebsiella species produce 77 capsular antigens. Each serotype only produces one type of polysaccharide, and the expression is usually stable. However, lysogeny of *Klebsiella* strains with certain bacteriophages may alter the capsular phenotype. The loss of original K antigen by lysogenised *Klebsiella* K59 and expression of a new non-typable capsular polysaccharide is one example (Pruzzo and Satta, 1988). The instability of the mucoid phenotype shown by irreversible loss of capsule formation has also been reported in some serotypes (Barr, 1981). Spontaneous loss of capsule

formation has been reported in other bacterial species such as *H. influenzae*. The reversion of mucoid *Pseudomonas* into nonmucoid form at high frequency *in vitro* culture is not uncommon (Govan *et al*, 1979).

The mechanism of loss of capsule production in *Klebsiella* is not known. Barr (1981) suggested that the loss of capsular antigen in *Klebsiella* K21 was associated with the loss of plasmid(s). In another observation (Allen *et al*, 1988), the transfer of *Klebsiella* K36 genes by conjugation into *E. coli* K12 resulting in induction of mucoid phenotype was mediated by chromosomal transfer and was not plasmid-borne.

The *rcsA* gene from *Klebsiella* K21b (Allen *et al*, 1987) and K20 (McCallum and Whitfield, 1991) has been characterised. When introduced into *E. coli* K12, the *Klebsiella rcsA* gene positively regulates the synthesis of colanic acid. A second positive regulator gene, *rcsB* has also been identified from *Klebsiella* K21b. The *Klebsiella rcsA* gene can also regulate the expression of serospecific capsular polysaccharide (McCallum and Whitfield, 1991).

In *Klebsiella* K1 and K2 serotypes a high molecular weight plasmid has been associated with a mucoid phenotype and virulence (Nassif *et al*, 1989). Plasmid-encoded genes, *rmpA* and *rmpB* have been cloned and seem to show functional similarities to *rcsA* and *rcsB* (Nassif *et al*, 1989). The authors suggested that the synthesis of capsule in *Klebsiella* could be positively regulated by *rcs* genes while the mucoid phenotype may be under the control of *rmp* genes.

The structural genes that determine the biosynthesis of *Klebsiella* K2 capsules have been cloned by Arakawa and his colleagues (1991). A 23 kb chromosomal DNA fragment from K2 complemented noncapsulated mutants and all the strains expressed K2-specific capsule on their surfaces. Based on DNA hybridisation, complementation,

and immunological analyses, a cps_k (capsule polysaccharide synthesis) gene cluster was proposed as the structural genes for the biosynthesis of *Klebsiella* K2 capsule. The cps_k gene cluster (more than 15 kb size) is conserved among the strains of serotype K2. The hybridisation of this gene cluster with 43-47 minute portion of the *E. coli* K12 chromosome (Arakawa *et al*, 1991) may also corroborate with the idea that the genetic mechanisms of the biosynthesis of colanic acid, *Klebsiella* and group I *E. coli* capsules may be similar (Jann and Jann, 1990; Gottesman and Stout 1991).

1.5.7. Lipopolysaccharide

The biosynthesis of LPS is determined by several genes located at different chromosomal loci. The genes with their functions and phenotypes are listed in Table 1.2. There are two different mechanisms of LPS biosynthesis, *rfe*-dependent and *rfc*-dependent. Both mechanisms use *rfb* genes. The *rfb* gene cluster which is closely linked to *his* operon is responsible for the synthesis of several sugar nucleotides and the assembly of the O-specific repeating units (Orskov *et al*, 1977).

The synthesis of O antigen by many *Salmonella* spp and *E.coli* is *rfc*-dependent and *rfe*-independent. The *rfc* gene is located between *trp* and *gal* on the *E. coli* chromosomal map. The *rfc*-dependent synthesis of the O antigen is assembled by *rfb* gene products from sugar nucleotide donors on an undecaprenol pyrophosphate (carrier lipid) in the cytoplasmic membrane. The polysaccharide (O antigen) grows at the reducing end by adding nascent O antigen to the non-reducing terminus of the newly synthesised repeating unit (Jann and Jann, 1984). This polymerisation is mediated by a polymerase encoded by *rfc*. A defect in the *rfc* region results in a semi-rough (SR) mutant instead of the normal smooth (S) form due the presence of only one single

repeating unit attached to the core. A defect in *rfb* region results in a rough (R) phenotype.

The *rfe* gene located near *ilv* is known to be involved in the synthesis of enterobacterial common antigens (Meier and Mayer, 1985). It encodes N-acetyl-D-glucosamine-1-phosphate transferase which is involved in the synthesis of lipid I (Meier *et al*, 1992). The *rfe*-dependent synthesis of O antigens has been reported in *E. coli* O8, and O9 (Jann *et al*, 1982; Jann and Jann, 1987) and recently in *K. pneumoniae* O1 (Clarke and Whitfield, 1992), although its role is not clear. The *rfe*-dependent O polysaccharides either lack N-acetyl-D-glucosamine (GlcNAc) completely or contain GlcNAc in which lipid I is not involved in the synthesis. In these group antigens, polymerisation of the polysaccharide occurs by sequential transfer of the sugar constituents from GDP-mannose directly on to the non-reducing end of the growing polysaccharide. Lipid intermediates are not involved in the synthesis.

In *K. pneumoniae*, a *his*-linked *rfb* gene cluster is known to be responsible for the expression of the D-galactan I, a component of the O polysaccharide. The cloned rfb_{O1} gene cluster (7.3 kb) from *Klebsiella* K20 was sufficient to direct the synthesis of D-galactan I in *E. coli* or *S. typhimurium*, or to complement mutants of *Klebsiella* that were defective in producing D-galactan I (Whitfield *et al*, 1991; Clarke and Whitfield, 1992). However, *Klebsiella* mutants defective in producing D-galactan II were not complemented, suggesting that non-linked genes might be required.

Table 1.2. Genetic determinants of LPS (Jann and Jann, 1990).

Gene	gene products	Phenotype of mutation
rfa	Glycosyl transferase in core synthesis	R-LPS with complete core (O polysaccharide synthesised but not exported)
rfb	Nucleotide sugar synthetases and glycosyl transferase operating in the synthesis of repeating oligosaccharides	R-LPS with complete core (O polysaccharide is not synthesised)
rfc	Enzyme polymerising repeating oligosaccharides to O polysaccharides	SR-LPS with only one repeating unit on core lipid A
rfe	Unknown	R-LPS in bacteria with *rfe*-dependent synthesis, no polysaccharide synthesised (in *Salmonella* C1 and E and *E coli* O8 and O9
rfaL *rfbT*	Translocase, transferring the O-specific polysaccharide from lipid carrier to core lipid A	R-LPS with complete core (O polysaccharide synthesised but not exported)

Production of D-galactan I requires both *rfe* and *galE* genes (Clarke and Whitfield, 1992). In *S. typhimurium*, the *kdsA* and *kdsB* genes are involved in the synthesis of the inner region of R-core (Rick and Osborn, 1977), while the *rfa* gene cluster and some genes such as *galE*, *galU*, and *pgi* direct the synthesis of the inner core region (Makela and Stocker, 1984). The transfer of O-specific polysaccharide to the core lipid A, and the translocation of LPS are the same in both biosynthetic pathways. The O-specific polysaccharide is transferred by the *rfaL* and *rfbT* gene products (Sugiyama *et al*, 1991). The synthesised LPS is finally transported from its site of synthesis (cytoplasmic membrane) and exported to the outer membrane. Details of biosynthesis, translocation, export across the outer membrane, and regulation of LPS synthesis are not well understood.

1.6. Importance of polysaccharides

1.6.1. Industrial importance

Polysaccharides that possess novel and highly functional properties have been discovered from a number of microorganisms. Because of their ability to thicken, suspend, or stabilise aqueous systems, polysaccharides have been commercially exploited. They can form gels, act as binders, film-formers, lubricants, friction reducers, and emulsion stabilisers (Baird *et al*, 1983).

In the past three decades a number of bacterial polysaccharides such as xanthan gum (from *Xanthomonas campestris*), dextran (from *Leuconostoc* spp), curdlans (from *Alcaligenes faecalis*), and bacterial alginate (from *Pseudomonas* and *Azotobacter*) have been used commercially. Other exopolymers have reached a semi-commercial stage (Paul *et al*, 1986).

1.6.2. Medical importance

Because of the emergence of antimicrobial resistant pathogens, the treatment of some diseases is still not possible by antimicrobial agents. To date development of vaccines for the prevention of microbial diseases is the best alternative. In recent years significant progress has been made in preventing diseases caused by capsulated bacterial pathogens.

Most polysaccharides are immunogenic and provide effective immunity in prevention of diseases. Bacterial polysaccharide vaccines from *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* have proved effective and have been licensed. Immunoprotective studies using purified CPS vaccines from pathogenic bacteria such as *Klebsiella* (Jones and Roe, 1984; Cryz *et al*, 1985 and 1986; Roe *et al*, 1986; Trautmann *et al* 1988) and *Pseudomonas* (Garner *et al*, 1990) have been shown to protect experimental animals from infections. A polyvalent vaccine from pathogenic strains of *Klebsiella* serotypes has been tested in animal models and is currently on clinical trial (Cryz, 1990).

There are still many problems with regard to the efficacy of some CPS vaccines. These include the cause of vaccine failure due to acquired or genetic deficiencies in the host, and the age-related response such as the poor immunogenecity of K-antigen to children under the age of 2 years (Jennings 1990).

Because vaccination is one of the most effective ways of prevention, the basic research and the development of new and more effective polysaccharide vaccines have been enhanced. There is a sense of optimism that advances in the area of biochemistry, immunology and genetics will unravel the problems concerning polysaccharide vaccines.

1.7. Aims of the project

Although the structure of the capsule and its role in virulence has been well documented, the physiological conditions and the genetic regulation of the production of capsule is still not well understood. The aims of this work were characterise the physiological conditions that affect the production of polysaccharide capsule in *Klebsiella* to analyze the genetic determinants that regulate the biosynthesis of serospecific CPS in *K. pneumoniae*.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and enzymes

All chemicals used were analytical grade and obtained from either Sigma or BDH. Endonuclease enzymes, T4 DNA ligase, T4 polynucleotide kinase, Klenow enzyme, random hexanucleotide primer labelling kit, and DNA *in vitro* packaging extracts were obtained from Boehringer, Mannheim. DNA Sequenase kit was supplied by United States Biochemical Corporation. Oligonucleotide primers were synthesised at King's College, London.

2.1.2. Antibiotics and supplements

Stock solutions of antibiotics or amino acids were filter sterilised by passing through a sterile 0.2 μm membrane filter. Stock solutions of antibiotics were stored at -20°C in aliquots and the amino acids at 4°C. Antibiotics were added to media at the following final concentrations (μg ml^{-1}): ampicillin (50); chloramphenicol (30); kanamycin (50); nalidixic acid (20); streptomycin (100); tetracycline (15). Unless otherwise stated amino acids were added to a final concentration of 40 μg ml^{-1}.

2.1.3. Buffers and solutions

20 x SSC

NaCl	3 M
Trisodium citrate	0.3 M
	pH 7.0

TE buffer

Tris-HCl	10 mM
EDTA	1 mM
	pH 8

Tris-acetate buffer (TAE)

Tris acetate	40 mM
EDTA	1 mM
	pH 8

Tris-borate buffer (TBE)

Tris	89 mM
Boric acid	89 mM
EDTA	2 mM

Agarose gel loading buffer

Urea	4 M
EDTA	50 mM
Ficoll	8%($^{w}/_{v}$)
Bromophenol blue	0.1%($^{w}/_{v}$)

SM buffer

NaCl	100 mM
$MgSO_4.7H_2O$	8 mM
Tris-HCl, pH 7.5	50 mM
Gelatin	0.001($^{w}/_{v}$)

Phosphate buffer saline (PBS)

NaCl	150 mM
KCl	2.7 mM
NaH_2PO_4	9.23 mM
KH_2PO_4	1.8 mM
	pH 7.4

Tris buffer saline (TBS)

Tris-HCl	10 mM
NaCl	150 mM
	pH 7.5

Saline tris EDTA (STE)

Tris-HCl	10 mM
NaCl	100 mM
EDTA	1 mM
	pH 8

Solution I

Glucose	50 mM
Tris-HCl, pH 8	25 mM
EDTA	10 mM

Solution II

NaOH	0.2 M
SDS	1%

Solution III

Potassium acetate	3 M
Glacial acetic acid	11.5%(v/v)

TEN buffer

Tris-HCl	20 mM
NaCl	1 M
EDTA	5 mM
	pH 8

Denaturing solution

NaOH	0.5 M
NaCl	1.5 M

Neutralising solution

Tris-HCl	1 M
NaCl	1.5 M
	pH 7.4

Prehybridisation buffer

6 x SSC

0.5% SDS

5 x Denhardt's reagent

100 μg ml^{-1} denatured calf thymus DNA.

Hybridisation buffer

Prehybridisation buffer containing 10 mM EDTA.

SDS-PAGE

1. Stacking gel solution

Acrylamide	4%($^{w}/_{v}$)
Tris-HCl, pH 6.8	0.125 M
SDS	1%($^{w}/_{v}$)
Ammonium persulphate	0.1%($^{w}/_{v}$)
TEMED	0.07%($^{w}/_{v}$)

2. Separating gel solution

Acrylamide	10%
Tris-HCl, pH 8	0.3 M
SDS	1%($^{w}/_{v}$)
Ammonium persulphate	0.1%($^{w}/_{v}$)
TEMED	0.07%($^{v}/_{v}$)

3. 2 x Gel loading buffer

Tris-HCl, pH 6.8	0.125 M
SDS	4%($^{w}/_{v}$)
Glycerol	20%($^{v}/_{v}$)
2-Mercaptoethanol	10%($^{v}/_{v}$)
Bromophenol blue	0.002%($^{w}/_{v}$)

4. Running buffer

Tris-HCl pH 8.3	0.025 mM
Glycine	0.125 M
SDS	1%($^{w}/_{v}$)

5. Staining solution

Coomassie blue R-250	0.125%($^{w}/_{v}$)
Methanol	50%($^{v}/_{v}$)
Acetic acid	10%($^{v}/_{v}$)

6. Destaining solution

Methanol	50%($^{v}/_{v}$)
Acetic acid	10%($^{v}/_{v}$)

Transfer buffer

Tris-HCl, pH 8	0.1 M
Glycine	0.15 M
Methanol	20%($^{v}/_{v}$)

2.1.4. Growth media

All growth media were sterilised by autoclaving at 121°C for 15 min unless otherwise specified. All sterile antibiotics and supplements were added to the sterile and cooled media when necessary.

Luria broth (L-broth)

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Distilled water to	1000 ml
	pH 7.0

Luria agar (L-agar)

L-broth containing 1.5%($^{w}/_{v}$) agar.

Nutrient broth (NB-2)

Nutrient broth No.2	25 g
Distilled water to	1000 ml

Nutrient agar (N-agar)

NB-2 containing 1.5%($^{w}/_{v}$) agar.

MacConkey agar

MacConkey agar No.2	51.5 g
Distilled water to	1000 ml

M9 Minimal medium

Na_2HPO_4	40 mM
KH_2PO_4	20 mM
NaCl	8.6 mM
NH_4Cl	20 mM
Glucose	44 mM

M9 minimal agar

Minimal medium containing 1.5%($^w/_v$) agar.

K-medium

M9 medium supplemented with 0.2 mM MgSO4.$7H_2O$, 1 mM $CaCl_2.2H_2O$, 0.2%($^w/_v$), and 0.001%($^w/_v$) thiamine.

Hershey medium

NaCl	100 mM
KCl	40 mM
NH_4Cl	20 mM
$CaCl_2.2H_2O$	0.1 mM
$MgCl_2.6H_2O$	1 mM
$FeCl_3.6H_2O$	0.8 mM
KH_2PO_4	0.6 mM
Tris-HCl, pH 7.4	100 mM

Supplemented with 0.4%($^w/_v$) glucose, 0.02%($^w/_v$) arginine, 0.02%($^w/_v$) proline, 0.01%($^w/_v$) leucine, 0.01%($^w/_v$) threonine, and 0.001%($^w/_v$) thiamine.

Minimal salt medium A (MSM-A)

$(NH_4)_2SO4$	11.35 mM
K_2HPO_4	8.6 mM
$MgSO_4.7H_2O$	6 mM
$CaCl_2.2H_2O$	0.34 mM

$Na_3C_6H_5O_7.2H_2O$	0.17 μM
$FeSO_4.7H_2O$	3.6 μM
$MnCl_2.4H_2O$	5 μM
Na_2MoO_4	5 μM
$ZnSO_4.7H_2O$	3.5 μM
Glucose	83.3 mM

Minimal salt medium B (MSM-B)

MSM-A containing 3.8 mM $(NH_4)_2SO_4$.

Minimal salt medium C (MSM-C)

MSM-A containing 0.76 mM $(NH_4)_2SO_4$.

Minimal salt medium D (MSM-D)

MSM-A containing 5.6 mM glucose.

2.1.5. Other materials

Membrane filters pore size 0.2 μm and Whatman (1 and 3 MM) papers were from Whatman Limited. Nitrocellulose filters (Grade BA85, 0.45 μm pore size) were supplied by Schleicher and Schull. All fermenter accessories were obtained either from F.T. Biotechnology or L.H. Fermentation Limited. Agarose (electrophoresis grade) was supplied by FMC Corporation. Polaroid type 655 films and X-Omat X-ray films were from Kodak Limited. X-ray film developer (Ilford PQ universal developer) and fixer (Ilford Hypam fixer) were from Ilford Limited. DNA sequencing gel concentrate and buffer were supplied by National Diagnostics, Atlanta, USA. Plasmid extraction kits were obtained from Qiagen Limited.

2.1.6. Bacterial strains

Bacterial strains used in the study are listed in Table 2.1 and 2.2.

2.1.7. Plasmids

Plasmids used or constructed in this study are listed in Table 2.3.

Table 2.1. *Klebsiella pneumoniae* strains.

Strain	Serotype/genotype	Reference
3L100	O1:K1, reference strain	PHLS*, London
3L101	O1:K1 Km^r	Gift from Dr. R.J. Jones
3L102	O1:K1, Km^r, nonmucoid, derivative of 3L101	This work
3L103	O1:K1, cured (Km^s), derivative of 3L101, Nal^r	This work
KNM101 to 103	01:K^-, derivatives of 3L100, Tc^r	This work
KNM1 to 23	O1:K^-, derivatives of 3L103, Km^r	This work
3L104	01:K2, reference strain	PHLS, London
M10	O1:$K2^-$	Poxton and Sutherland (1976)
3L105	O1:K3 reference strain	PHLS, London
3106 (889/50)	O1:K20	Laakso *et al* (1988)
3L107 (KD2)	01:K^-, Sm^r	"
3L108	O1:K21, reference strain	PHLS, London

*: Public Health Laboratory Services

Table 2.2. *Escherichia coli* strains.

Strain	Relevant genotypes.	Reference
HB101	F⁻, *hsd*S20(*rk*⁻,*mk*⁺), *rec*A13, *leu*, *pro*, *thi*, *lac*Y1, *gal*K2, *ara*14, *mtl*, *sup*E44, *xyl*-5, *rps*L20, *end*A, Sm^r	Boyer and Rouland-Dussoix (1969)
DH1	F⁻, *hsd*R17(*rk*⁻, *mk*⁺), *rec*A1, *gyr*A96, *thi*, *sup*E44,	Hanahan (1983)
LE392	F⁻, *hsd*R514(*rk*⁻,*mk*⁻), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*55,	Sambrook *et al* (1989)
AB1157	F⁻, *thr*-1, *leu*-6, *thi*-1, *lac*Y1, *gal*K2, *ara*-14, *xyl*-5, *pro*A2, *his*-4, *arg*E3, *tsx*-33, *str*-31	Sambrook *et al* (1989)
JC12186	*rec*B21, *rec*C22, *sbc*B15, *rec*J154, and all AB1157 markers	Horii and Clarke (1973)
JC7623	*rec*B21, *rec*C22, *sbc*B, and all AB1157 markers	"
PA360	(O⁻K⁻), *thi*-1, *leu*-B6, *his*G1, *ser*A1, *arg*H1, *thr*-1, *lac*Y1, *gal*-6, *mal*A1, *xyl*-7, *mtl*-2, *rps*L9 *ton*A2, *sup*E44, Sm^r	Laakso *et al* (1988)
CSH26▲F6	(*lac*, *pro*), *ara*, *thi*, (*rec*A, *srl*) *rps*L	Stocker *et al* (1984)
SM10	*thi*, *thr*, *leu ton*A, *lac*Y1, *rec*A::*RP4-2-Tc::Mu*::	Simon *et al* (1983)
PA360A and PA360B	*E. coli* PA360 transconjugant containing R-prime plasmid, *his*⁺, Tc^r, Ap^R, Km^r	This work
JC7623A and JC7623B	*E. coli* JC7623 transformant containing *his* region of K1 in the chromosome	This work

Table 2.3. Plasmids.

Plasmid	Characteristics	Reference
pBR322	Tc^r, Ap^r	Bolivar *et al* (1977)
pACYC184	Tc^r, Cm^r	Chang and Cohen (1978)
pEMBLCOS4	Tc^r, Ap^r, *cos*λ	Roberts *et al* (1986)
pHC79	Tc^r, Ap^r, *cos*λ	Hohn and Collins (1980)
pLV59	Tc^r, Cm^r	O'Connor and Humphreys (1982)
pUC19	Ap^r	Yanisch-Perron *et al* (1985)
R64-drd-11	R64, Tc^r, Sm^r	Meynell and Datta (1967)
pHSG415	ts, Ap^r, Km^r, Cm^r	Hashimoto-Gotoh *et al* (1981)
pHSG415::Tn10	ts, Tc^r, Km^r, Cm^r	Gacesa *et al* (1987)
pRT733	*Ori*R6K, *tra*$^-$, *mob*$^+$, Ap^r, Km^r	Tayler *et al* (1989)
pULB113 (RP-mini-Mu)	*tra*$^+$, Ap^r, Km^r, Tc^r(Mu3A)	Van Gijsegem and Toussaint (1982)
pLV213	Cm^r, pLV59 containing *rcsA* from K21b	Allen *et al* (1987)
pCOS47	Ap^r, 42 kb DNA fragment of K1 in pEMBLCOS4	This work

Plasmid	Characteristics	Reference
pCOS222	Ap^r, 40 kb DNA fragment of K1 in pEMBLCOS4	This work
pCOS261	Ap^r, 35 kb DNA fragment of K1 in pEMBLCOS4	This work
pCOS596	Ap^r, 40 kb DNA fragment of K1 in pEMBLCOS4	This work
pCOS474	Ap^r, 28 kb subclone of pCOS47	This work
pCOS474A	Ap^r, pCOS474 containing 2.4 kb *BglII* fragment of pLV591	This work
pACC6	Cm^r, 14 kb subclone of pCOS596 in pACYC184	This work
pACC7	Cm^r, 2.5 kb, subclone of pCOS596 in pACYC4	This work
pLV591	Cm^r, 12 kb DNA fragment of K1 in pLV59	This work
PLV592	Cm^r, 2.5 kb subclone of pLV591 in pACYC184	This work
pLV5962	Cm^r, 1.5 kb subclone of pLV591 in pACYC184	This work
pLV593	Tc^r, 1.5 kb *PstI* fragment of pLV592 in pBR322	This work

Plasmid	Characteristics	Reference
pLV5964	Tc^r, 1 kb *BglII/ClaI* fragment of pLV5962 in pACYC184	This work
pKLS1	65 kb, resident plasmid from 3L01	This work
pKC50	Cm^r, 4 kb *Bam*HI fragment of pKLS1 in pACYC184	This work
pKLS12	90 kb, Km^r, conjugative plasmid from 3L101	This work
pACTN103	Km^r, Tc^r, 22 kb	This work
pACTN12	Km^r, Tc^r, 28 kb	This work
pACTN9	Km^r, Tc^r, 16 kb	This work
pACTN15	Km^r, Tc^r, 18 kb	This work
pACTN18	Km^r, Tc^r, 30.5 kb	This work

Table 2.3 - continued.

2.2. Methods

2.2.1. Growth and maintenance of bacteria

Bacteria were grown in liquid or solid media supplemented with appropriate antibiotics and/or amino acids as required. Bacteria were stored at 4°C for short periods (one week) or at -70°C in 30%($^{v}/_{v}$) glycerol broth.

2.2.2. Flask shake culture

An overnight culture (1 ml) was inoculated into a series of 250 ml flasks containing 100 ml of MSM-A. All the flasks were incubated at 37°C for 18 to 48 h with shaking at 200 rpm.

2.2.3. Batch fermenter culture

A 1.5 litre capacity vessel with fermentation modules (Biolab minifermenter, F.T. Biotechnology) for the automatic control of pH, temperature, foaming, impeller speed, and gas flow rates was used for batch fermentation studies. To prevent phosphate precipitation, the pH of the medium in the vessel was adjusted to below 6. The vessel and the corrective solutions (2M KOH, 2M H_2SO_4, and 1%($^{v}/_{v}$) PPG 2025 as antifoam) were sterilised at 121°C for 30 min. Glucose was sterilised separately and added to the sterilised and cooled medium. The pH and temperature were set at 7±0.1 and 37°C respectively. Air was pumped into the culture vessel through a sterile 0.45 μm Acro filter at 1500 cm^3 min^{-1}. An autoclavable electrode (Russel) was used to measure oxygen tension which was maintained above 20% saturation.

One ml of an overnight culture (grown in minimal medium) was inoculated into the vessel containing 1.5 litre of MSM-B. The culture was continuously agitated at 800 rpm. Samples were taken at regular time intervals through the sample port.

2.2.4. Continuous culture

Continuous culture studies used a 1 litre culture vessel attached to fermentation modules (L.H. Fermentation) for controlling pH, temperature, aeration, agitation, and foaming. A reservoir of minimal salts medium (20 litres) was prepared and sterilised at 121°C for 1 h. The vessel with the assembled tubing and the corrective solutions were sterilised at 121°C for 30 min. The glucose component was sterilised separately and added to the sterile and cooled reservoir medium. The sterile medium was pumped into the vessel with an LKB peristaltic pump. The flow rate of the medium was determined by varying the speed of the pump. From each calibration the dilution rate could be determined according to the following equation:

$$D = f/v$$

where D= Dilution rate per hour.

f= flow rate (ml h^{-1}).

v= volume of culture in the vessel (ml).

The rate of air flow was maintained at 750 cm^3 min^{-1}. The culture was grown at 37°C, agitated at 1000 rpm, and the pH was initially maintained at 7.0±0.1.

An overnight culture grown in MSM-A was inoculated into the vessel containing 750 ml MSM-C or MSM-D which was grown until late exponential phase before pumping sterile medium into the vessel was initiated. The pump was then turned on and the culture grown at a constant dilution rate. After 4 turnovers (volume

replacements) samples were taken and new growth parameters were adjusted. When nonmucoid cells were detected in the culture, a new chemostat was set up. Mutants that grew in the culture were scored by serially diluting the sample and plating on to nutrient agar.

2.2.5. Quantitative determination of cell growth

Cell growth was measured by determining the optical density at 550 nm using an LKB spectrophotometer. The biomass (dry weight) was also determined from 50 ml culture sample by pelleting the cells at 12,000 rpm for 15 min at 4°C. The cells were then washed with distilled water, repelleted, and suspended in 2-3 ml distilled water. The suspension was transferred on to a pre-dried and weighed aluminium foil cap, dried at 100°C in an oven for 24 h, cooled and weighed.

2.2.6. Quantitative determination of exopolysaccharide

The exopolysaccharide was precipitated from cell-free supernatant culture with 3 volumes of cold ethanol at 4°C. The precipitate was dissolved in water, dried and weighed as above.

The exopolysaccharide was also determined by a colorimetric method that could detect up to 5 μg ml^{-1} of carbohydrate (section 2.2.8). To remove any sugar contaminant, the dialysis tubing was pre-washed in 10 mM EDTA containing 1%($^{w}/_{v}$) sodium carbonate. One ml of cell-free culture supernatant was dialysed against distilled water for 24 h.

2.2.7. Purification of capsular polysaccharide

Capsular polysaccharide was isolated from *K. pneumoniae* or mucoid *E. coli* by alkaline precipitation followed by chloroform extraction (Roe *et al*, 1989). Bacteria were grown in minimal salts medium or L-broth for 24-48 h at 37°C. The culture was centrifuged at 12,000 rpm for 1 h. The polysaccharide was precipitated with 0.5%($^{w}/_{v}$) N-acetyl-N,N,N-trimethylammonium bromide from the cell-free supernatant culture. After stirring for 1 h, the polysaccharide was collected by centrifugation at 4,000 rpm, for 15 min at 4°C. The crude polysaccharide was dissolved in 1 M $CaCl_2.2H_2O$. The nucleic acid was precipitated by addition of 25%($^{v}/_{v}$) ethanol and the mixture centrifuged at 4,000 rpm for 15 min at 4°C after which the supernatant was retained. The ethanol concentration was increased to 80%($^{v}/_{v}$) and the polysaccharide was precipitated at 4°C overnight. The precipitate was recovered by centrifugation and dissolved in distilled water. The protein was removed by extraction with an equal volume of chloroform: butanol (5:1). The aqueous layer was dialysed against tap water for 24 h and freeze-dried.

The cell pellet was freeze-dried and the capsule was isolated from cell surfaces by the phenol extraction method (Hancock and Poxton, 1988) and purified as above.

2.2.8. Determination of total carbohydrate

The total carbohydrate concentration was determined by a phenol-sulphuric acid method (Dubois *et al*, 1956) using dextran T-500 (Pharmacia) as a standard. Sample dissolved in 1 ml distilled water was mixed with 25 µl of 80%($^{w}/_{v}$) phenol solution in a glass test tube. To the mixture, 2.5 ml of concentrated H_2SO_4 was added and mixed. The reaction was kept for 20 min at room temperature and the optical

density was determined at 490 nm. For microassay, 100 µl of sample solution was mixed with 2.5 µl of 80% phenol solution and 250 µl of concentrated H_2SO_4. Dextran, as a standard solution was used prepared at a concentration of 5 to 100 µg ml^{-1} and was treated as above.

2.2.9. Determination of total protein

The protein concentration was estimated by the method of Bradford (1976) using Biorad reagent and BSA (Sigma) as a standard. 100 µl of sample solution was mixed with 5 ml protein reagent (Biorad). The mixture was mixed and the optical density was determined at 595 nm. BSA prepared at a concentration of 10 to 100 µg ml^{-1} was used to prepare a standard calibration curve.

2.2.10. Determination of 2-keto-3-deoxyoctonate

Lipopolysaccharide level was estimated by determining 2-keto-3-deoxyoctonate (KDO) following the procedure of Karkhanis *et al* (1976). One ml of 0.2 N H_2SO_4 was added to a glass test tube containing 1 to 2 mg of polysaccharide sample. The solution was mixed and heated for 30 min at 100°C. The sample was cooled and centrifuged for 5 min. 0.5 ml of clear solution was transferred into another test tube and 0.25 ml of 0.04 N periodic acid (HIO_4) in 0.125 N H_2SO_4 was added. After 20 min at room temperature, 0.25 ml of sodium arsenite ($NaAsO_2$) in 0.5 N HCl was added. The solution was mixed and kept at room temperature until the brown colour disappeared. Then 0.5 ml of 0.6%($^w/_v$) thiobarbituric acid was added, mixed and heated for 15 min at 100°C. To the hot mixture, 1 ml of DMSO was added and allowed to cool. The optical density was then determined at 548 nm against a blank treated as

above. Concentrations representing 0.5 to 10 µg of KDO (Sigma) were treated as above and used to prepare standard calibration curve.

2.2.11. Determination of residual glucose

The level of residual glucose in cultures was determined by the method of Gerhardt *et al*, (1981) using the glucose oxidase enzyme kit (SIGMA) and glucose as a standard. 500 µl of cell-free supernatant culture (diluted with water if required) was transferred into a test tube and 1 ml of enzyme-colour-reagent solution was added. The reaction was incubated for 30 min at 37°C in the dark. A drop of 2 N HCl was added and the optical density determined at 400 nm.

Combined-enzyme-colour reagent was prepared according to the manufacturers' recommendation. A standard glucose solution was prepared at a concentration of 50 to 300 µg ml^{-1} and treated as above.

2.2.12. Determination of ammoniacal nitrogen

The ammoniacal nitrogen in the culture was measured colorimetrically by a phenolate-hypochlorite method (Wainwright and Pugh, 1973). Phenolate solution was prepared on the day by mixing 20 ml of phenol solution (62.4 g phenol, 25 ml ethanol, and distilled water to 100 ml) and 20 ml of 0.675 M NaOH. One ml of cell-free culture supernatant was mixed with 3.5 ml distilled water. Phenolate solution (3.5 ml) and 1.5 ml of 0.9%($^{v}/_{v}$) sodium hypochlorite were added and the solution was thoroughly mixed. The optical density of the solution was then determined at 630 nm after 20 min. A standard solution of ammonium sulphate was prepared and used in the range of 5 to 50 µg ml^{-1} ammonia.

2.2.13. Gel filtration

A pre-packed Superose 6 HR 10/30 FPLC column (Pharmacia) was equilibrated with phosphate buffer, pH 7.0 containing 0.1 M NaCl. Purified polysaccharide (1 mg ml^{-1}) was applied to the column and the running buffer was set at a flow rate of 0.5 ml min^{-1} with continuous monitoring through a recorder at 280 nm. 0.5 ml fractions were collected and the total carbohydrate concentration of each fraction was determined by the phenol-sulphuric acid method (Dubois *et al*, 1956) as described earlier. Dextran T-200, T-500 , T150, and T-75 (Pharmacia) were also used as markers for molecular size estimation.

2.2.14. Paper chromatography

Ten mg of purified polysaccharide was hydrolysed in 3 M HCl at 100°C under a nitrogen atmosphere for 3 h. The solution was neutralised with 3 M NaOH and freeze-dried. The freeze-dried sample was dissolved in 100 µl distilled water. The hydrolysed products (25 µl each) were applied on to chromatography paper (Whatman No.1) 1.5 cm apart and dried at room temperature. The descending chromatography was run in butanol-1: acetic acid:1 M NaOH (2:3:1) solvent for 18 h. The sugars were detected by spraying with alkaline silver nitrate solution (Trevelyan *et al*, 1950; Sage *et al*, 1990).

2.2.15. Absorption of antiserum

Antiserum against *K. pneumoniae* K1 (whole bacterial cells) was raised in rabbit or mouse. Antiserum purified through 0.45 µm membrane filter was stored at -20 °C. Undiluted or diluted antiserum (1:200) was used for typing or screening.

Cross-reacting antibodies were removed by absorbing with whole cells. Cells from an overnight culture (50 ml) were suspended in 1.5 ml of antiserum and the suspension was stored at 4°C overnight. The cells were removed by centrifugation. The absorbed antiserum was stored at 4°C in the presence of 0.1% sodium azide.

2.2.16. Serotyping of strains

Serotyping of capsules was performed by a combination of various methods; counter-current immunoelectrophoresis (Palfreyman, 1978), phage typing (Gaston *et al*, 1987) and double immunodiffusion (Hancock and Poxton. 1988). Strains were also independently serotyped by PHLS, at Colindale, London.

Double immunodiffusion was performed in 1%($^{w}/_{v}$) agarose in 0.15 M NaCl. The gel was poured on to a 50 x 100 mm diameter gelbond sheet (Gelbond^{TM}, Marine colloid) to a thickness of 3 mm. Peripheral wells (3 mm diameter) and a central well (5 mm) were cut 5 mm apart. The rabbit antiserum raised against *Klebsiella* K1 was placed in the centre and the antigens in the peripheral wells. The plate was incubated at 4°C in a humid chamber for 24-72 h. The gel was placed on flat surface covered with several sheets of Whatman No.1 and compressed by covering with a glass plate which had a 1 kg weight on top of it. After 15 min, the press was dismantled and the gel washed in 10 mM NaCl and distilled water. The pressing was repeated, the gel dried, stained with Coomassie blue solution for 15 min after which the background was destained with several changes of destaining solution.

2.2.17. Enzyme-linked immunosorbent assay

ELISA technique was used for initial screening of cosmid clones as described previously (Roe and Jones, 1985; Roe *et al*, 1989). Cosmid clones were transferred into sterile microtiter plates (Nunc II) containing 250 µl of broth with 50 µg ml^{-1} ampicillin. The plates were incubated at 37°C for 24 h. The culture was discarded and the wells were washed 3x with PBS-T20 (PBS containing 0.05%($^{v}/_{v}$) Tween 20). *E. coli* absorbed antiserum was diluted to 1:200 with PBS containing 1%($^{w}/_{v}$) BSA and 100 µl was added to each well. The plate was incubated at 37°C for 1 h. The wells were washed as above and 100 µl of 1:1000 diluted peroxidase conjugated anti-rabbit antiserum (Dakopatts, Denmark) was added. After 1 h at room temperature, the wells were washed 3x with PBS-T20. To each well 100 µl substrate containing OPD (0.5 M citric acid, 0.1 M Na_2HPO_4, pH 5; 0.04 mg ml^{-1} OPD; 0.006% H_2O_2) was added and kept at room temperature for 30 min in the dark The reaction was stopped by addition of 100 µl of 2.5 M H_2SO_4. Colour development was visually detected and/or by reading at 490 nm using a Dynatech microreader.

2.2.18. Immunoblotting

A. Colony immunoblotting

Colonies were transferred on to a nitrocellulose filter and lysed in chloroform vapour for 30 min at room temperature. The filter was blocked with 2%($^{w}/_{v}$) BSA in TBS for 3 h at room temperature and transferred into absorbed antiserum diluted 1:200 in TBS containing 2% BSA. The reaction was incubated at room temperature for 1 h and washed 4 x with TBS. The filter was soaked in substrate buffer containing

0.6 mg of 4-chloro-1-naphthol, 0.1% H_2O_2, and 16.5% methanol in TBS. The reaction was stopped by washing briefly in distilled water.

B. Western blotting

Proteins were separated by polyacrylamide gel electrophoresis and analyzed by Western blotting as described by Hancock and Poxton (1988). Proteins isolated from whole cell lysates were electrophoresed in 15% polyacrylamide gels. The gel was transferred into transfer buffer and soaked for 45 min. Two sheets of 3 MM Whatman paper and nitrocellulose filter paper were cut larger than the size of the gel and wetted in the same buffer. The gel was placed on Whatman paper and the nitrocellulose paper was placed on top of the gel. The filter was then covered with a sheet of Whatman paper. The set up was then sandwiched between Scotchbrite pads and placed in the electroblotting apparatus (Biorad) filled with transfer buffer. Protein was transferred from the gel to the nitrocellulose filter for 4 h at 150 volts. After electroblotting, the filter was briefly washed and probed with anti-*Klebsiella* antiserum as described above.

2.2.19. Immuno-gold labelling

Cells were negatively stained by gold conjugated antiserum according to the method of Hancock and Poxton (1988). A loopful of overnight culture was suspended in distilled water and a formvar-coated copper grid placed in the suspension for 15 min. The grid was sequentially transferred into PBS (1 min), PBS containing 1% BSA (15 min), and anti-K1 antiserum (2 h). It was then briefly washed in PBS and placed into 15 *nm* gold particle conjugate antiserum. After 15 min, the grids were briefly

washed in PBS and stained with 1% phosphotungstic acid (pH 6) for 1 min. The grid was allowed to dry in air and observed under a Cora M1232 transmission electron microscope.

2.2.20. Isolation of chromosomal DNA

Large scale quantities of chromosomal DNA were prepared from 500 ml of overnight culture following the procedures of Marmur (1961).

Small scale chromosomal DNA was also prepared by a modification of Moxon *et al* (1984). The cell pellet from 5 ml of overnight culture was suspended in 0.1 M NaCl, 10 mM tris-HCl, and 10 mM EDTA. SDS was added to a final concentration of 1%($^{w}/_{v}$) and the mixture was incubated at 60°C for 10 min. The lysate was digested with proteinase K (1 mg ml^{-1}) at 37°C for 1 h. The protein was removed by extracting twice with phenol/chloroform and once with chloroform. The DNA was precipitated with 2 volumes of ice-cold ethanol and dissolved in TE buffer containing RNase at a concentration of 40 μg ml^{-1}.

2.2.21. Isolation of plasmid DNA

Large or small scale isolation of plasmid DNA was by the method of Sambrook *et al* (1989). Large scale quantities of plasmid DNA were prepared from 500 ml overnight cultures by the alkaline lysis method and purified by equilibrium centrifugation in a caesium chloride gradient containing ethidium bromide.

Miniplasmid preparations of plasmid DNA were performed as follows: 1.5 to 3 ml of an overnight culture was harvested by centrifugation at 12,000 rpm for 1 min, the cells were washed in 0.5 ml STE and suspended in 100μl ice-cold solution and

lysed by addition of freshly made 200 µl of solution II. To the lysed mixture, 150µl of ice-cold solution III was added, mixed, and kept on ice for 2 min. The suspension was centrifuged at 12,000 rpm for 5 min and the supernatant was extracted with phenol/chloroform. Finally, the DNA was precipitated with 2 volumes of ethanol washed in 70% ethanol, dried and dissolved in 50 µl TE buffer with 20 µg ml^{-1} RNase.

2.2.22. Quantitative determination of purified DNA

The concentration of purified chromosomal or plasmid DNA was determined by measuring the absorbance at 260 nm using a Perkin-Elmer spectrophotometer. An absorbance of 1 at 260 nm was assumed to contain 50 µg ml^{-1} double stranded DNA.

2.2.23. Agarose gel electrophoresis

DNA samples were run on horizontal agarose slab gels (0.4 to 1.5%$^{w}/_{v}$ containing 0.1 µg ml^{-1} ethidium bromide in TAE or TBE buffer. DNA solution was mixed with gel loading dye, loaded into the agarose gel slots, and separated by electrophoresis for 1 to 3 h at 50-100 volts. DNA bands were visualised using a 302 nm UV transilluminator and photographed on polaroid 665 film using a polaroid camera. Molecular sizes of DNA fragments were calculated by comparison of their mobilities with λ DNA digested with *Hin*dIII, SPPI DNA digested with *Eco*RI, or pBR328 DNA digested with *Bgl*II + pBR328 DNA digested with *Hin*fI.

2.2.24. Digestion and purification of DNA fragments

DNA samples were digested with endonucleases in the presence of recommended buffers at 37°C for 1 to 3 h. Chromosomal DNA was partially digested

with restriction enzyme. The digested DNA was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 500 µl TE buffer. A glucose gradient in the range of 10-to 40%($^{w}/_{v}$) in TEN was prepared in a 12 ml centrifuge tube. The DNA solution was loaded onto the gradient and centrifuged at 26,000 rpm for 24 h at 20°C. The bottom of the tube was punctured and 500 µl of gradient fraction collected. The fragment size of each fraction was analyzed by agarose gel electrophoresis. Fractionated samples containing 25 to 45 kb DNA fragments were pooled and dialysed gainst TE buffer (pH 7.8) at 4°C overnight. The sample was then extracted with phenol/chloroform and precipitated with ethanol and dried in a small volume of TE.

Small scale DNA fragments were also purified from agarose gels using a Qiaex-gel-extraction kit (Qiagen). The DNA fragment was excised from the gel and dissolved in solubilising buffer QX1. The DNA was bound with QIEAX suspension. The suspension was centrifuged and the pellet was washed with washing buffer QX2 and QX3. The DNA was finally eluted with small volume of distilled water.

Large size DNA fragments were also purified from the agarose gel by elution into dialysis tubing by the method of Sambrook *et al* (1989). The excised gel containing the DNA band of interest was placed in prewashed dialysis tubing and eluted by electrophoresis for 1 h at 150 volts. The current was reversed for 20 sec and the solution was transferred into a 1.5 ml tube. The solution was extracted once with phenol/chloroform and the DNA precipitated with ethanol and dissolved in TE.

2.2.25. Dephosphorylation and ligation of DNA

Cleaved vector or chromosomal DNA was treated with calf intestinal alkaline phosphatase for 30 min at 37°C according to the manufacturers' recommendation. The

enzyme was inactivated by heating at 56°C for 30 min in the presence of 50 μg ml^{-1} proteinase K. The mixture was extracted with phenol/chloroform, precipitated in ethanol in the presence of 0.1 volume of 3 M sodium acetate (pH 5.2) and the precipitate dissolved in a small volume of TE buffer.

Vector DNA (plasmid or cosmid) was ligated with insert DNA (5:1 molar ratio) using T4 DNA ligase in the presence of 1 x T4 ligase buffer and 1 mM ATP at 16°C for overnight.

2.2.26. Packaging and transfection

The ligated cosmid was packaged into bacteriophage heads using the DNA *in vitro* packaging kit (Boehringer, Mannheim). The packaged cosmid was diluted with 0.5 ml SM buffer and stored at 4°C with a drop of chloroform. *E. coli* was grown in 10 ml L-broth for 18 h in the presence of 0.2%($^{w}/_{v}$) maltose and 10 mM $MgCl_2$. The culture was centrifuged at 4,000 rpm for 10 min and the cells were suspended in 0.5 volume of sterile 10 mM $MgSO_4.7H_2O$. 100 μl of packaged cosmid and 200 μl of cell suspension were mixed together and incubated at 37°C for 20 min. One ml of fresh broth was added and further incubated for 45 min. The culture was then plated on L-agar containing the appropriate antibiotic.

2.2.27. Transformation

Competent cells were prepared as follows. A 10 ml volume of an overnight culture was inoculated into 1 litre of sterile L-broth and incubated at 37°C until OD 600 of 0.75 had been reached. The culture was chilled on ice for 30 min and centrifuged at 4000 rpm for 15 min. Cells were centrifuged 2 x with ice-cold sterile

deionised water and once with 10%($^{v}/_{v}$) ice-cold sterile glycerol. The cells were then suspended in 1.5 ml of 10% glycerol and 40 µl was dispensed into sterile cold tubes which were stored at -70°C.

Electroporation, a more efficient transformation method, was used to transform ligated plasmids into cells. 40 µl competent cells were thawed in ice and mixed with DNA (1-3 µg in 3 µl of TE buffer) and kept on ice for 1 min. The suspension was transferred into an ice-cold cuvette (0.2 cm electrode gap) and pulsed using a Biorad gene pulser apparatus set at 25 µF and 2.5 *k*V. One ml of fresh broth was added, transferred into a sterile universal tube, and incubated at 37°C for 1 h. The culture was diluted and plated on selective media.

2.2.28. Conjugation

The transfer of conjugative plasmids was performed as described by Taylor *et al* (1989). Samples (0.2 ml) of overnight broth cultures (or loopful of cells from an agar plate) of donor and recipient were mixed and plated on nutrient agar. The plate was incubated at 30 or 37°C for 6-18 h. The culture was suspended in 10 mM MgSO4.7H_2O, diluted and plated on selective media.

2.2.29. Plasmid curing

Cells (10^{-2} to 10^{-3}) were inoculated into fresh broth containing 1%($^{w}/_{v}$) SDS and incubated at 37°C for 4 days without shaking. Dilutions were made and plated on to non-selective media and incubated at 37°C for overnight. Individual colonies were patch-plated and the loss of antibiotic resistance marker determined

2.2.30. Southern hybridisation

A. Transfer of DNA to nitrocellulose filter

DNA from agarose gels was transferred to a nitrocellulose filter as described by Southern (1975). DNA samples were separated by electrophoresis. The gel was immersed in 0.2 M HCl for 15 min and briefly washed in distilled water. The gel was denatured in denaturing solution for 30 min. After briefly washing with distilled water, the gel was immersed in neutralising solution for 45 min and placed on 3 sheets of 3 MM Whatman paper pre-wetted with 10 x SSC and supported by a glass plate. The filter was placed on top of the gel and covered with 3 sheets of pre-wetted Whatman paper followed by 7 dry sheets of paper and a stack of paper towels. The blot was covered with a glass plate and a 0.5 kg weight was placed on top and blotting allowed to occur overnight. After dismantling, the filter was briefly washed in 6 x SSC, dried and baked at 80°C under vacuum for 1½ h.

Recombinant clones were also screened by *in situ* hybridisation following the procedure of Sambrook *et al* (1989). Colonies were transferred from the master plates on to nitrocellulose filters. Colonies were lysed for 5 min by placing on to 3 MM Whatman paper impregnated with 10% SDS. The filter was then sequentially transferred on to each 3 MM Whatman paper saturated with denaturing solution, neutralising solution, and 2x SSC. The filter was dried at room temperature for 30 min and baked at 80°C in a vacuum for 1½ h.

B. Radiolabelling of DNA probes

Plasmid DNA fragments separated by electrophoresis on 1%($^{w}/_{v}$) low melting agarose gel were cut out, melted, and diluted with distilled water. The DNA was

denatured at 100°C for 5 min and labelled with α^{32}P-dCTP (Amersham) using a random hexanucleotide primer labelling kit. Oligonucleotide probe was labelled with γ^{32}P-ATP using T4 polynucleotide kinase.

C. Hybridisation of immobilised DNA with the probe

The nitrocellulose filter was transferred into a sandwich box and 20 ml pre-hybridisation solution added. After 2 h incubation at 68°C the solution was replaced with 20 ml hybridisation buffer. The labelled probe was heated to 100°C for 5 min, chilled on ice and added to the filter immersed in hybridisation buffer. The box was sealed and incubated overnight at 68°C. The labelled oligonucleotide was hybridised with the DNA at 42°C in hybridisation buffer containing 25%($^{v}/_{v}$) formamide.

The filter was washed with 2 x SSC, 0.5% SDS for 5 min and with 2 x SSC, 0.1% SDS for 15 min at room temperature. The filter was further washed 4 x 30 min at 68°C with high stringent washing buffer (0.1 x SSC, 0.1% SDS). DNA hybridised with oligonucleotide probe was washed for only 15 min at room temperature. After washing briefly with 0.1 x SSC at room temperature, the filter was dried (or the wet filter if reprobing was required), wrapped in Saran wrap and set for autoradiography. The filter was attached to a 3 MM Whatman paper (24 x 18 cm) and placed on a cassette fitted with intensifying screens. The Kodak X-Omat AR film was exposed to the filter at -70°C for 18-72 h, developed and fixed.

2.2.31. Maxicell analysis

Plasmid-encoded proteins were selectively labelled in whole bacterial cells by the maxicell system of Sancar *et al* (1979) as outlined by Stocker *et al* (1984). Plasmid DNA was introduced into *E. coli* CSH26▲F6 which is defective in repairing DNA damaged by UV light. Plasmid-harbouring cells were grown in K-medium overnight and the culture diluted to 1:50 with fresh K-medium and grown to an optical density of 0.5 at 550 nm. A 2.5 ml portion of culture was transferred into a sterile petri dish (4.5 cm diam) and irradiated at 1 joule sec^{-1} mm^{-3} for 12 sec at 256 nm. The irradiated culture was incubated in the dark for 1 h at 37°C. D-cycloserine was added to a final concentration of 300 μg ml^{-1}. The culture was further incubated overnight with shaking and 1.5 ml was harvested, resuspended in 0.5 ml Hershey medium, and labelled with 25 μCi of [^{35}S] methionine for 1 h at 37°C. The cells were harvested and suspended in 40 μl of SDS sample buffer and the mixture heated at 80°C for 10 min and the proteins analyzed by SDS-PAGE. The gel was fixed in glacial acetic acid: methanol: water (10:20:70) for 30 min, dried at 80°C in a vacuum for 1½ h and analyzed by autoradiography.

2.2.32. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using a discontinuous buffer system described by Laemmli (1970). The vertical gel slab (140 x 130 x 1.5 cm) contained 4%($^{w}/_{v}$) acrylamide as a stacking gel and 10%($^{w}/_{v}$) acrylamide. The acrylamide and bisacrylamide was used at constant ratio of 37.5:1.

After electrophoresis at 150 volts for 4-6 h, the gel was stained in staining solution for 2 h. The background was destained with destaining solution.

2.2.33. DNA sequencing

Cloned DNA was sequenced by direct chain termination method using a Sequenase kit (US Biochemicals). The entire sequence of the cloned DNA was determined using the newly sequenced regions as primers.

A. Preparation of template

Recombined plasmid was purified by caesium chloride density centrifugation and denatured according to the method of Sambrook *et al* (1989). Three µg of plasmid DNA was denatured in 0.2 M NaOH for 5 min at room temperature. The mixture was neutralised with 0.1 volume of 3 M sodium acetate (pH 5.2), the DNA was precipitated in 2.5 volume of ice-cold ethanol, and washed in 1 ml of 70% ethanol and dried before dissolving in 7 µl of deionised water.

B. Sequencing reactions

All the sequencing reactions were performed following the manufacturer's instruction. The denatured DNA (7 µl) and the primer (1 µl) in equimolar ratios were annealed at 65°C for 2 min in the presence of 1 x sequencing reaction buffer. The mixture was cooled to below 30°C before labelling and the annealed template-primer was (10 µl) labelled by addition of 1 µl of 0.1 M DTT , 2 µl of 1 x labelling mix, 0.5 µl of 10 µCi/µl [α-^{32}S]dATP and 2 µl of diluted sequenase version 2 enzyme. After incubation at room temperature for 3 min, 3.5 µl of the labelling reaction was transferred in to tubes containing 2.5 µl of ddGTP, ddATP, ddTTP, or ddCTP termination mix. The termination reaction was incubated at 37°C for 5 min and the reaction was stopped by addition of 4 µl of stop solution (95% formamide, 20 mM

EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The samples were heated at 80°C for 3 min and 3 µl was loaded on to an acrylamide sequencing gel.

C. Polyacrylamide sequencing gel

Labelled nucleotide sequences were analyzed on 6% polyacrylamide gels prepared from the commercial stock solutions of Sequagel concentrate. The gel was run at 2000 volts for 2-4 h in TBE buffer, fixed in 10%($^{v}/_{v}$) acetic acid for 30 min and dried at 68°C for 1½ h. The dried gel was directly exposed to X-ray film for 18-72 h at room temperature in the dark which was then developed and fixed.

CHAPTER III

EXOPOLYSACCHARIDE PRODUCTION BY *KLEBSIELLA PNEUMONIAE* K1 IN BATCH AND CONTINUOUS CULTURES

3.1. Introduction

Bacterial polysaccharides are not only diverse and complex in structure but also their production is highly influenced by the environment, probably more than any other cellular components. The functional role of the exopolysaccharides is dependent on the natural environment of the organisms producing them.

There are several parameters that commonly influence the nature of microbial exopolysaccharides. The influence of chemical and environmental conditions on exopolysaccharide production has been studied for a number of bacterial species in batch and continuous culture. Certain nutrient limitations such as nitrogen, phosphorus or sulphur in the presence of excess carbon drastically influences the level of polysaccharide production (Sutherland, 1988). The production of polysaccharide by different bacteria may also vary depending on the carbon substrate or the optimal temperature and pH of the medium in which the organism is grown (Jarman, *et al*, 1978; Masson and Holbein, 1985; Linton *et al*, 1987).

The involvement of capsular polysaccharide in the invasiveness of *K. pneumoniae* is well established. Most studies in the past concentrated on seroepidemiology, chemistry, and on pathology. In contrast to these areas of investigation, little is understood with regard to the physiology of capsule production in *K. pneumoniae*. As part of this study, the production of capsular polysaccharide by *K. pneumoniae* K1 was studied in batch and continuous culture. The influence of medium composition, pH, temperature, aeration, and growth rate on polysaccharide production were examined.

3.2. Polysaccharide production in flask shake culture

The production of CPS by *K. pneumoniae* K1 was studied under a range of nutritional and environmental conditions in a series of shake flask experiments. The organism was grown in minimal salts medium (MSM-A) for 18 to 48 h at 37°C with shaking at 200 rpm. The dry weight of cells and polysaccharide were quantitatively determined and the Ycps expressed as the amount of polysaccharide produced per unit cell biomass (Y= yield: i.e., mg of CPS per mg cell dry weight) was used as an index of polysaccharide production. For each nutritional limitation, the mean value of three culture samples was determined.

3.2.1. Ammonium sulphate

Ammonium sulphate as a sole source of nitrogen was added into MSM-A at a final concentration ranging from 0.01 to 2.0 mg ml^{-1}. Low concentrations of ammonium sulphate in the medium were favourable for the production of polysaccharide. As shown in Fig 3.1, the increase in cell biomass was followed by a reduction in the level of precipitable polysaccharide as the concentration of the nitrogen source was increased in the medium.

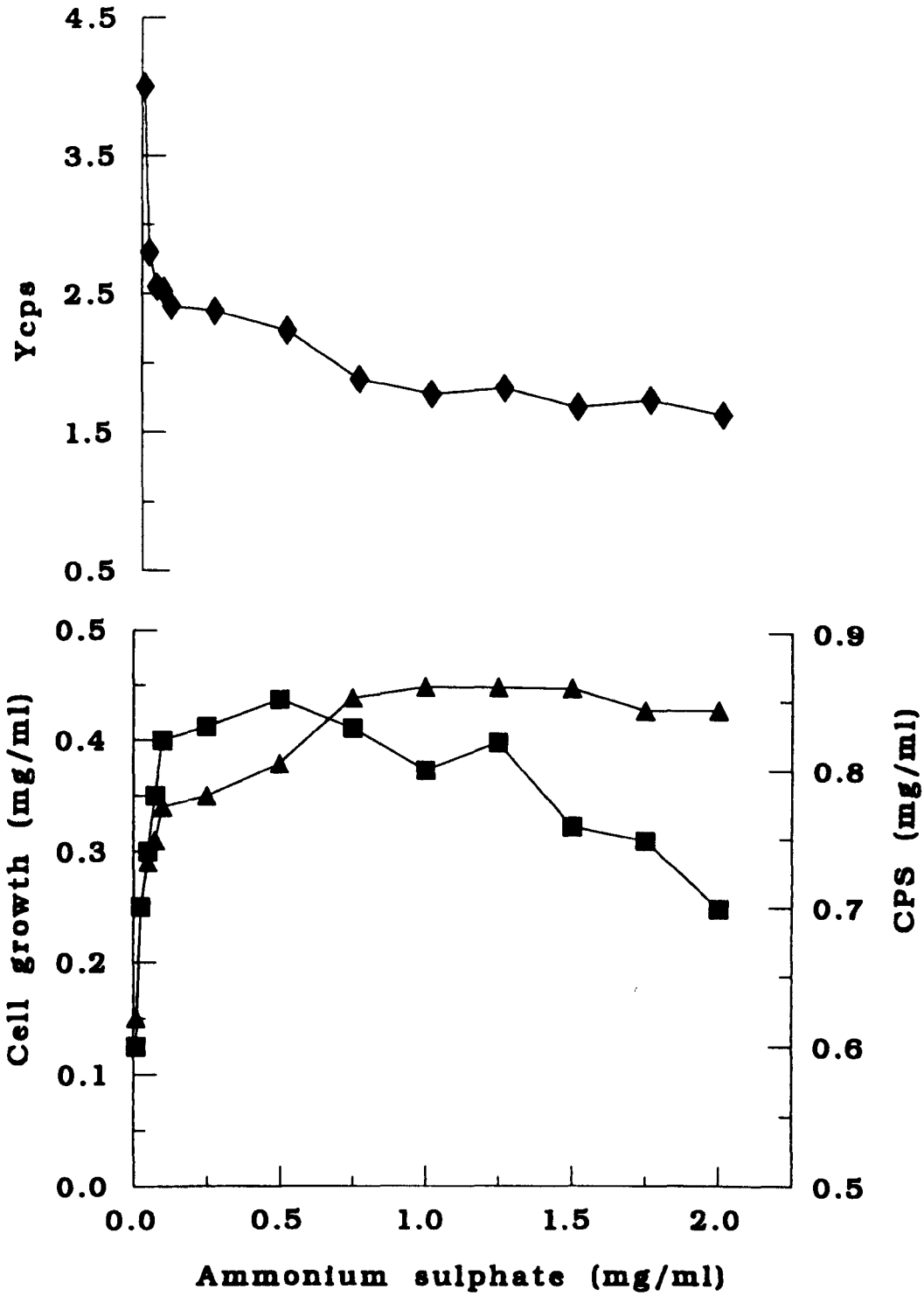


Figure 3.1. Effect of ammonium sulphate concentration on polysaccharide production in shake flask culture. (▲) Cell dry weight mg ml^{-1}; (▬) CPS mg ml^{-1}; (♦) Ycps.

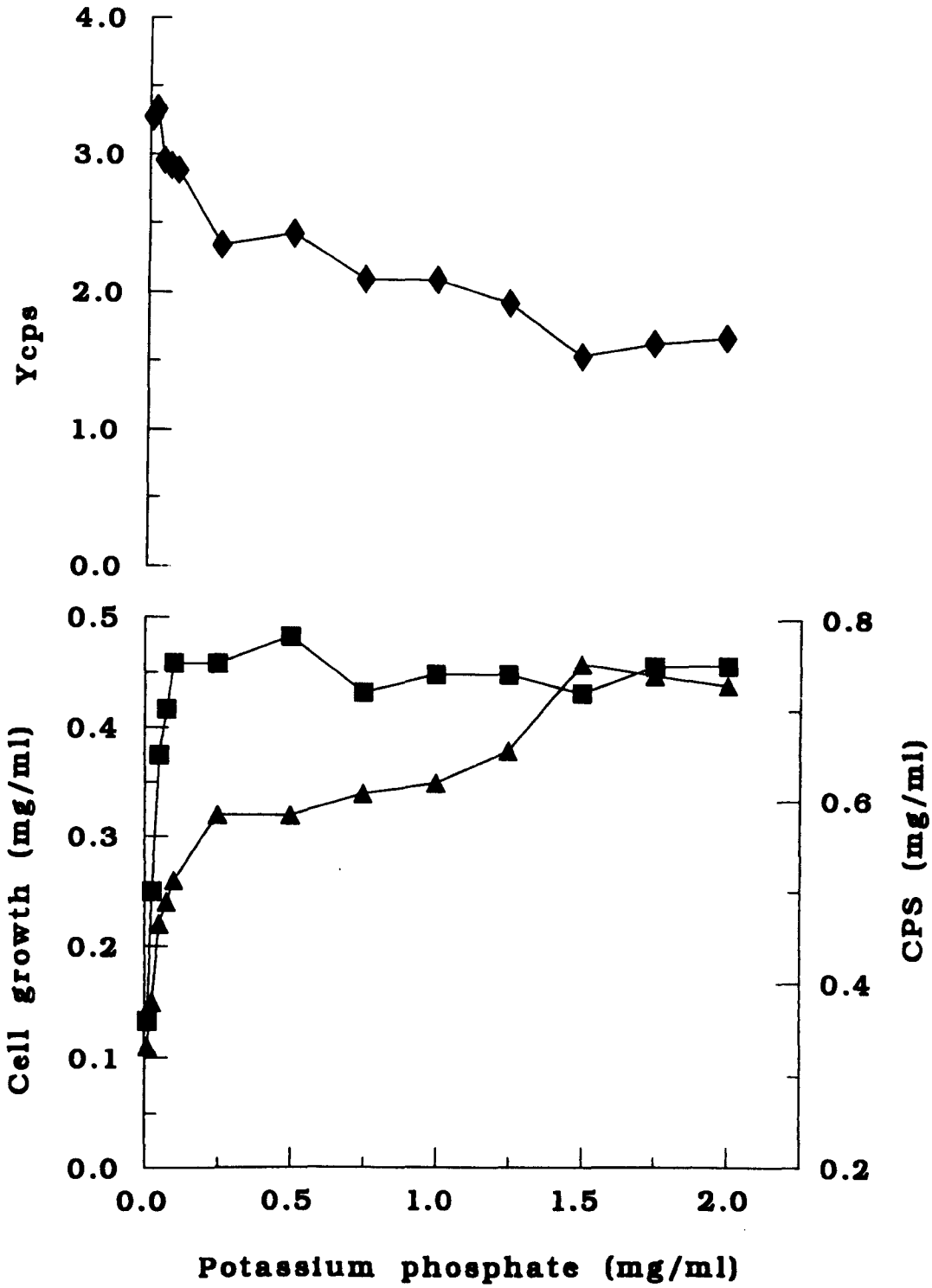


Figure 3.2. Effect of potassium phosphate concentration on polysaccharide production in shake flask culture. (▲) Cell dry weight mg ml^{-1}; (▬) CPS mg ml^{-1}; (♦) Ycps.

3.2.2. Potassium phosphate

Potassium phosphate as a sole source of phosphate was used at a final concentration of 0.01 to 2.0 mg ml^{-1}. Media containing low concentrations of phosphate were buffered to pH 7.4 with 0.5 M Tris-HCl (pH 7.4) to offset the reduction in buffering capacity of the phosphate. Initial concentration of phosphate in the medium had a direct effect on the level of total cell dry weight recovered. The yield of polysaccharide obtained in media containing low phosphate concentrations was greater than phosphate sufficient media (Fig 3.2).

3.2.3. Magnesium sulphate

Magnesium sulphate (0.01 to 2 mg ml^{-1}) was used as a source of sulphate and ammonium sulphate was replaced by ammonium chloride. Low concentrations of magnesium sulphate in the medium had similar effects to that of low concentrations of nitrogen or phosphate in growth media. The maximum precipitable polysaccharide was obtained in media containing low concentrations of magnesium sulphate (Fig 3.3).

3.2.4. Glucose

Glucose as carbon source was added to a final concentration ranging from 0.1 to 3.0%($^{w}/_{v}$). There was a direct relationship between the initial concentration of glucose and the amount of polysaccharide produced. Cell growth could be stopped as a result of depletion of nutrients while the available glucose might be utilised for the synthesis of polysaccharide. As Fig 3.4 shows, the yield of polysaccharide was not greatly enhanced at concentrations of glucose above 2%. This could be due to metabolic control reactions and a feedback repression or product inhibition.

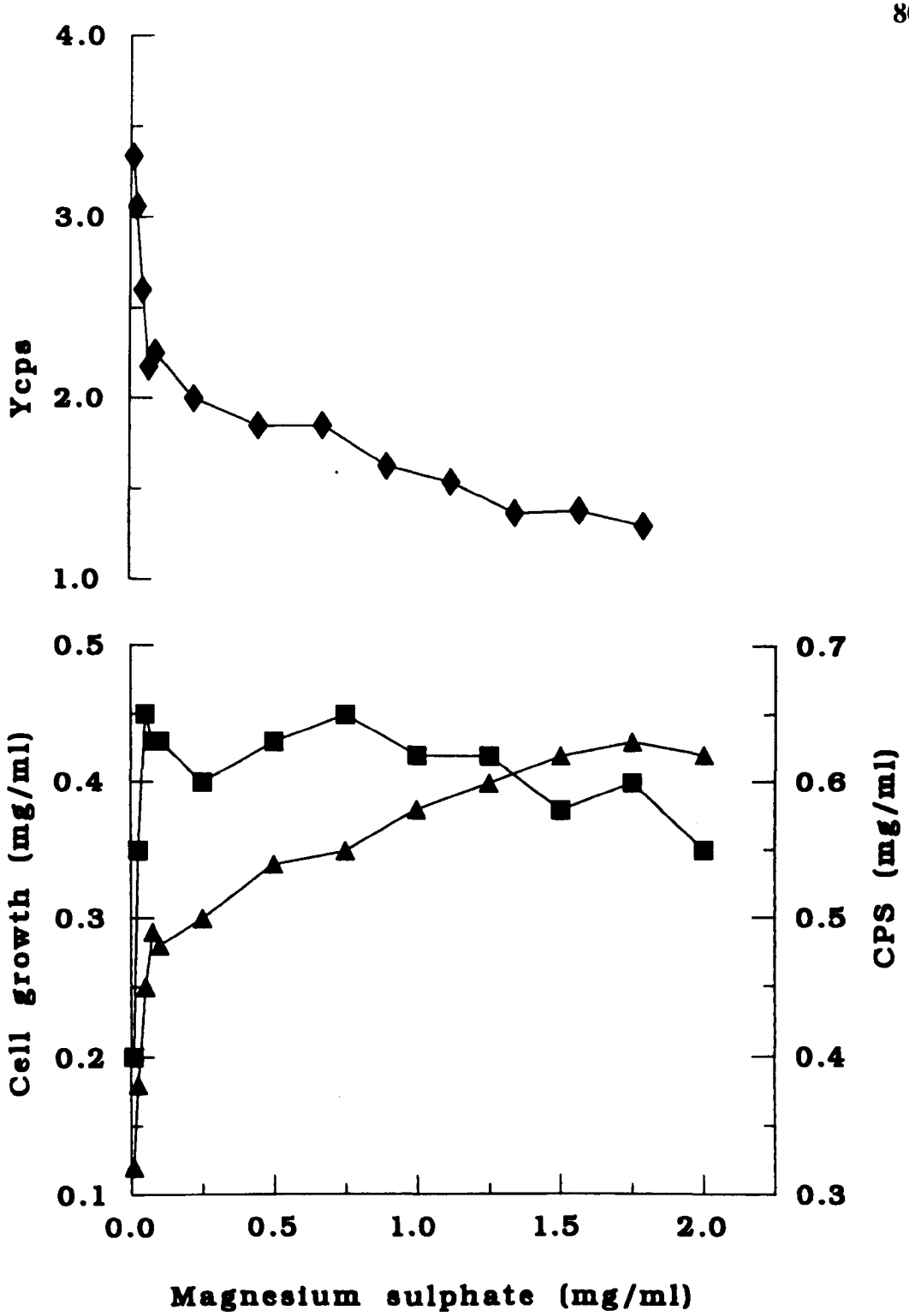


Figure 3.3. Effect of magnesium sulphate concentration on polysaccharide production in shake flask culture. (▲) Cell dry weight mg ml^{-1}; (▬) CPS mg ml^{-1}; (♦) Ycps.

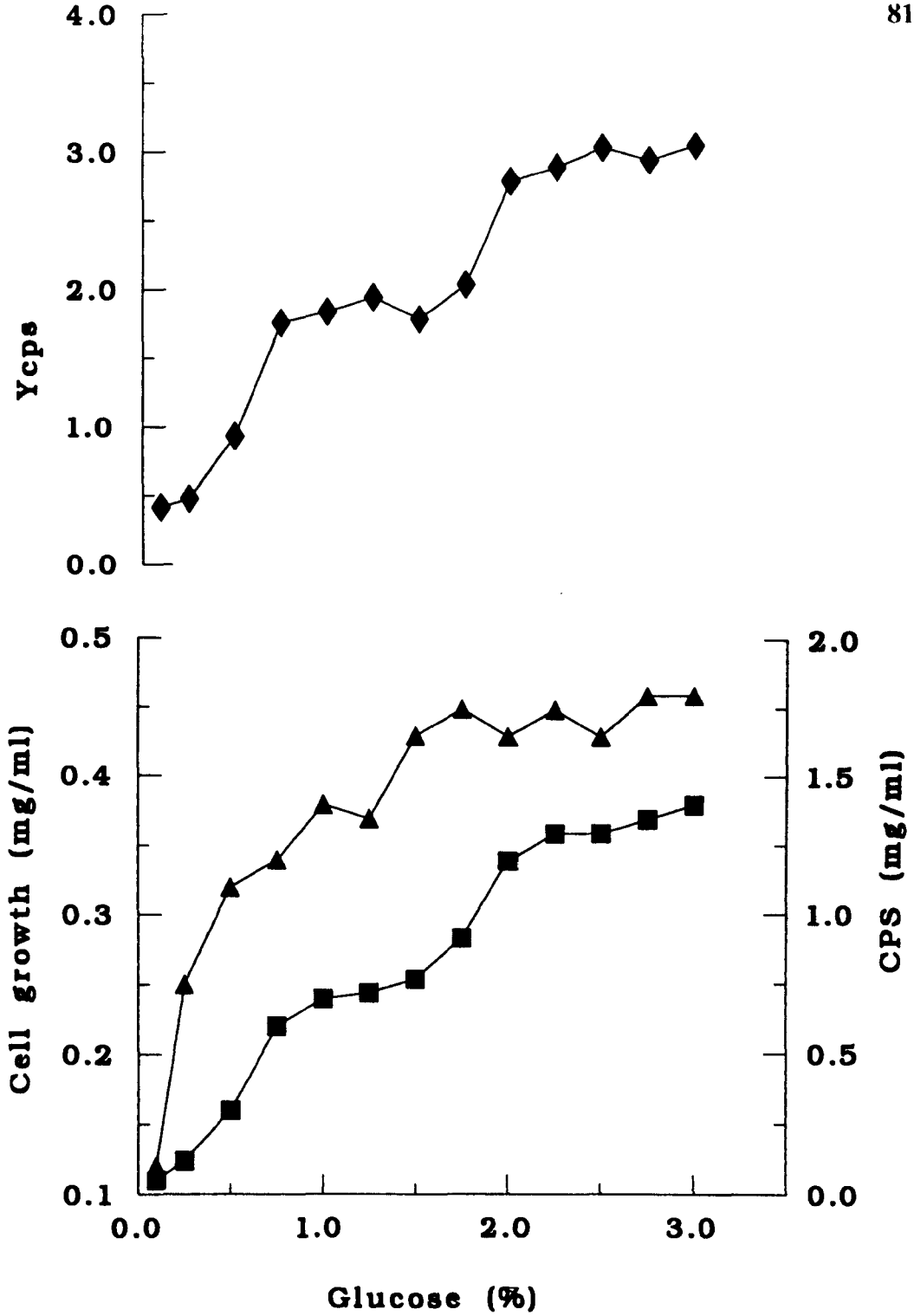


Figure 3.4. Effect of glucose concentration on polysaccharide production in shake flask culture. (▲) Cell dry weight mg ml^{-1}; (▬) CPS mg ml^{-1}; (♦) Ycps.

3.2.5. Carbon source

The production of polysaccharide by *Klebsiella* K1 was also investigated in media containing different types of carbon sources. The carbon substrate was added to the basal medium (MSM-A) to a final concentration of 2%($^{w}/_{v}$). The addition of each carbon substrate increased the final yield of polysaccharide. Maximum polysaccharide synthesis was observed in media containing glucose followed by sucrose. For other carbon sources, there was no significant difference (Table 3.1). This suggests that *Klebsiella* produces polysaccharide in the presence of excess sugar irrespective of the type of carbon source utilised.

3.2.6. Growth temperature

The production of polysaccharide by *Klebsiella* was examined in cultures grown at various temperatures for 48 h after initiation of growth. The yield of CPS was better at lower growth temperatures (≤30°C, Table 3.2). Polysaccharide production was also observed at 40°C, although the amount produced was relatively small.

Table 3.1. Effect of carbon source on polysaccharide production.

Carbon source (2% $^{w}/_{v}$)	Final yield, Cell mg ml^{-1}	Final yield, CPS mg ml^{-1}	Ycps
Glucose	0.42	1.2	2.86
Sucrose	0.45	1.2	2.67
Lactose	0.42	0.92	2.2
Galactose	0.38	0.85	2.2
Maltose	0.35	0.75	2.01
Mannitol	0.40	0.89	2.25

Ycps: mg CPS mg $cell^{-1}$ dry weight.

Table 3.2. Effect of temperature on polysaccharide production.

Growth Temperature	Final yield, Cell mg ml^{-1}	Final yield, CPS mg ml^{-1}	Ycps
25°C	0.4	0.98	2.45
30°C	0.45	1.2	2.67
35°C	0.42	0.92	2.2
37°C	0.38	0.85	2.2
40°C	0.35	0.58	1.66

Ycps: mg CPS mg $cell^{-1}$ dry weight.

3.3. Polysaccharide production in batch fermentation

3.3.1. Time course of polysaccharide production

Growth and polysaccharide production by *K. pneumoniae* K1 was followed in MSM-B in a fermenter for 24 h, at 37°C and at a controlled pH of 7.0±0.1. A batch fermentation profile of cell growth and polysaccharide production is presented in Fig 3.5. Growth continued for 17 h until the nutrients available were apparently depleted. Exponential growth was observed for 5 hours (12 to 17 h) at a growth rate of μ_{max}= 0.44 h^{-1}. After 17 h growth stopped, probably due to the depletion of nitrogen in the medium. At the end of exponential growth, maximum cell density was obtained and amounted to 2.17 mg ml^{-1}.

K. pneumoniae K1 synthesised polysaccharide throughout growth. The mean rate of polysaccharide produced during exponential growth was 0.48 mg ml^{-1} h^{-1}. The synthesis of polysaccharide continued after exponential growth with a mean production rate of 1 mg ml^{-1} h^{-1} until the glucose was completely utilised. The polysaccharide produced caused the culture broth to become greenish-yellow in colour. Although actual measurement was not performed, increased viscosity of the culture was also apparent.

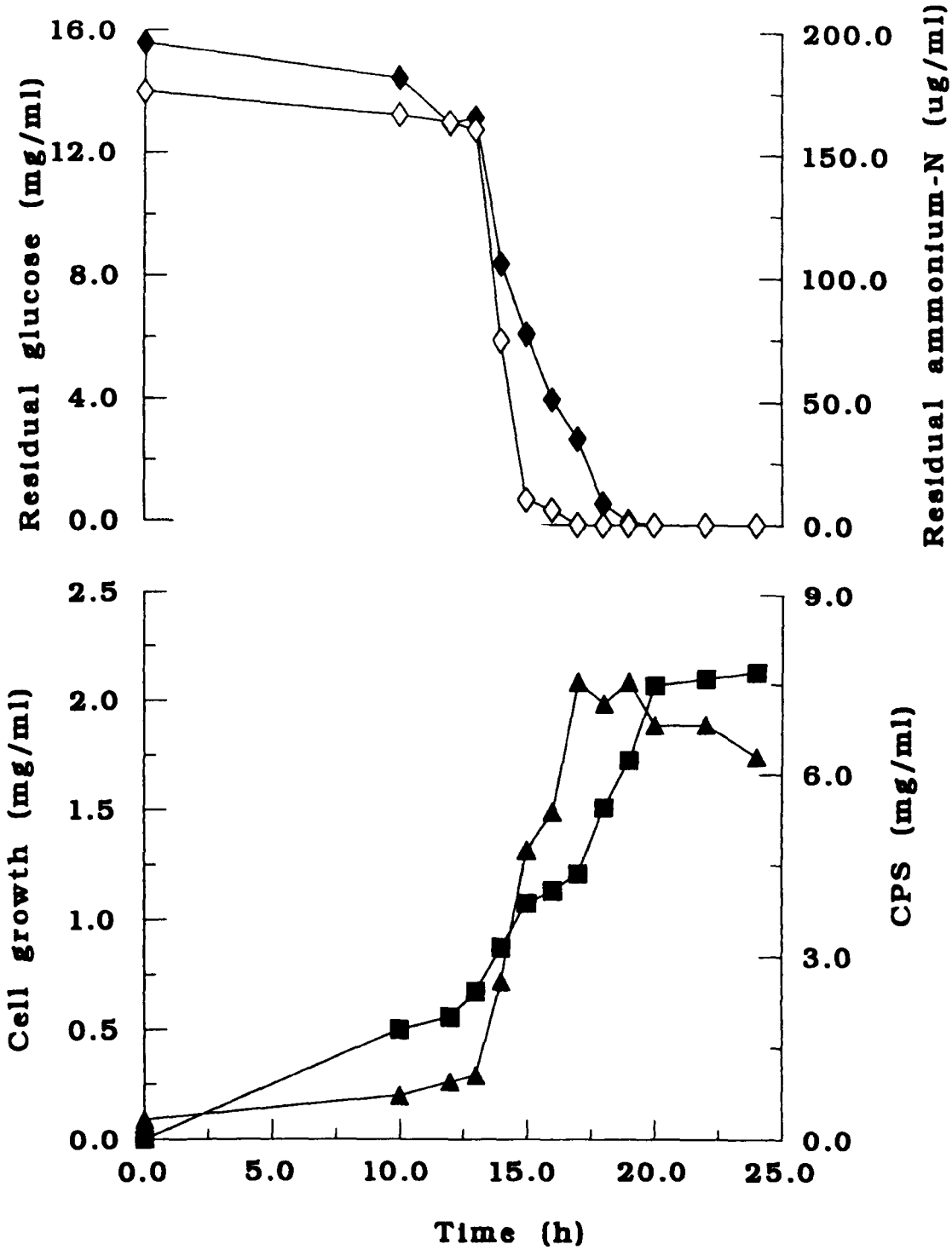


Figure. 3.5. Growth and polysaccharide production in batch fermenter culture. (▲) cell dry weight mg ml^{-1}; (▬) CPS mg ml^{-1}; (♦) residual glucose mg ml^{-1}; (◊) residual ammoniacal nitrogen, µg ml^{-1}.

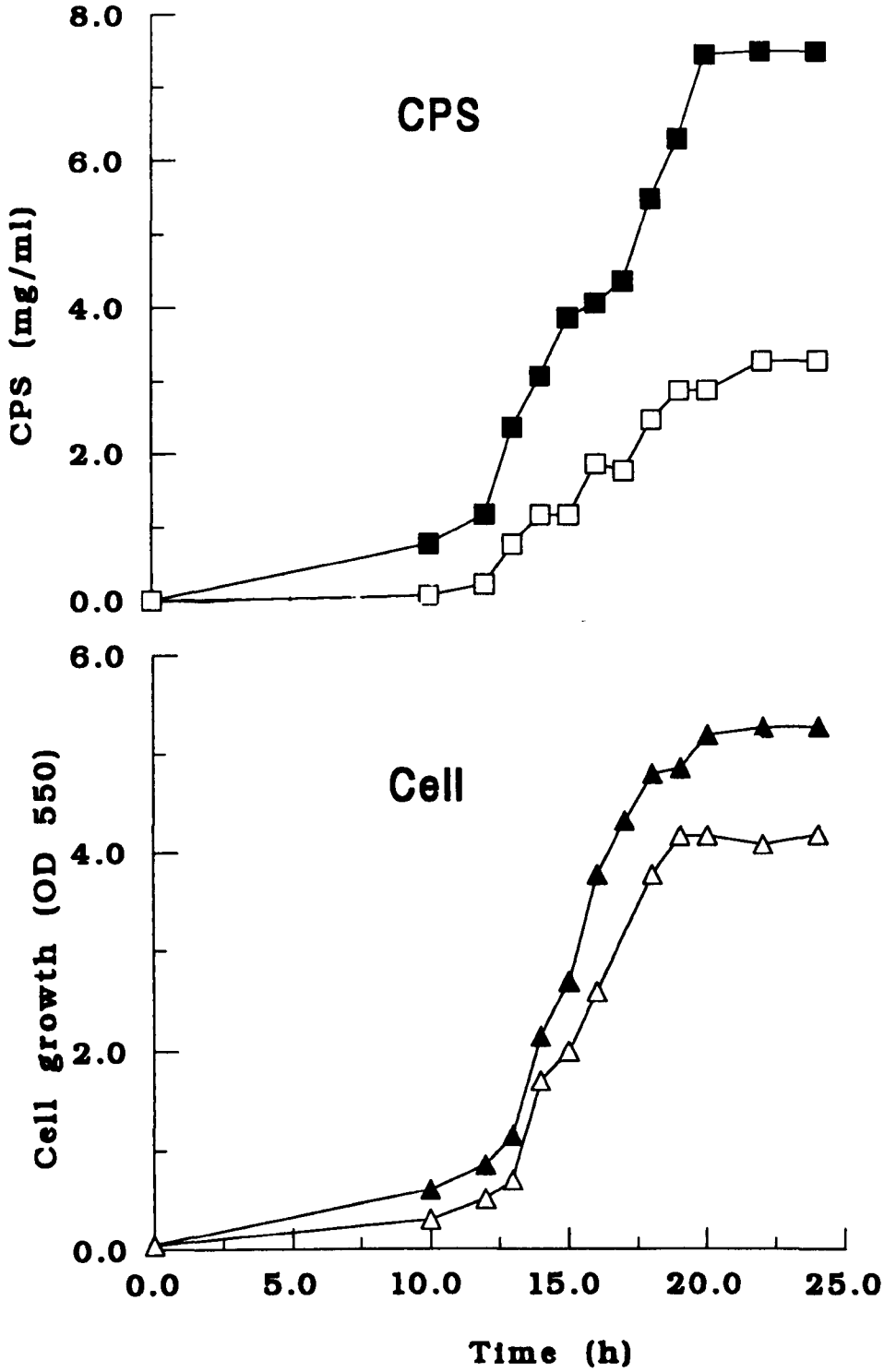


Figure 3.6. Cell growth and polysaccharide production during anaerobic batch fermenter culture. Dark-shaded and non-shaded symbols represent aerobic and anaerobic culture conditions respectively. The data for aerobic culture growth was from Fig 3.5.

3.3.2. Aeration

The synthesis of polysaccharide in batch fermentation was observed under anaerobic condition where air was replaced by nitrogen gas and the culture was incubated for 24 h in a fermenter. Culture grown with aeration had a higher yield of cell biomass and polysaccharide (Fig 3.6). The final yield of polysaccharide after anaerobic culture was two fold lower than that observed under aerobic culture. Although *K. pneumoniae* is a facultative anaerobe, the energy that can be produced from a given carbon source is less under anaerobic conditions. The transport of substrates such as glucose into the cell and the subsequent biosynthesis of capsular polymer is an energy-requiring process (Sutherland, 1979). Thus a reduction in the level of ATP production might have a direct influence on the amount of polysaccharide produced as well as on cell yield.

3.4. Polysaccharide production in continuous culture

The organism was grown in continuous culture with nitrogen- or glucose-limited MSM. To reduce the viscosity of the culture and for better separation of the cells from the exopolysaccharide, the concentration of limiting nutrient was adjusted to give an optical density of 0.7 to 1.0 at 550 nm in chemostat culture. Bacterial growth was followed by measuring the optical density of an appropriately diluted culture sample and/or by determining the dry weight of the cells. An optical density of 1 at 550 nm had a mean value of 1.8×10^9 cfu ml^{-1} and a mean value of 0.53 mg ml^{-1} cell dry weight. Such a relation was observed in both batch and continuous cultures except for nitrogen-limited continuous cultures. In nitrogen-limited culture the dry weight of the cell biomass was at least twice the corresponding optical density of

the culture. This was as a result of the viscosity of the culture and the presence of large quantities of capsular material that increased the total dry weight.

It was assumed that steady state in the chemostat would be reached after 3-4 times the culture volume had been replaced. Measurements were taken twice at each growth rate before setting new parameters. The culture was routinely examined for contamination. Chemostat cultures were stopped when nonmucoid cells were detected in culture so that effects of CPS production would be properly assessed on cultural conditions.

3.4.1. Dilution rates

The production of polysaccharide by *K. pneumoniae* was studied in nitrogen-limited culture (MSM-C) at 37°C, pH 7±0.1 with volume/volume aeration and with stirring at 1000 rpm. Dilution rates of the culture were varied between D= 0.05 to 0.5 h^{-1}. The production of polysaccharide was higher at low dilution rates and lower at high dilution rates.

As shown in Fig 3.7, at low dilution rates polysaccharide production was maximal (2.0 to 2.3 mg ml^{-1}). Increasing the dilution rate to D= 0.5 h^{-1} resulted in decreased amounts of polysaccharide to 0.35 mg ml^{-1}. The level of polysaccharide in the effluent was also determined by the colorimetric method as total carbohydrate content. The level of carbohydrate concentration in the effluent was also correspondingly decreased from 325 to 40 μg ml^{-1} as the dilution rates increased. Cell growth was relatively similar when the dilution rates were maintained between 0.05 to 0.25 h^{-1}. Residual glucose was detected in the effluent at increasing concentrations at high dilution rates. Ammonium sulphate as the growth-limiting nitrogen source was

not detected in the effluent at any of the dilution rates. A reduction in the viscosity of the culture was also apparent at higher growth rates.

3.4.2. Growth temperature

The effect of growth temperature on polysaccharide production was characterised in nitrogen-limited cultures at a fixed dilution rate (D=0.2 h^{-1}). The growth temperature was varied between 25 and 40°C. Polysaccharide was produced optimally at ≤30°C while cell growth was optimal at 37°C. As shown in Fig 3.8, increasing the temperature of the culture vessel to 40°C decreased the levels of cell biomass and polysaccharide produced. This was also observed in shake flask cultures incubated at different growth temperatures (Table 3.2).

3.4.3. pH

Cell growth and polysaccharide formation were also determined at various pH values (pH 4.5 to 8.0) in nitrogen-limited cultures at 37°C and at a dilution rate of 0.2 h^{-1}. The pH of the culture medium influenced both cell growth and polysaccharide production. Although maximum production was obtained at neutral pH, capsule was still produced at a reduced rate under low pH (Fig 3.9).

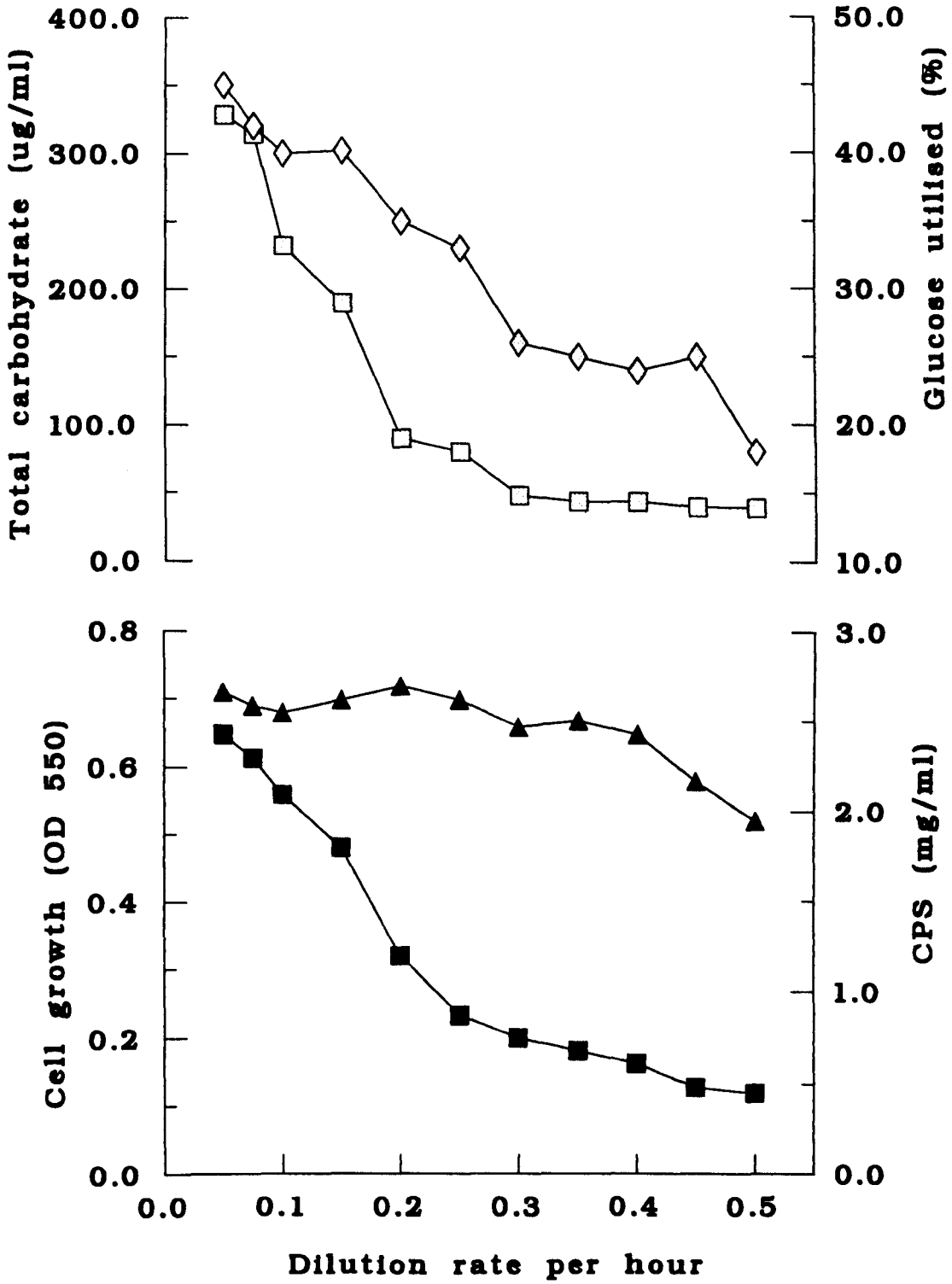


Figure 3.7. Effect of dilution rate on steady state polysaccharide production in nitrogen-limited culture. (▲) cell growth OD 550 nm; (▬) CPS mg ml^{-1}; (□) total carbohydrate, μg ml^{-1}; (◇) % glucose utilised.

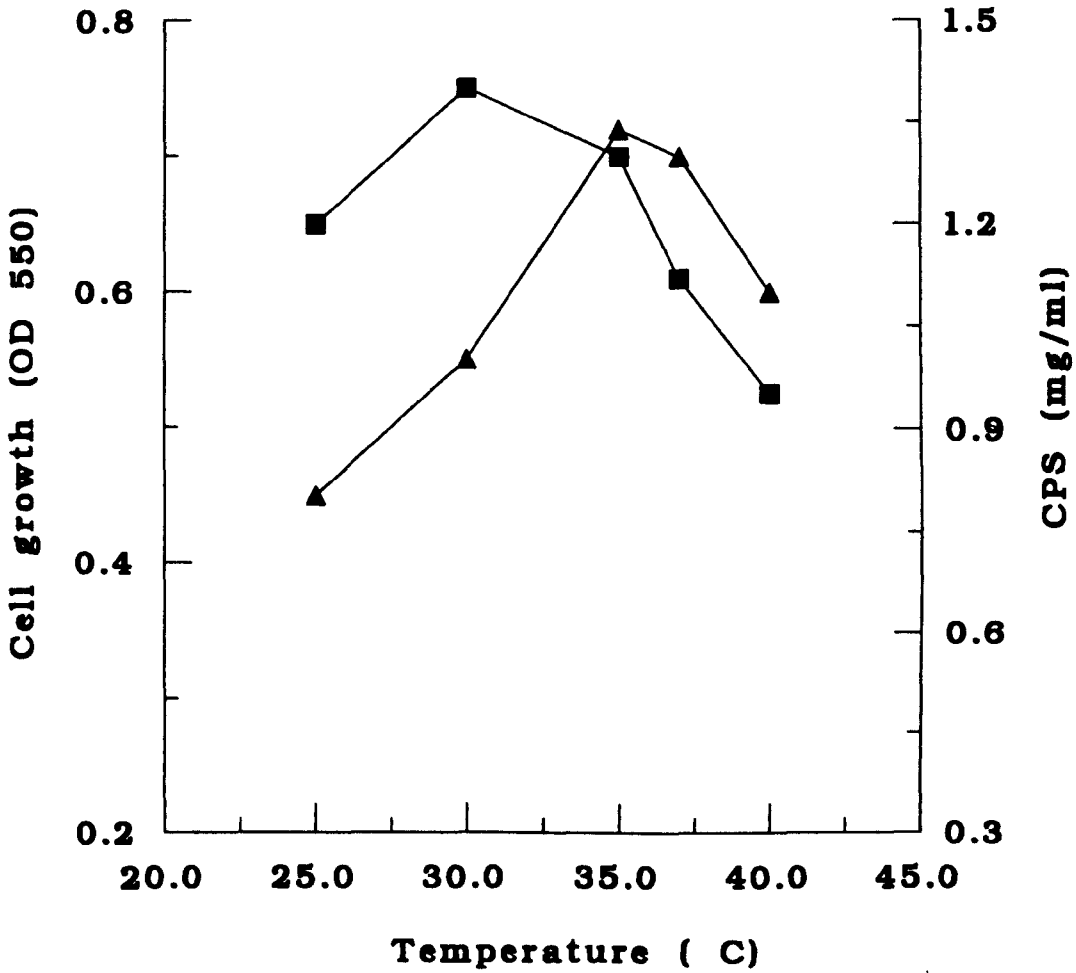


Figure 3.8. Effect of growth temperature on steady state polysaccharide production in nitrogen-limited culture. (▲) cell growth OD 550 nm; (▬) CPS mg ml^{-1}.

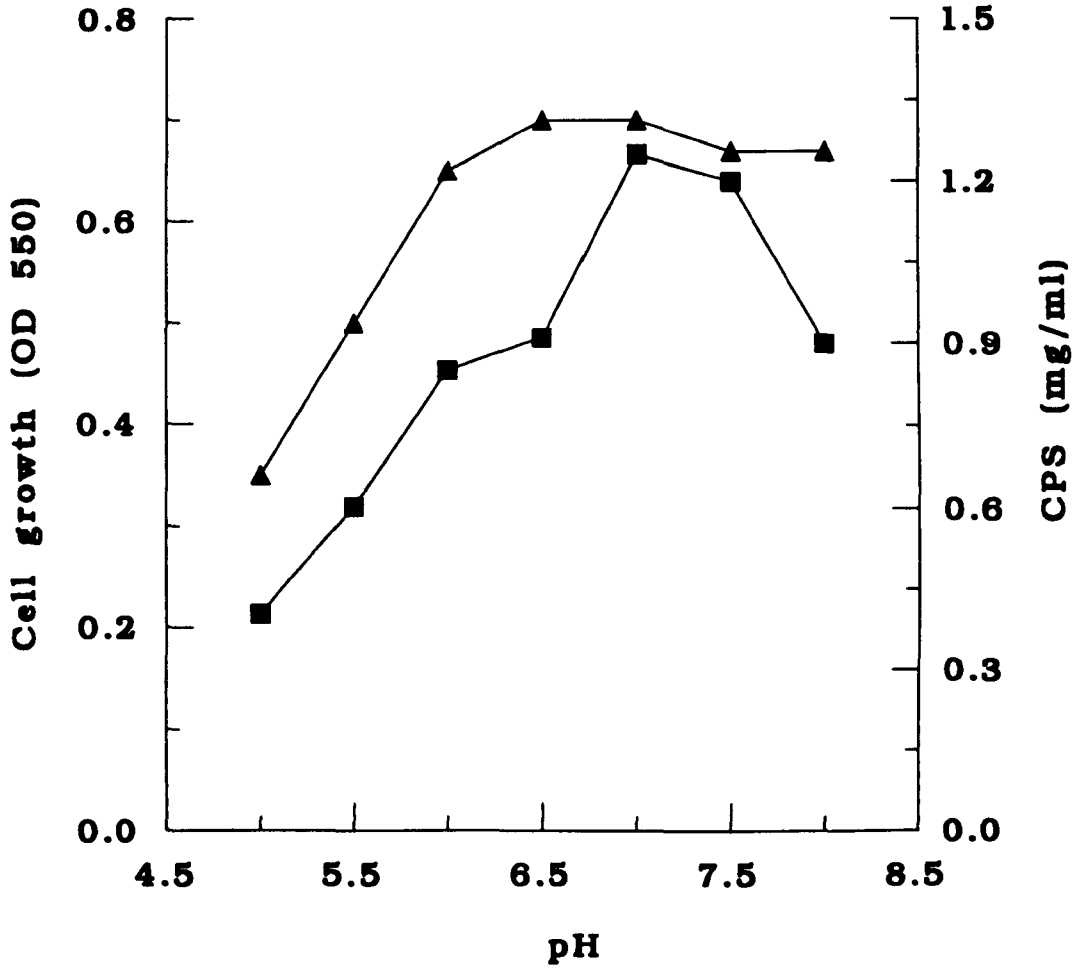


Figure 3.9. Effect of pH on steady state polysaccharide production in nitrogen-limited culture. (▲) cell growth OD 550 nm; (▬) CPS mg ml^{-1}.

3.4.4. Agitation speed

The effect of oxygen availability on CPS production was examined in nitrogen-limited culture at 37°C, pH 7.0 and at a fixed dilution rate of 0.2 h^{-1}. The fermenter agitation speed was altered between 200 and 1000 rpm so that the rate of oxygen transfer into the culture varied. As shown in Fig 3.10, both cell growth and polysaccharide levels were increased when the fermenter agitation speed was increased.

3.4.5. Steady state polysaccharide production in nitrogen-limited culture

Steady state cell yields and polysaccharide production were maintained in nitrogen-limited chemostats at a dilution rate of D= 0.2 h^{-1} for more than 240 h (Fig 3.11). The level of residual glucose in the culture effluent was similar for most of the growth period. However, the polysaccharide level progressively reduced with time and fell by approximately 27%. At the same time nonmucoid variants arose in the chemostat to comprise about 18% of the total cell population after 240 h (Fig 3.11). The appearance of the nonmucoid cell population during continuous culture might be the result of selective pressure that gives them an advantage over the mucoid cell population.

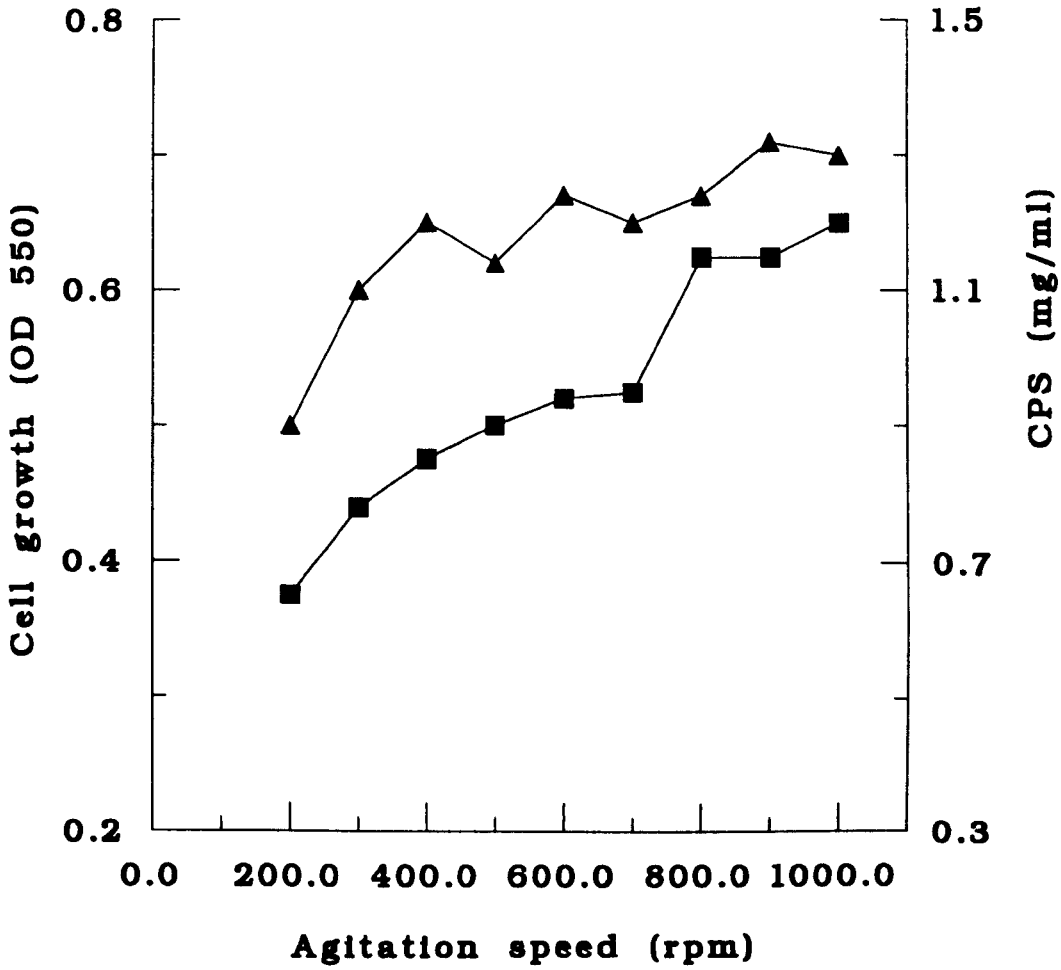


Figure 3.10. Effect of fermenter agitation speed on polysaccharide production in nitrogen-limited culture. (▲) cell growth OD 550 nm; (▬) CPS mg ml^{-1}.

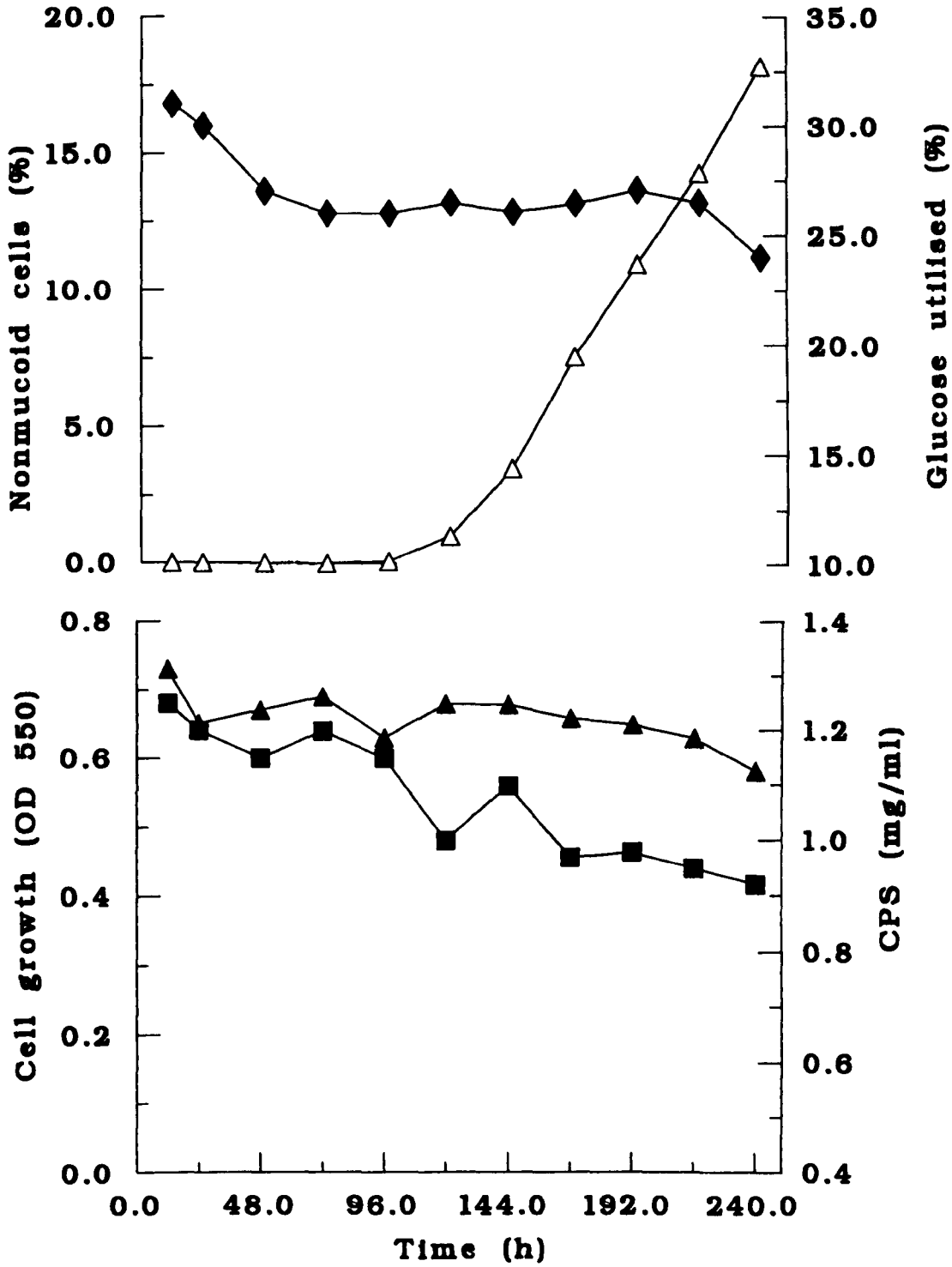


Figure 3.11. Steady state cell growth and polysaccharide production in nitrogen-limited culture. Dilution rate as maintained at D=0.2 h^{-1}. (▲) Cell growth OD 550 nm; (▬) CPS mg ml^{-1}; (△) % nonmucoid cell population; (♦) % glucose utilised.

3.4.6. Steady state polysaccharide production in glucose-limited continuous culture

Steady state cell growth and polysaccharide formation was also followed in glucose-limited continuous cultures at a dilution rate of 0.2 h^{-1}. The glucose-limited medium (MSM-D) supported cell growth to an optical density of 1.0 at 550 nm (equivalent to 0.53 mg ml^{-1} cell dry weight). About 36% of the available nitrogen source (ammonium sulphate) was utilised while glucose was completely used.

As shown in Fig 3.12, steady state growth and polysaccharide formation was observed for a period of 240 h. Unlike nitrogen-limited cultures where culture viscosity was a problem and pelleting of the cell required longer centrifugation, the culture grown in glucose-limited medium was less viscous and the cells were easily pelleted. Alcohol-precipitable exopolysaccharide was gravimetrically determined and found to be appreciably less than in nitrogen-limited cultures. The polysaccharide determined as total carbohydrate was eight fold less than the total carbohydrate obtained in nitrogen-limited conditions. Although the level of exopolysaccharide in the effluent was extremely low, the mucoid phenotype was stably maintained and there was no apparent change in colonial morphology when grown in solid media. This may suggest that the strain used in this study constitutively produces capsule that is firmly attached to the cell irrespective of the cultural conditions. However, large quantities of capsular polysaccharide could also be produced under favourable conditions and constantly shed into the surrounding medium.

The population of nonmucoid cells (<1%) detected in glucose-limited cultures was less than that observed in nitrogen-limited conditions.

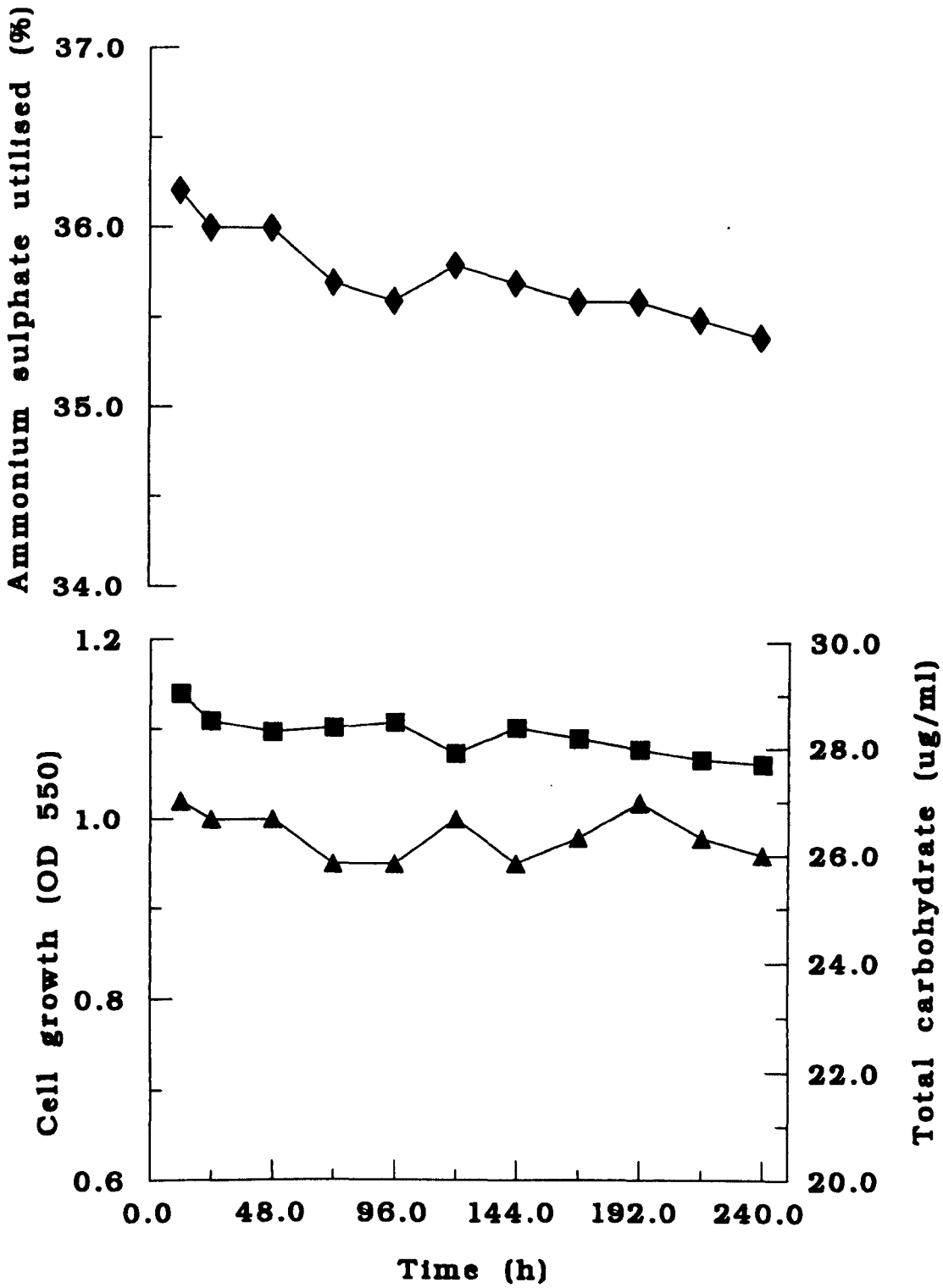


Figure 3.12. Steady state cell growth and polysaccharide production in glucose-limited continuous culture. (▲) cell growth OD 550 nm; (▬) total carbohydrate μg ml^{-1}; (♦) % ammonium sulphate utilised.

3.4.7. Characterisation of a nonmucoid variant

Capsulated strains of *Klebsiella* are stable and rarely mutate to nonmucoid variants. During routine laboratory subculturing, a nonmucoid mutant was isolated. *Klebsiella* K1 (3L101) that was used most frequently in this study was found to irreversibly mutate to nonmucoid phenotype at a frequency of less than 0.01%.

During continuous culture, especially under nitrogen-limited conditions, the appearance of nonmucoid variant cells was more frequent. The nonmucoid variant that was isolated had the same growth rate as the parent cell and was not different in nutritional requirements. The Km^r conjugative plasmid (pKLS12) integrated within the chromosome and the resident plasmid pKLS1 carried by the parent cells were also both stably maintained in the nonmucoid variant.

Although the nonmucoid variant was phenotypically nonmucoid when grown on solid medium (Fig 3.13), it still produced K antigen that was identical with the parent antigenic determinant when analyzed by double immunodiffusion. In addition, the presence of a distinct capsule layer was also confirmed in a negatively stained electron microscope preparation (Fig 3.14).

Unlike the parent strain which was not susceptible to any of the *Klebsiella* specific-bacteriophages, the nonmucoid variant was sensitive to four of the phages tested (Table 3.3). The resistance of the mucoid parent to phage may be due to increase thickness of the capsule that might protect the bacterial surface from the bacteriophage. The reduction in the thickness of the capsule in the nonmucoid variant might have rendered it susceptible to infection by bacteriophage through exposure surface receptor sites.

Table 3.3. Phage typing of *Klebsiella pneumoniae.*

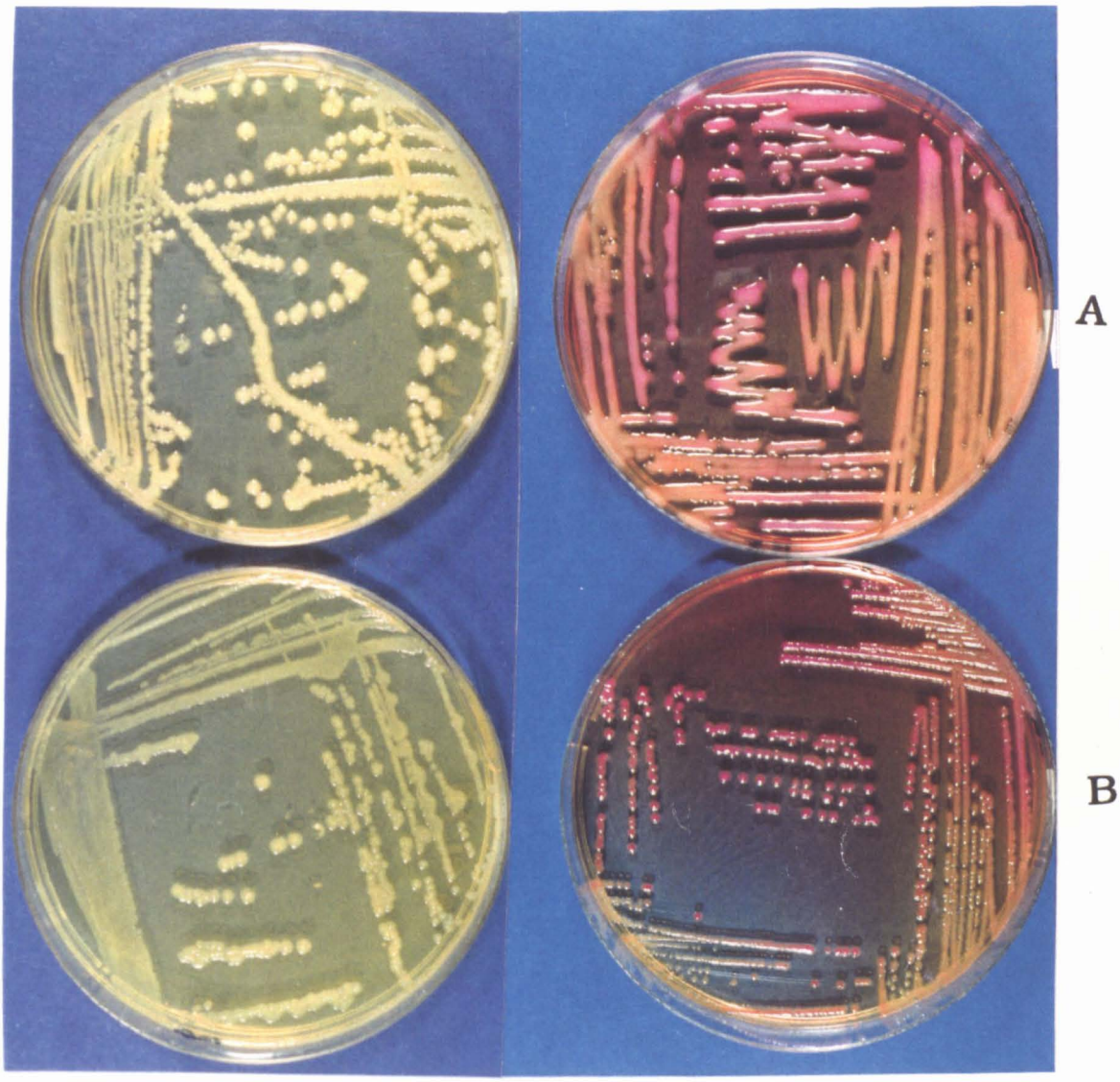
Strain	phenotype	Sensitivity to phage type								
		1	2	3	4	5	6	7	8	9
3L101	O1:K1, mucoid	-	-	-	-	-	-	-	-	-
3L102	O1:K1, nonmucoid	-	+	+	-	-	+	-	-	±

+: Strongly positive

±: Weakly positive

-: Not typable.

Figure 3.13. Colonial morphology of mucoid and nonmucoid cells of *Klebsiella pneumoniae* K1. **A.** Mucoid colonies (3L101) grown on nutrient agar (top left) or on MacConkey agar (top right). **B.** Nonmucoid variant (3L102) grown on nutrient agar (bottom left) or on MacConkey agar (bottom right).


A
B

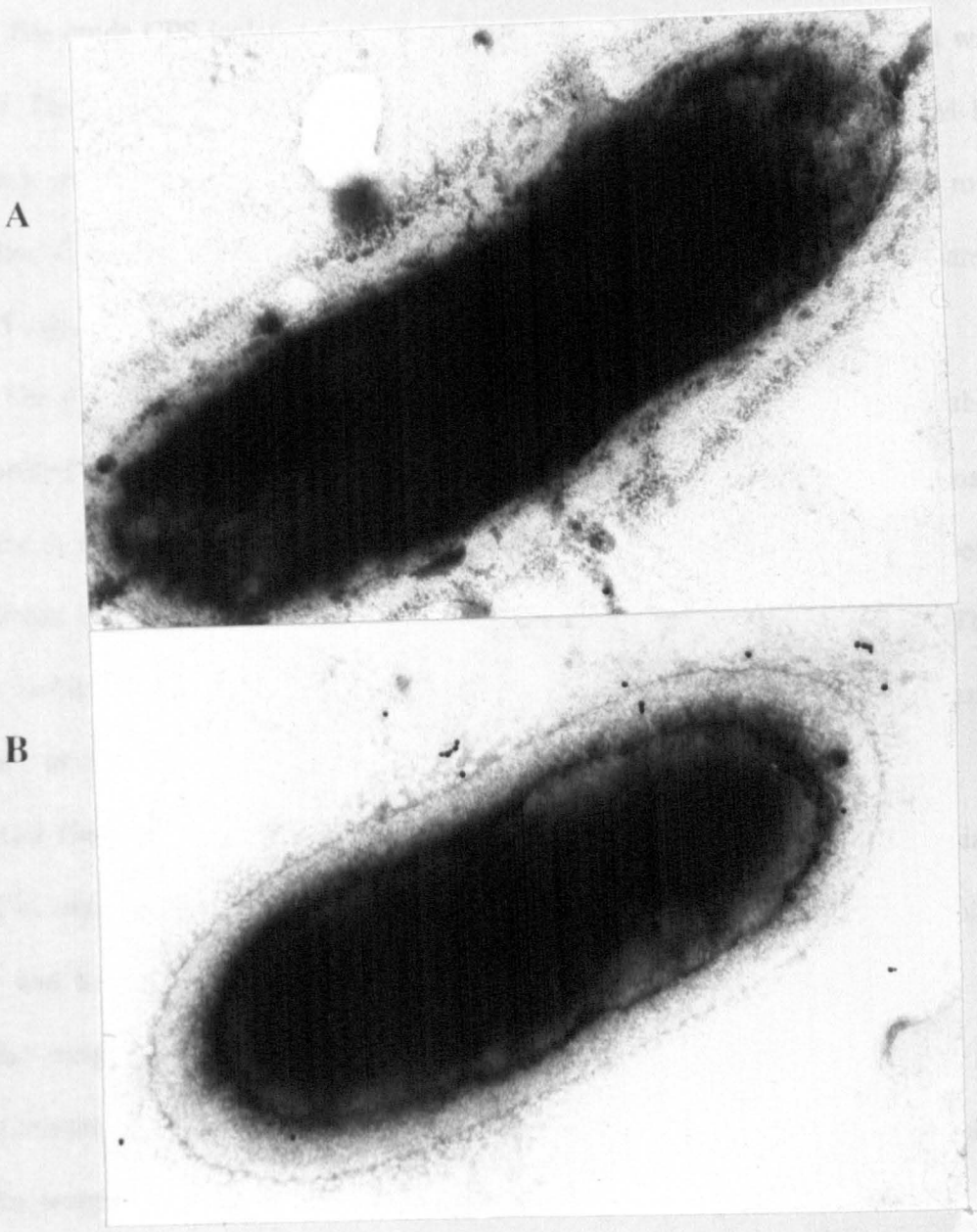


Figure 3.14. Negatively stained electron microscope preparation using immuno-gold labelled antibody. **A**. Mucoid parent cell (3L101). **B**. nonmucoid cell (3L102). The antiserum was absorbed with whole cell suspension of the nonmucoid variant. The absence of labelled antibody on the cell surface is an indication of the removal of all K1-specific primary antibody. The secondary antibody was conjugated with 15 *nm* gold particles. Magnification: 50,000x.

3.5. Analysis of capsular polysaccharide

The crude CPS isolated from the cell surfaces and/or culture supernatant was purified. The yield of polysaccharide from cells grown in minimal medium (MSM-B) was much greater than that of cells grown in complex medium (L-broth). 1.5 mg ml^{-1} of purified CPS was obtained from the culture grown in minimal medium compared with 0.5 mg ml^{-1} of CPS after growth in L-broth.

The chemical composition of the CPS was also analyzed. As shown in Table 3.4, purified CPS was primarily composed of carbohydrate (>60%) and small quantities of nucleic acid (<2%) and protein (<0.1%). Small quantities of LPS were also present in the capsular material as indicated by KDO-positive material. The capsule isolated from the cell surfaces and culture supernatant did not show any difference in chemical composition or antigenicity.

Gel filtration of K1 CPS through Superose 6 HR 10/30 (FPLC, Pharmacia) resulted in two peaks (Fig 3.15). The major peak (I) was eluted at or near the void volume and had a molecular weight of $>10^6$. The second small peak (II) had a molecular weight of $<10^5$. The polysaccharide fraction corresponding to peak I strongly reacted with anti-K1 capsular antibody indicating that it was CPS. The small molecular weight fraction could possibly be the O polysaccharide.

Table 3.4. Chemical analysis of CPS isolated from *Klebsiella pneumoniae.*

Chemical	%, total weight	
	A	B
Total carbohydrate	67.4	68.2
KDO	0.65	0.56
Nucleic acid	1.7	1.4
Protein	0.08	0.06

A: CPS isolated from cell surfaces

B: CPS isolated from culture supernatant

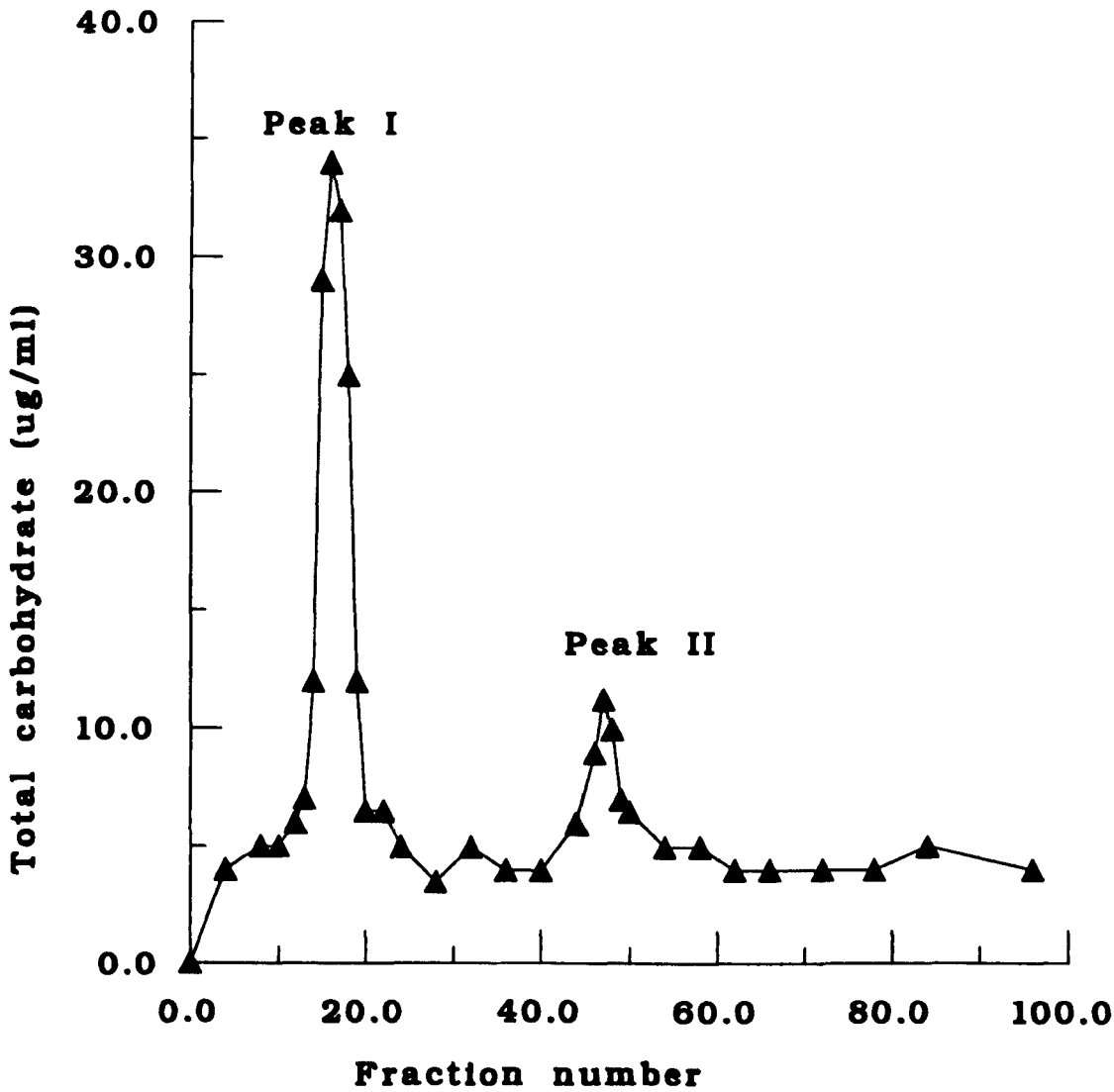


Figure 3.15. Gel filtration of *K. pneumoniae* K1 CPS on Superose 6 HR 10/30 column. Fractions (0.5 ml/tube) were monitored for total carbohydrate by phenol sulphuric acid method.

3.6. Discussion

Bacterial exopolysaccharides occur in two forms, as capsule and slime polysaccharide. Most bacteria show a preference for producing one form over another, although some may produce both. Production of exopolysaccharide especially in the form of capsules is widespread in pathogenic bacteria. The type of polysaccharide, the amount produced, and the rate of production may all have an effect on the pathogenicity of the organism.

Batch culture studies on *P. aeruginosa* (Goto *et al*, 1973; Williams and Wimpenny, 1977; Mian *et al*, 1978) have indicated the enhancement of exopolysaccharide formation under nutrient-limited condition in the presence of sufficient carbon. This study has also shown increased CPS production by *K. pneumoniae* in nitrogen-, phosphate-, or sulphate-limited culture in the presence of excess carbon source.

It is possible that the deficiency of an essential nutrient other than the carbon source may limit cell growth and thus the energy requirement for growth could be used for the biosynthesis of the polysaccharide. Kinetic studies with *K. aerogenes* (Neijssel and Tempest, 1975), *Agrobacterium radiobacter* (Linton *et al*, 1987) have also shown that synthesis of exopolysaccharide is the result of over-production of ATP under condition of excess carbon.

Previous studies have demonstrated that exopolysaccharide formation is dependent on the available carbon, although the amount and composition of the exopolysaccharide can be affected depending on the type of bacteria producing them (Sutherland, 1988; Linton, 1990). The organism used in this study was capable of producing polysaccharide from the available carbon source irrespective of the type of

the carbon source utilised. The multiplicity of the catabolic pathways and the ability of *Klebsiella* to utilise different carbon sources under both anaerobic and aerobic conditions might explain this lack of influence of any one carbon source on polysaccharide production (Neijssel and Tempest, 1975; Streekstra *et al*, 1987).

The growth temperature is also a major factor for optimal production of bacterial exopolysaccharide. Most bacteria produce copious amounts of polysaccharides at low temperature and suboptimal growth (Williams and Wimpenny, 1978; Alves *et al*, 1991). In this study, maximum levels of polysaccharide production by *Klebsiella* were also observed in both batch and chemostat culture conditions at suboptimal growth temperatures.

The high amount of exopolysaccharide production at suboptimal growth temperatures might be as a result of the increased activities of enzymes that are involved in the synthesis of polysaccharide precursors. One example that can be cited is the increased activities, below the optimal growth temperature of enzymes involved in the synthesis of GDP-mannuric acid, a precursor of alginic acid (Leitao *et al*, 1992).

However, other reports have also demonstrated temperature-dependent synthesis of capsular antigen. Mutant strains of *K. aerogenes* that produce less capsule at low temperature (30°C) than the parent strain have been reported by Norval and Sutherland (1969). A temperature-dependent K antigen of *E. coli* that can be expressed at 37°C but not at 18°C has also been documented (Orskov *et al*, 1984). An *E. coli* K1 strain which does not express K antigen at 22°C was also susceptible to phagocytosis when incubated at this temperature (Bortolussi *et al*, 1983). Whether the temperature-dependent K antigens that are expressed at 37°C might be more associated with

virulence and causation of diseases in humans and animals than the temperature independent K antigens is not known.

In batch fermentation study, *Klebsiella* produced polysaccharide throughout the growth phases although about 40% of the polysaccharide was produced after exponential growth. This observation was similar to other organisms such as *S. aureus* (Dassy *et el*, 1991), *A. vinelandii* (Horan *et al*, 1983), and *P. aeruginosa* (Mian, 1978) which produce exopolysaccharide throughout all growth phases. The result of this study, however contrasted with other studies where the synthesis of exopolysaccharide was observed only after the exponential phase of growth (Phillips *et al*, 1983; Williams and Wimpenny, 1978). The idea that exopolysaccharide is a secondary metabolite and its production is the result of an "over-flow metabolism" (Neijssel and Tempest, 1975) is arguable.

Production of exopolysaccharide in continuous culture is also dependent on the dilution rate of the culture. *Klebsiella* produced maximum levels of polysaccharide in nitrogen-limited culture at low dilution rate. Such an observation is in agreement with previous studies (Williams and Wimpenny, 1978; Phillips *et al*, 1983). At low dilution rates the mean residence time of the organism increases and there exists an opportunity to utilise the excess carbon thereby producing significant quantities of polysaccharide. The surface polysaccharides are thought to share common carrier systems at least in the initial stages of biosynthesis. At high growth rate it would appear that synthesis of peptidoglycan and LPS may take precedence over polysaccharide synthesis. This could be due to insufficient availability of the isoprenoid lipid for simultaneous synthesis of all surface polymers (Sutherland, 1977). When this constraint is removed as a result of either nutrient limitation (other than

the carbon and energy source) or slow growth rate, then polysaccharide synthesis will increase.

In carbon-limited cultures very low levels of polysaccharide were synthesised. This may suggest that the organism may constitutively produce CPS and the effect of carbon-limitation could be possibly quantitative rather qualitative. This observation was similar to that of Evans *et al* (1979); Jarman *et al* (1978); Mian *et al* (1978), but contrasted to other studies where exopolysaccharide synthesis was not observed under carbon-limited conditions (Phillips *et al*, 1983; Williams and Wimpenny, 1978).

The influence of pH on the production of exopolysaccharide is well known. Most bacteria produce large quantities of polysaccharide when grown in media at neutral pH (Dassy *et al*, 1991; Williams and Wimpenny, 1978; Goto *et al*, 1973). In other studies, low pH has been reported to favour the production of CPS and increase the virulence of *N. meningitidis* (Masson and Holbein, 1985). The influence of pH and the increased productivity of polysaccharide formation by *K. pneumoniae* K1 at pH 6.5 to 7.0 was not exceptional compared to such previous observations.

Amongst the environmental factors that control the biosynthesis of exopolysaccharide is the availability of oxygen. The oxygen supply and the rate at which the culture was mixed had a direct effect on the production of polysaccharide by the organism used in this study. Increase in the physical dispersion of cell and cell-bound polysaccharide in the culture could improve oxygen and nutrient uptake. It is also possible that the polysaccharide which surrounds the cell may act as a barrier for nutrient and/or oxygen transfer. Thus mixing of the culture at higher speed may increase the availability of nutrient and/or dissolved oxygen, thereby increasing the growth rate of mucoid cells. The reduction in synthesis of polysaccharide under

anaerobic conditions may be as a result of a decrease in the amount of available energy from the given supply of sugar.

The increase in exopolysaccharide production by *S. aureus* (Dassy *et al*, 1991), *P. aeruginosa* (Bayer *et al*, 1990), *Xanthomonas* (Suh *et al* , 1990) and *Alcaligenes* (Lawford and Rousseau, 1989) as a result of the increase in the supply of oxygen supported the results described here.

The observation that nonmucoid variants of *Klebsiella* arise during continuous culture is consistent with previous reports on the instability of mucoid *Pseudomonas* when grown in continuous culture (Govan, 1975; Cadmus *et al*, 1975; Mian 1978). When microorganisms are grown under constant nutrient-limited condition new strains (mutants) will develop that are well adapted to the particular environment. Although the precise mechanism of mutational lesion involved is far from understood, the emergence of nonmucoid variants during continuous cultivation may be due to a selective advantage of the non-exopolysaccharide producing cells over exopolysaccharide-producing cells.

The results of this study demonstrated that large quantities of polysaccharide capsule can be recovered using the minimal salts medium formulated in this study. In addition, the recovery of pure capsular polysaccharide using this simple medium could also be helpful for clinical investigation and vaccine development.

CHAPTER IV

CLONING OF *KLEBSIELLA PNEUMONIAE* K1 CAPSULE BIOSYNTHETIC GENES

4.1. Cosmid cloning of Klebsiella chromosomal DNA

4.1.1. Introduction.

The mechanisms of capsule synthesis, translocation across the cell membrane, and export to the cell surface is not known in *Klebsiella*. In addition, the regulatory mechanisms that are involved in the expression of capsule are poorly explained. This could be partly attributed to the complexity and diversity of *Klebsiella* capsule types.

K. pneumoniae serotype 1 was chosen for cloning capsule biosynthetic genes. Since the number and the nature of *Klebsiella* capsule biosynthetic genes are not known, a cosmid cloning strategy was exploited to increase the probability of cloning the entire capsule biosynthesis genes from large fragments of chromosomal DNA. A cosmid vector pEMBLCOS4 that has been used for cloning group II CPS biosynthesis genes (Roberts *et al*, 1986) was used to clone large fragment of chromosomal DNA. A polyclonal antiserum against *Klebsiella* K1 antigen was used to screen the cosmid library for the expression of the K1 antigen in *E. coli* K12.

4.1.2. Construction of cosmid library

The cosmid library was constructed in *E. coli* HB101 using pEMBLCOS4. Random selection and analysis of cosmid clones showed the presence of an insert DNA of 35 to 50 kb in addition to the vector DNA. The probability that any gene sequence was represented in the library was determined by the equation of Clarke and Carbon (1976). Assuming that the genome of *Klebsiella* is the same size as *E. coli* (4.2×10^6 bp) 500 cosmid clones with an average insert size of 40 kb would be predicted to have a 99% probability that any gene would be represented in the library.

4.1.3. Screening of the cosmid library

More than 2000 cosmid clones were screened for the expression of K antigen of *Klebsiella* K1 by ELISA. Ten clones reacted positively and gave a reading of 1.0 to 1.25 at OD 490 nm. The positive control gave a reading of >2.0 while *E. coli* HB101 (pEMBLCOS4) gave an OD of less than 0.45. The entire library and the ELISA positive clones were also screened by colony immunoblotting. Only three of the clones containing recombinant cosmid designated as pCOS47, pCOS22, pCOS261 showed a positive reaction (Fig 4.1).

The library was also constructed in different *E. coli* K12 strains using either cosmid pHC79 or pLAFR3. Although alternative approaches were attempted, cloning of the structural genes that direct the biosynthesis of *Klebsiella* capsular antigen was not possible. Previous work has also indicated the difficulty of direct cloning of capsule biosynthesis genes. The structural genes may be too big to be accommodated in a single vector or are not closely linked together. Alternatively, the clones that carry the genes may only grow very slowly making it difficult to distinguish them from other satellite colonies.

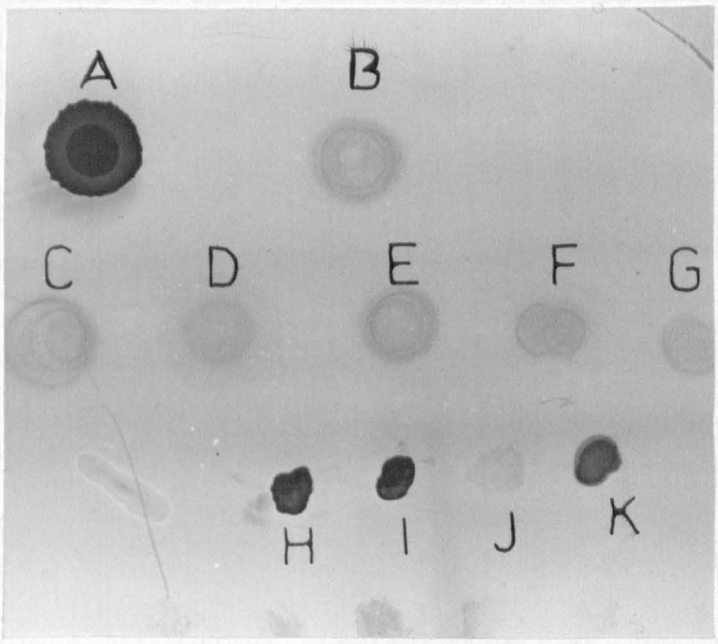


Figure 4.1. Immuno-blot analysis of antigens encoded by recombinant cosmid clones. *Klebsiella* K1 (A); *E. coli* HB101/pEMBLCOS4 (B); HB101/pCOS47 (H); HB101/pCOS222 (I); HB101/pCOS261 (K); *E. coli* cosmid clones which did not react positively (C to G and J).

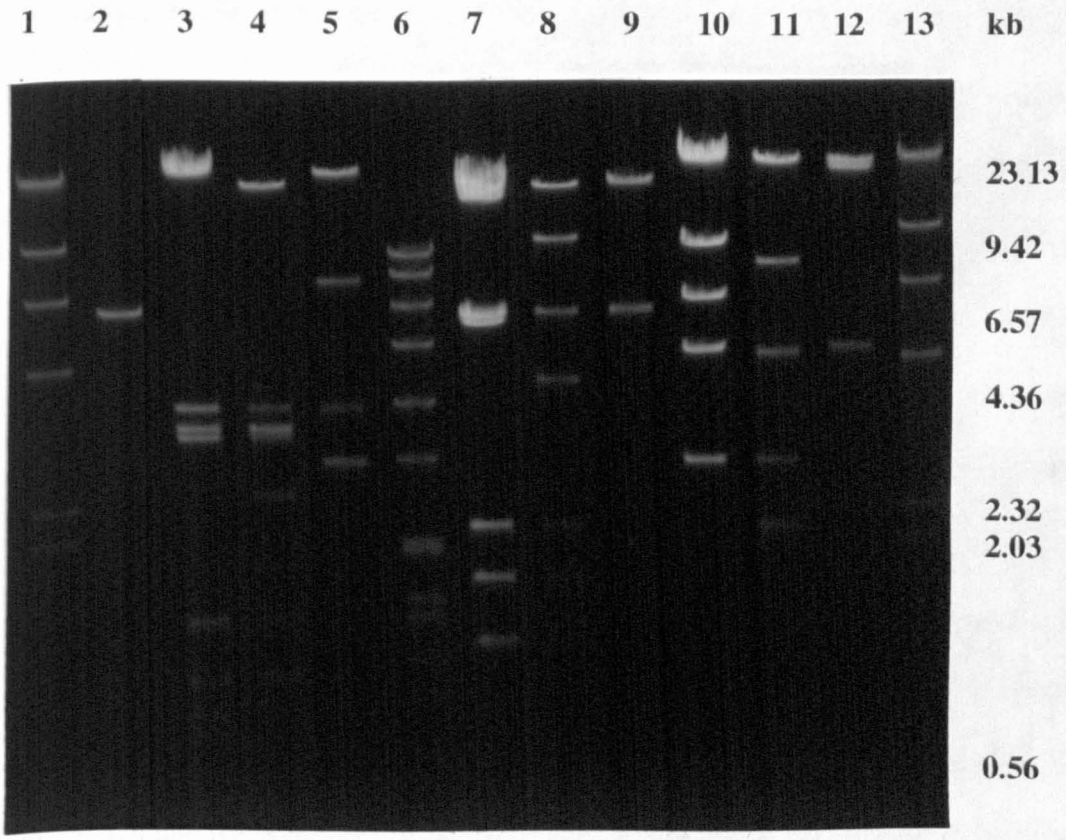


Figure 4.2. Agarose gel electrophoresis of recombinant cosmids. pCOS47 digested with *Bam*HI (3), *Eco*RI (7), *Hin*dIII (10); pCOS222 digested with *Bam*HI (4), *Eco*RI (8), *Hin*dIII (11); pCOS261 digested with *Bam*HI (5), *Eco*RI (9), *Hin*dIII (12); pEMBLCOS4 *Bam*HI digest (2); λ DNA *Hin*dIII digest (1, 13); SPPI DNA *Eco*RI digest (6).

4.1.4. Subcloning of plasmid pCOS47

The three recombinant cosmids designated as pCOS47, pCOS222, pCOS261 contained 36, 34, and 29 kb insert DNA respectively (Fig 4.2). As shown in the partial linear restriction map (Fig 4.3), the recombinant cosmids shared at least a common 20 kb DNA fragment. The *Bam*HI fragment of pCOS47, also common to pCOS222 and pCOS261 was subcloned by self-ligating and transforming into *E. coli* HB101. The subcloned cosmid was designated as pCOS474 contains a 22 kb DNA fragment. A partial linear map of pCOS474 is also shown in Fig 4.3. The clone containing pCOS474 expressed *Klebsiella*-specific antigen, suggesting that the DNA fragment which expresses the antigen was localised within this region.

To investigate the effect of *rcs* genes on the expression of the antigen, a hybrid plasmid was also constructed. The *Bgl*II fragment of pLV592 that contains both *rcsA* and *rcsB* from *K. pneumoniae* K1 (chapter V) was ligated into the *Bam*HI site of pCOS474 and the resulting recombinant plasmid (pCOS474A; Fig 4.4) was introduced into *E. coli* HB101. The clone containing pCOS474A became mucoid as a result of induction by *rcs* genes. In addition to the production of the colanic acid, the clone expressed the protein antigen. Since both the protein antigen and colanic acid polysaccharide were expressed, *rcs* genes had no influence in the expression of pCOS474A-encoded antigens.

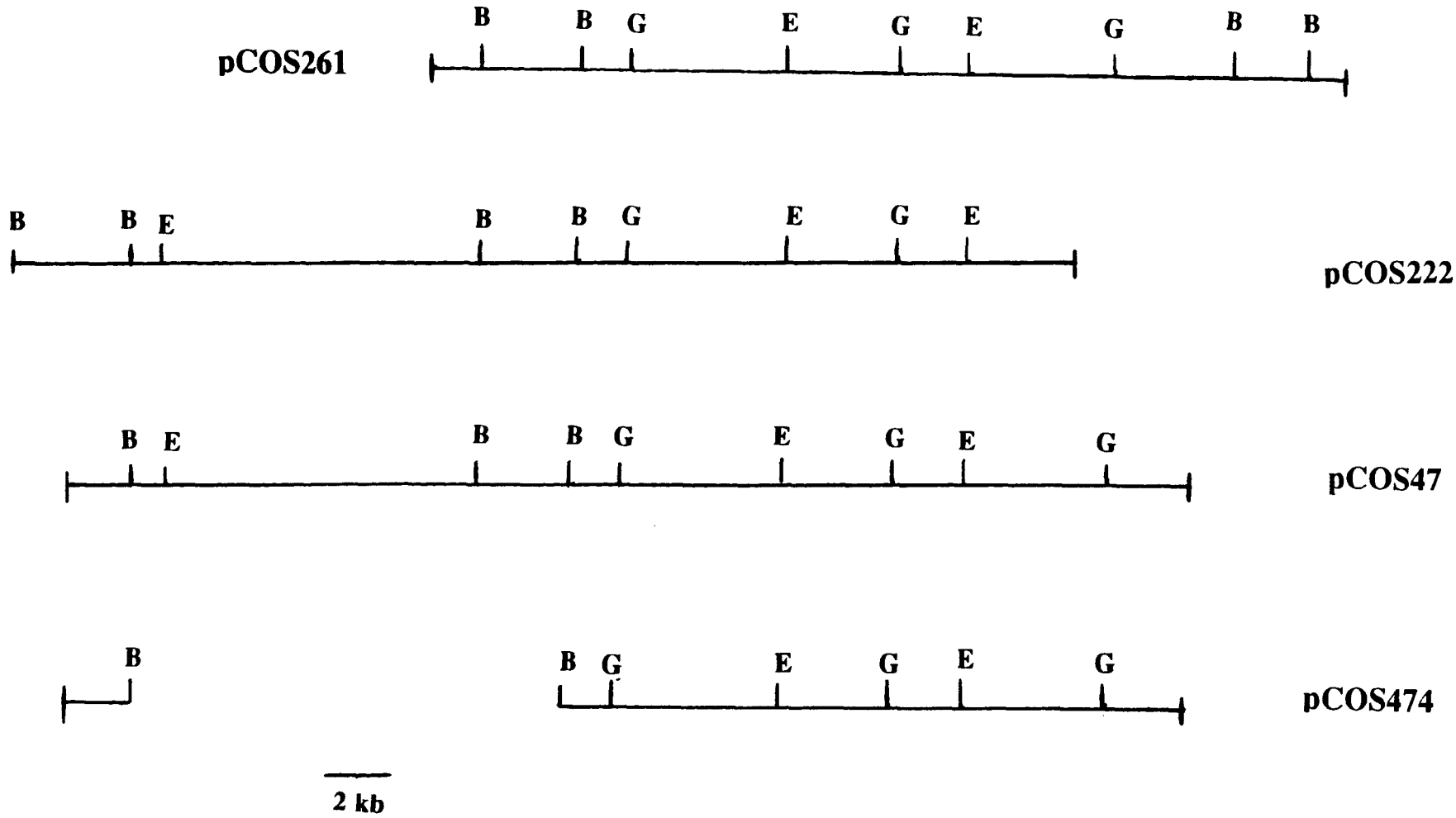


Figure 4.3. Partial restriction map of recombinant cosmids. B= *Bam*HI, E= *Eco*RI, G= *Bgl*II. Only the insert DNA is shown.

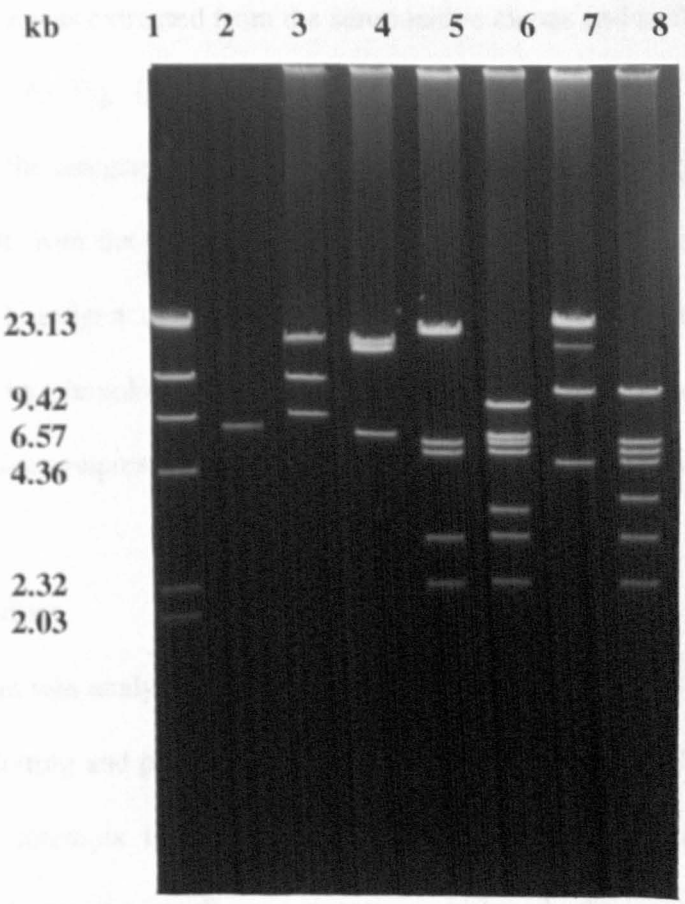


Figure 4.4. Agarose gel electrophoresis of the recombinant cosmid pCOS474A. Cosmid DNA digested with *Bgl*II (3), *Eco*RI (4), *Cla*I (5), *Eco*RI+*Cla*I (6), *Cla*I+*Hin*dIII (7). λ DNA *Hin*dIII digest (1); pEMBLCOS4 *Bgl*II digest (2).

4.1.5. Analysis of recombinant cosmid-encoded antigen

A. Immunodiffusion

The antigen was extracted from the seropositive clones and analyzed by double immunodiffusion. As Fig 4.5 shows the precipitin lines formed as a result of the reaction between the antigen expressed by the clones and the antibody were identical but were different from the precipitin lines formed as result of the reaction between the *Klebsiella* K1 antigen and the antibody. Since proteinase K treatment of the antigen followed by phenol/chloroform extraction resulted in the loss of antigenicity (Fig 4.5), the antigen expressed by these cosmid clones could be a protein antigen.

B. Western Blotting.

The protein was analyzed by SDS-PAGE and transferred on to a nitrocellulose filter by electroblotting and probed with *E. coli*-absorbed anti *Klebsiella* K1 antibody. Despite repeated attempts to transfer the antigenic component using a range of alternative procedures, the result was negative. Although the reason is far from understood, it may be due to the instability of the protein antigen which may have lost its antigenic epitope.

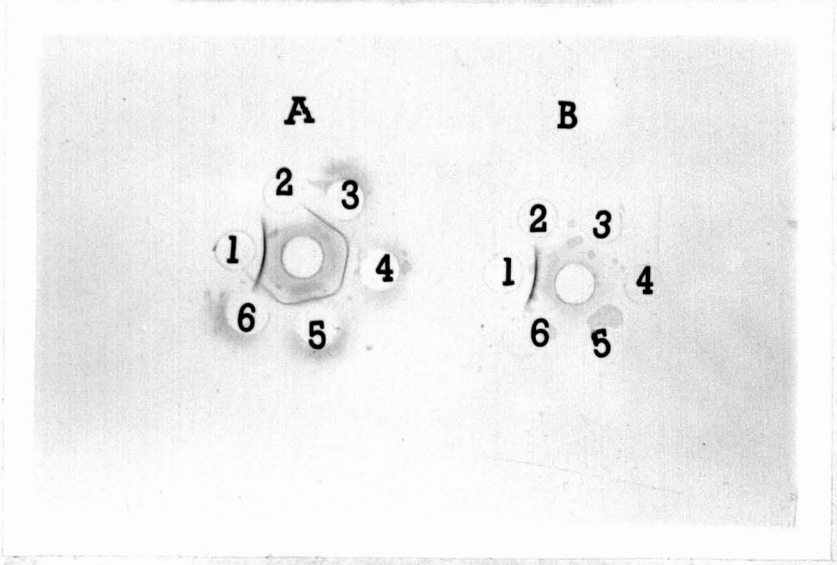


Figure 4.5. **A.** Double immunodiffusion analysis of antigens expressed by cosmid clones. Central well: anti *Klebsiella* K1 antiserum. Peripheral well: K1 CPS (1); HB101/pEMBLCOS4 (2); pCOS47 (3); pCOS222 (4); pCOS261 (5); pCOS474 (6). **B.** As above except all the antigens were treated with proteinase K and extracted with phenol/chloroform.

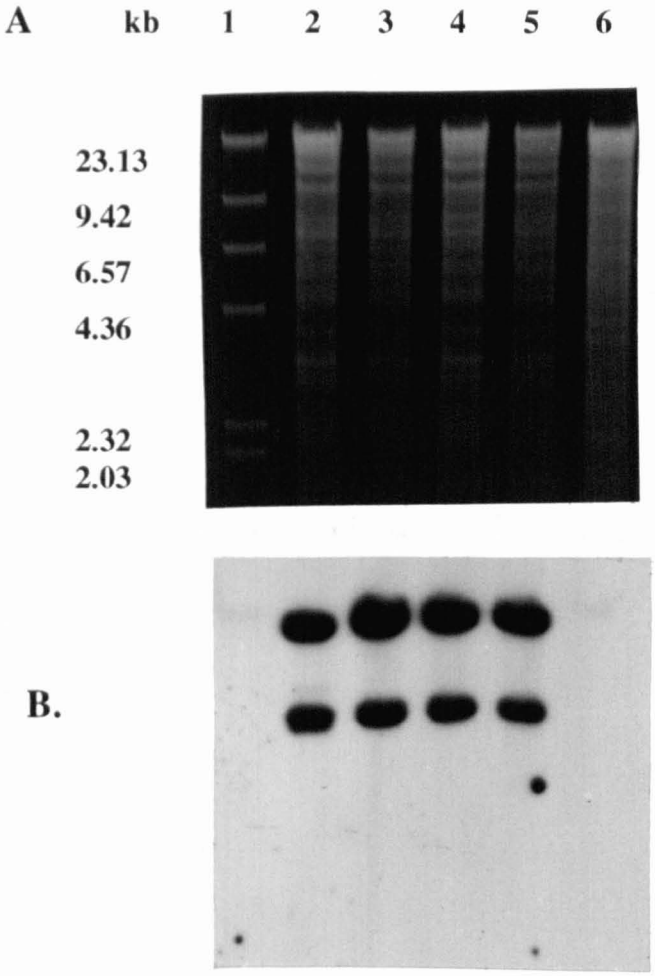


Figure 4.6. **A.** Agarose gel electrophoresis of *Eco*RI digests of chromosomal DNA. *Klebsiella* 3L100 (2), 3L101 (3), 3L104 (4), 3L105 (5), *E. coli* HB101 (6). λ DNA *Hin*dIII digest (1). **B.** Southern hybridisation analysis using labelled insert DNA from pCOS474.

4.1.6. Southern hybridisation analysis

To determine the location of the cloned genes which encode the protein antigen, chromosomal DNA from *Klebsiella* and *E. coli* strains was analyzed by Southern hybridisation using labelled insert DNA from pCOS474. As Fig 4.6 shows the cloned DNA that directs the expression of the protein antigen was restricted to *Klebsiella*. No homology was detected between the insert DNA and the chromosomal DNA from *E. coli* HB101.

4.2. Transposon mutagenesis of *Klebsiella* K1 capsule genes using Tn*10*

4.2.1. Introduction

Transposon elements have the ability to move in a random fashion from one genetic locus to another. The insertion of the transposon into a gene usually leads to the inactivation of that gene. Transposon mutagenesis techniques have been exploited for understanding the complex genetic organisation of bacteria. Transposons that carry antibiotic resistance genes are particularity useful in the positive selection of the mutants, for cloning, and mapping of genes (Saunders and Saunders 1987). Transposon-induced mutations have been previously applied in *Klebsiella* for the analysis of type I fimbriae (Purcell and Clegg, 1983), alginate lyase (Gacesa *et al*, 1987), the histidine utilisation gene cluster (Schwacha *et al*, 1990), mapping of the *his* region (Brujin *et al*, 1983), and cloning of the K2 capsule genes (Arakawa *et al*, 1991). Transposon mutagenesis was used in an attempt to generate noncapsulated mutants of *Klebsiella* and to clone the mutated capsule biosynthetic genes.

4.2.2. Transfer of pHSG415::Tn*10* to *Klebsiella* K1

Plasmid pHSG415 is a derivative of pSC101 containing a temperature sensitive gene for replication (Hashimoto-Gotoh *et al*, 1981). This plasmid can only replicate and be maintained at ≤30°C. Plasmid pHSG415::Tn*10* was previously constructed by ligating the *Pst*I fragment of Colb::Tn*10* into the *Pst*I site of pHSG415 (Gacesa *et al*, 1987). This plasmid was used for the delivery of the transposon Tn*10* into *Klebsiella* strains in order to generate noncapsulated mutants.

A. Mobilisation of pHSG415:Tn*10* using R64*drd*11

A mobilising plasmid R64*drd*11 (Meynell and Datta, 1967) was used to mobilise the Tn*10* donor plasmid into *Klebsiella*. *E. coli* LE392 containing both plasmids (R64*drd*11 and pHSG415::Tn*10*) was mated with *Klebsiella* 3L100 or 3L103 at 30°C. Transconjugants were selected on minimal medium containing Tc and Cm or Km. The transfer of pHSG415::Tn*10* by the conjugative plasmid was observed at a very low frequency (10^{-7}) in both 3L100 and 3L103 strains. Transfer of pHSG415 by R64*drd*11 has been reported to be extremely low and mobilisation might be due to passive transfer through the formation of cointegrate plasmids (Hashimoto-Gotoh *et al*, 1981).

All $Cm^rKm^rTc^r$ *Klebsiella* transconjugants were also Sm^r, a marker for R64*drd*11. An attempt to eliminate the conjugative plasmid R64*drd*11 was less effective.

B. Transformation of pHSG415:Tn*10*

The plasmid pHSG415::Tn*10* isolated from *E. coli* LE392 was transformed into different strains of *Klebsiella*. All of the strains failed to be transformed except for *Klebsiella* 3L100. This might be due to a difference in restriction barrier associated with the strains used. This phenomenon has also been observed by Gacesa *et al* (1987).

Transformants were selected on media containing Tc, Km, and Cm. The Tn*10* donor plasmid was eliminated from transconjugants by growing at 42°C. All transformants were tested for the Cm^r and Km^r markers of pHSG415. Transformants that were $Cm^sKm^sTc^r$ would have lost the plasmid and inserted Tn*10* in their chromosome. Out of 3,600 colonies that were $Cm^sKm^sTc^r$, 3(0.08%) were nonmucoid and 28(0.8%) were auxotrophs and did not grow on minimal medium. This indicated the random transposition of Tn*10* into different regions of the chromosome.

4.2.3. Analysis of noncapsulated mutants

The loss of K antigen production by the noncapsulated mutants was analyzed by double immunodiffusion. As judged by precipitation reaction, three of the nonmucoid mutants KNM101, KNM102, and KNM103 did not produce capsule.

Tn*10* insertion into the chromosome was analyzed by Southern hybridisation using a labelled Tn*10* probe. Since *Bam*HI has a single site in the transposon region, chromosomal DNA digested with this endonuclease was expected to give two bands if the chromosome had acquired a single insertion of Tn*10*. Positive hybridisation and the appearance of only two bands within the chromosome in all the noncapsulated mutants was an indication of a single transposon insertion mutation in each case. Tn*10*

insertion into the chromosome of KNM101 and KNM102 appeared to be at the same position, while in KN103 it was different (Fig 4.7).

4.2.4. Cloning of Tn*10*-mutated genes

In order to clone Tn*10* plus genes located adjacent to the insertion site, cosmid pEMBLCOS4 was used. *Sau*3A-digested chromosomal DNA isolated from the noncapsulated mutants was ligated into the *Bam*HI site of the cosmid and packaged into λ head particles. The cosmid library was then amplified in *E.coli* HB101.

Eight Ap^rTc^r clones (pCOSTN101, 102, 103, 104, 105, 106, 107 and 108) were isolated. Analysis of plasmid preparations revealed that only recombinant cosmid pCOS103 retained insert DNA with a size of 45 kb, including Tn*10*, while others lost all the insert DNA and part of the vector fragment (Fig 4.8). Although pCOSTN103 initially contained insert DNA, further growth of the clone in either broth culture or solid media revealed the loss of the entire insert DNA fragment and part of the vector fragment.

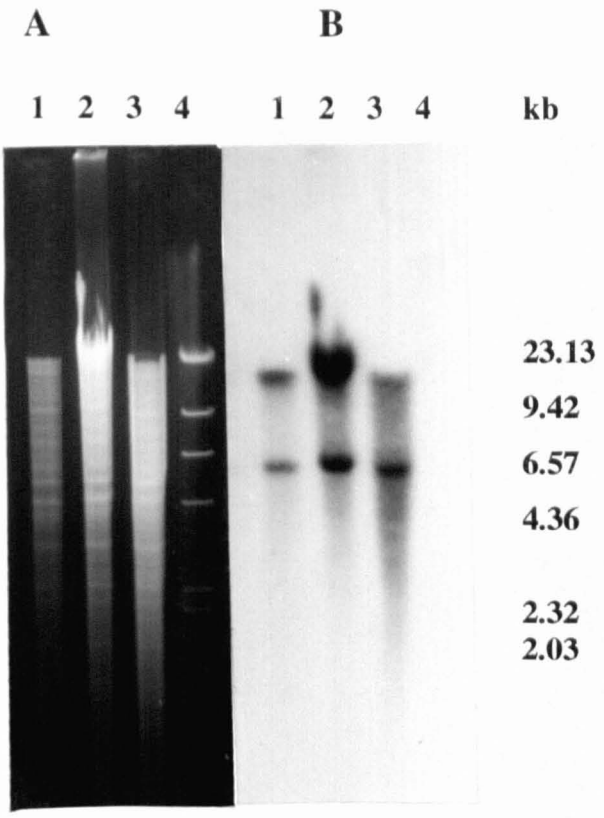


Figure 4.7. **A.** Agarose gel electrophoresis of *Bam*HI digests of chromosomal DNA. *Klebsiella* KNM101 (1), KNM102 (2), KNM103 (3); λ *Hin*dIII digest (4).

B. Southern hybridisation analysis using *Pst*I fragment of pHSG415::Tn*10* as a probe.

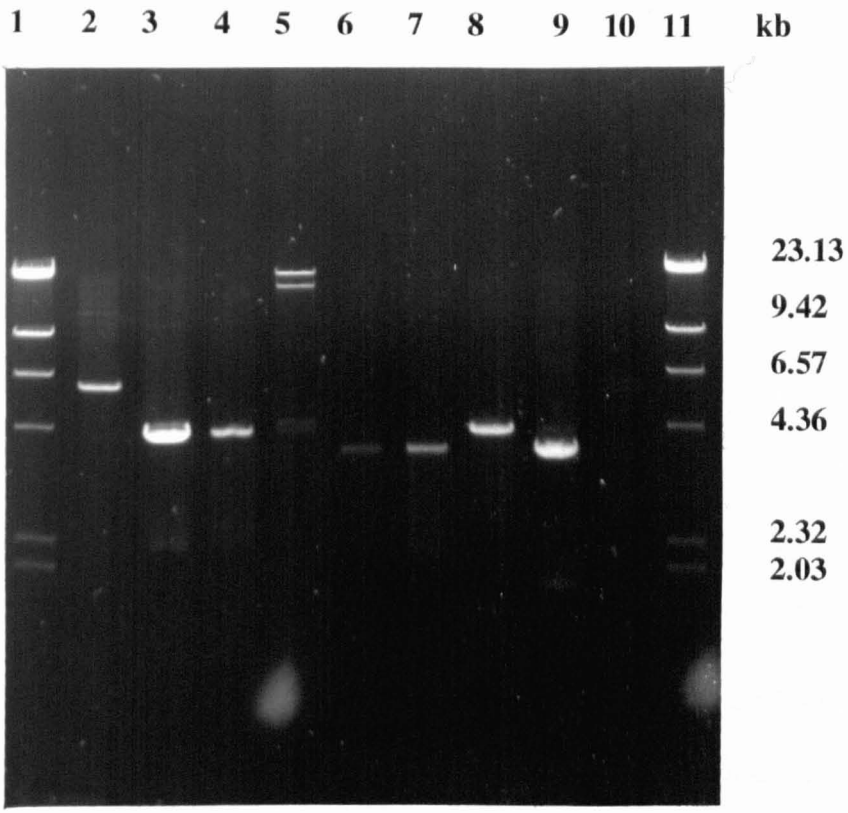


Figure 4.8. Agarose gel electrophoresis of *Pst*I digests of recombinant cosmids. pCOSTN101 (3), pCOSTN102 (4), pCOSTN103 (5), pCOSTN104 (6), pCOSTN105 (7), pCOSTN106 (8), pCOSTN107 (9), pCOS108 (10). pEMBLCOS4 *Pst*I digest (2). λ DNA *Hin*dIII digest (1 and 11).

An *E coli* strain with *recC*, *recB*, *sbc15*, and *recJ* mutations that is claimed to stabilise recombinant cosmids and prevent deletion (Ishiura *et al*, 1989) was also used to amplify the cosmid library. However, the recombinant cosmid was nevertheless not maintained without deletion of the insert DNA. Because of the instability of the cosmid clone further analysis was not possible.

An attempt has also been made to clone the Tn*10*-mutated genes using plasmid pUC19. *Sau3A* or *Pst*I partial digests of chromosomal DNA from KNM101/103 were ligated with *Bam*HI or *Pst*I linearised pUC19 respectively. The ligated plasmids were transformed into *E. coli* HB101, DH1, AB1157, or LE392. Initially Tc^r clones were isolated, but further growth was not possible due to loss of the Tc^r marker followed by deletion of the recombinant plasmids.

The temperature-sensitive low copy pHSG415 was constructed from pHSG415:Tn*10* by removing the *Pst*I fragment of DNA containing Tn*10* that was originally inserted at the *Pst*I site of pHSG415. Partially digested chromosomal DNA ligated into the *Bam*HI site of pHSG415 was transformed into *E. coli* HB101 or LE392. Although Ap^rTc^r, Cm^rT^r or Km^rTc^r transformants were initially observed after 24 h growth at 30°C, the clones were unstable and lost the Tc^r marker. Since deletion including that of Tn*10* was observed irrespective of the plasmid vector and genetic background of the host strain used, further analysis was not possible.

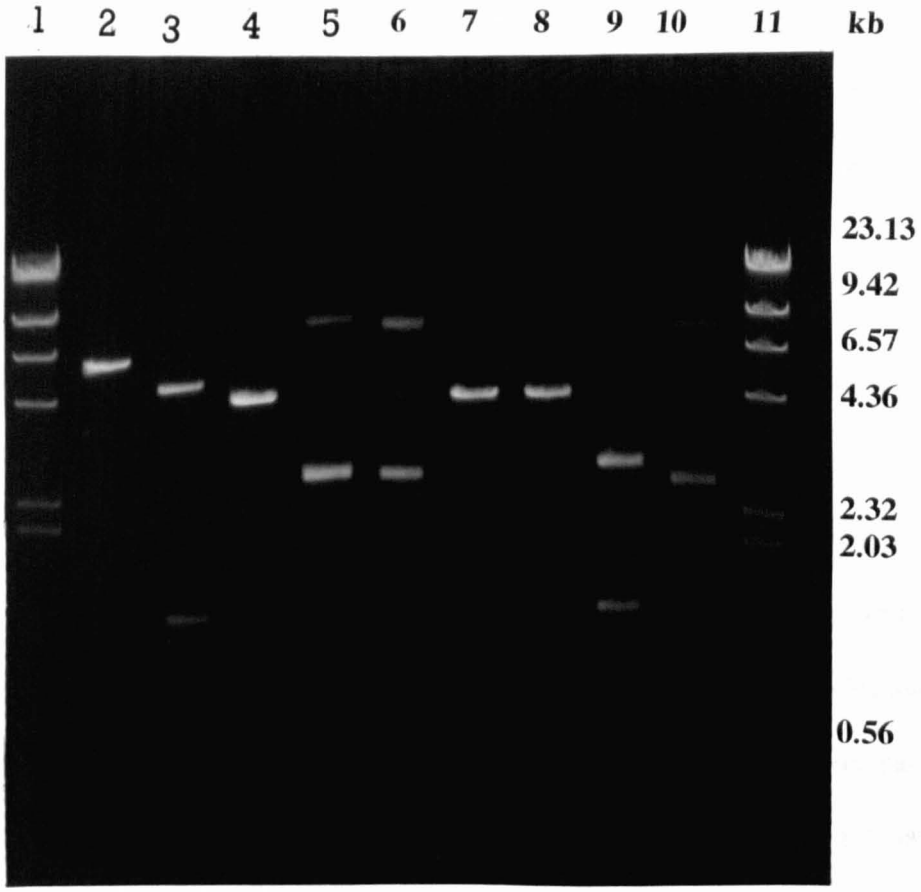


Figure 4.9. Agarose gel electrophoresis of cosmid pCOS103. *Nru*I (4), *Bgl*II (5), *Pvu*II (6), *Pst*I (7), *Sal*I (8), *Sal*I+*Pst*I (9), *Hpa*I (10). pEMBLCOS4 digested with *Bgl*II (2) *Nru*I (3). λ DNA *Hin*dIII digest (1, 11).

4.2.5. Analysis of cosmid pCOS103.

The deleted cosmid (pCOS103) from the recombinant pCOSTN103 was stably maintained when transformed into various *E. coli* K12 strains. This recombinant cosmid has a molecular size of about 4.5 kb and was less than the size of the original vector pEMBLCOS4 (Fig 4.9). Restriction endonuclease digestion of this cosmid revealed the deletion of the *cos* site downstream to the *tet* gene while all other functional regions were retained (Fig 4.10).

The cosmid pEMBLCOS4 has been used on different occasions for cloning of large fragments of chromosomal DNA in this work and by others. In such cases deletion was not observed (Roberts *et al*, 1986). Apart from inactivation of the gene as a result of insertion, Tn*10* is unlikely to mediate deletion of a gene unless by aberrant excision. Structural instability may be mediated by insertion elements or transposons present in the plasmid and may lead to deletion by aberrant excision (Jones *et al*, 1982; Rood *et al*, 1980). The deletion of the recombinant cosmid and loss of the part of the vector segment might also be influenced by the unstable cloned genes.

To determine whether the cosmid pCOS103 could be packaged into a λ head particle, the *Bam*HI linearised cosmid was ligated with a *Bam*HI digest of chromosomal DNA and subsequently packaged. Transfection of the packaged cosmid into *E. coli* HB101 resulted in a good deal of transformation. Random selection and plasmid analyses of Ap^r cosmid clones indicated the insertion of large insert DNA with an average insert size of 40 kb. A restriction digest of one of the selected recombinant cosmids (pCOS1031) is shown in Fig 4.11. The stability of the clone was also checked by repeated subculturing in broth. After several generations, plasmid

isolation and restriction digestion showed the stable maintenance of the recombinant cosmid without deletion. Since cosmid pCOS103 is not only stable but can also accommodate large insert DNA, it might be a better choice for future cosmid cloning purposes.

4.3. Transposon mutagenesis of *Klebsiella* K1 capsule genes using Tn*phoA*

4.3.1. Introduction

Plasmid pRT733, a derivative of the suicide vector pJM703.1 which contains Tn*phoA* was previously constructed by Taylor *et al* (1989). It can only replicate and be stably maintained in *E. coli* strains containing λ *pir* prophage. Tn*phoA* is a derivative of Tn*5* fused with the alkaline phosphatase (*phoA*) gene which was constructed for the analysis of protein export signals (Manoil and Beckwith, 1985). In this work, Tn*phoA* was also used to generate noncapsulated mutants of *K. pneumoniae* K1 for the analysis of capsule biosynthesis genes.

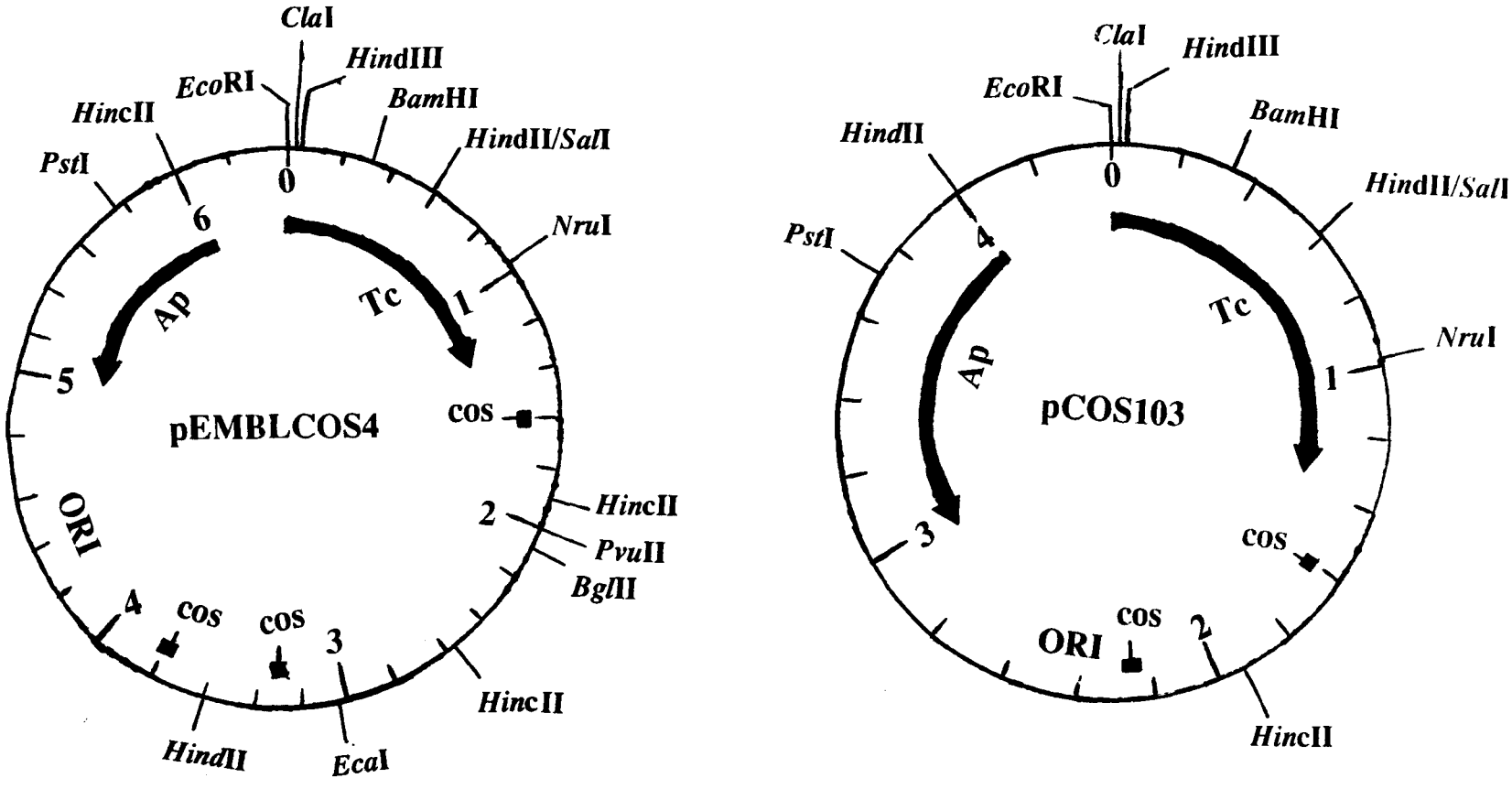


Figure 4.10. Partial restriction map of pEMLCOS4 and pCOS103. The arrows show the *bla* or *tet* gene position and the direction of transcription. Approximate size of the cosmid pCOS103 was determined by restriction endonuclease digestions.

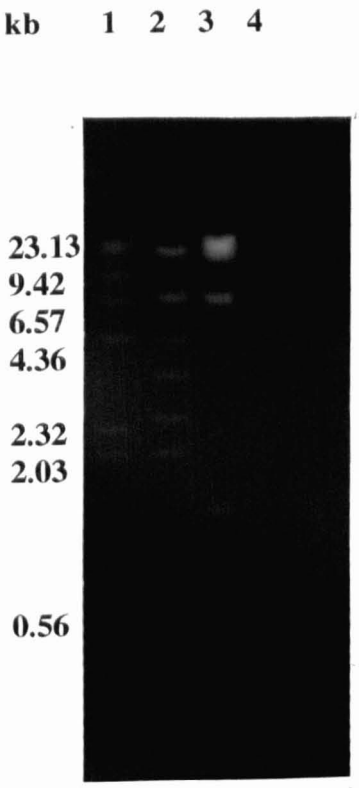


Figure 4.11. Agarose gel electrophoresis of recombinant cosmid pCOS1031. *Bam*HI (2), *Eco*RI (4). pCOS103 *Bam*HI digest (4). λ DNA *Hin*dIII digest (1).

4.3.2. Mobilisation of pRT733 into *Klebsiella* K1

A Nal^r mutant *K. pneumoniae* K1 (3L103) was isolated by growing in L-broth containing nalidixic acid. *E. coli* SM10 containing pRT733 was mated with *Klebsiella* on nonselective media for 10 h at 37°C. Transconjugants were selected by a single step of selection for both Km^r and Nal^r. Km^r transconjugants were isolated at a frequency of 10^{-5} recipient cells.

Random transposition of Tn*phoA* into the different regions of the chromosome of *Klebsiella* was confirmed by the emergence of auxotrophic transconjugants when patch-plated on minimal medium. Out of 1800 Km^r transconjugants that were patch-plated on minimal medium, 13 (0.7%) failed to grow on minimal media.

Ap^{r}Km^r transconjugants could be isolated at a frequency of up to 20% of the transposon fusion (Taylor *et al*, 1989). This was assumed to be the result of co-integrate formation between the chromosome and the vector plasmid. Since almost all *Klebsiella* isolates are resistant to ß-lactam antibiotics, cointegrate formation between the recipient chromosome and the vector plasmid can only be assessed by Southern hybridisation.

4.3.3. Analysis noncapsulated mutants

A total of 23 noncapsulated mutants were isolated by Tn*phoA* insertional inactivation into the chromosome. An anti *Klebsiella* K1 antiserum absorbed with noncapsulated mutant M10 strain (O1:K$^-$) was used to screen for loss of K1 capsule production. Immunodiffusion analysis of antigen extracted from whole cell lysate showed the absence of any *Klebsiella* K1 antigen.

The loss of capsule was also confirmed by immuno-electron microscopy. K1 capsule was indirectly labelled with an anti-mouse antibody-gold conjugate. The aggregation of gold particles on the surface of the parent mucoid cell was an indication of binding of the gold conjugate antibody to the capsule through anti-capsular antibody. The absence of the gold particles on the surface of the noncapsulated mutant (KNM52) was related to the loss of capsule (Fig 4.12).

4.3.4. Southern hybridisation analysis

Southern hybridisation analysis of chromosomal DNA with the labelled probe confirmed that 22 of the 23 nonmucoid mutants showed a single band (Fig 4.13). Since *Eco*RI endonuclease has no site within the transposon region, hybridised DNA that showed a single band was assumed to have acquired a single Tn*phoA* insertion into their chromosomes. The insertion of Tn*phoA* occurred on different *Eco*RI fragments of the chromosome. This also confirmed the transposition of Tn*phoA* at random. There are at least five different regions in which transposition has occurred. It is therefore possible that structural genes that direct the synthesis of CPS might be organised into at least five regions.

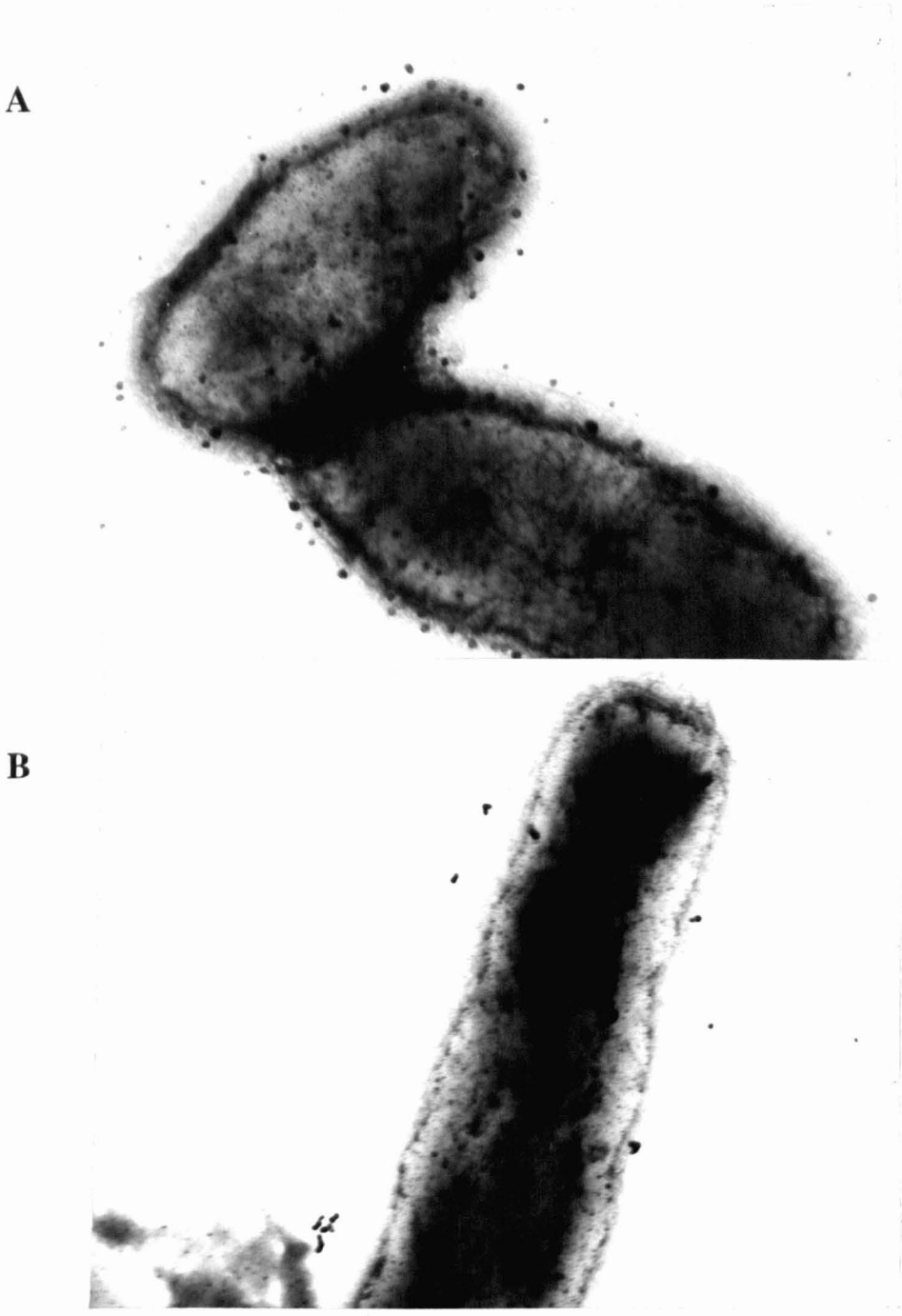
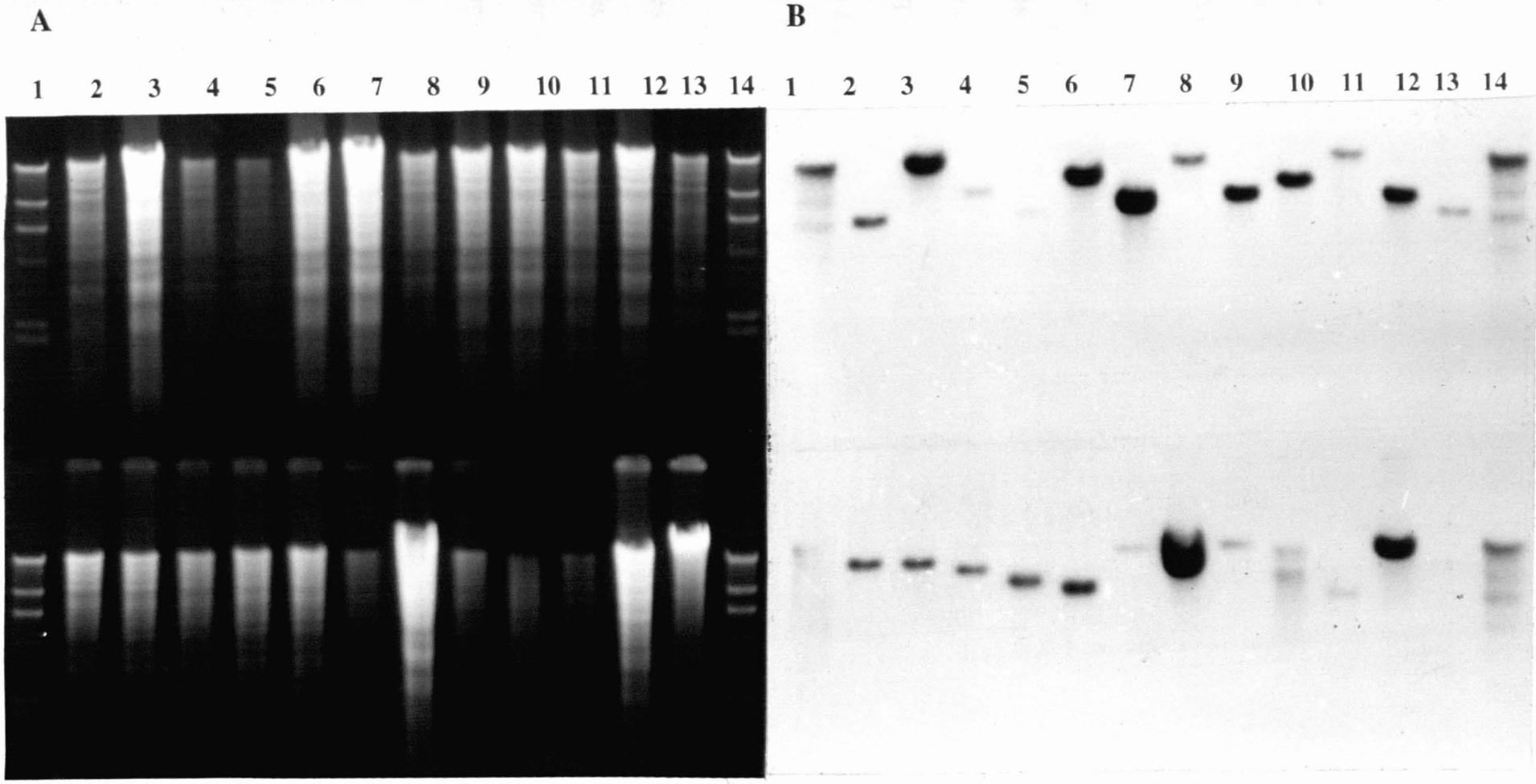


Figure 4.12. Negatively stained electron microscope preparation of *K. pneumoniae* labelled with immuno-gold. An antibody specific for K1 antigen was indirectly labelled with anti-mouse antibody-gold conjugate. **A**. Capsulated *Klebsiella* 3L103, (50,000X). **B**. Noncapsulated mutant *Klebsiella* KNM52 (50,000X).

Figure 4.13. **A**. Agarose gel electrophoresis of *Eco*RI digests of chromosomal DNA isolated from noncapsulated mutant strains: KNM51 (2), KNM52 (3), KNM53 (4), KNM54 (5), KNM55 (6), KNM56 (7), KNM57 (8), KNM58 (9), KNM59 (10), KNM510 (11), KNM511 (12) KNM512 (13), KNM513 (15), KNM514 (16), KNM515 (17), KNM516 (18), KNM517 (19), KNM518 (20), KNM519 (21), KNM520 (22), KNM521 (23), KNM522 (24), KNM523 (25), 3L103 (26). λ DNA *Hin*dIII digest (1, 14, 15, 28). **B**. Southern hybridisation analysis using the 0.92 kb *Pst*I internal fragment of Tn*phoA* as probe. The hybridised filter was reprobed using labelled λ DNA for approximate estimation of fragment size.


A
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
15 16 17 18 19 20 21 22 23 24 25 26 27 28

4.3.5. Cloning of Tn*phoA*-mutated genes

*Eco*RI which does not cut within the Tn*5* region was exploited for cloning Tn*pho*A plus DNA fragments flanked at either side of the fusion. *Eco*RI partial digests of chromosomal DNA (15-30 kb) were ligated into the *Eco*RI site of either plasmid pACYC184 or pBR322. The ligated DNA was transformed into *E. coli* HB101.

From the noncapsulated mutant KNM52, a gene library was constructed in *E. coli* HB101 using pACYC184 and 1250 clones were isolated. Tn*phoA* containing clones were positively selected by growing on L agar containing Km and Tc. Two clones were isolated and the corresponding recombinant plasmids were designated as pACTN12 and pACTN103. A gene library of KNM55 was also constructed. Out of 1500 *E. coli* clones, 3 Tc^rKm^r clones were isolated. The recombinant plasmid isolated from the three clones were designated as pACTN9, pACTN15, and pACTN18.

Plasmid isolation and restriction digestion analysis showed that all the km^rTc^r clones contained insert DNA between 11 to 26 kb including Tn*phoA*. The presence of Tn*phoA* was also confirmed by Southern hybridisation analysis of recombinant plasmids pACTN12 and pACTN18 (Fig 4.13). A partial restriction map of the recombinant plasmids was also done (Fig 4.14).

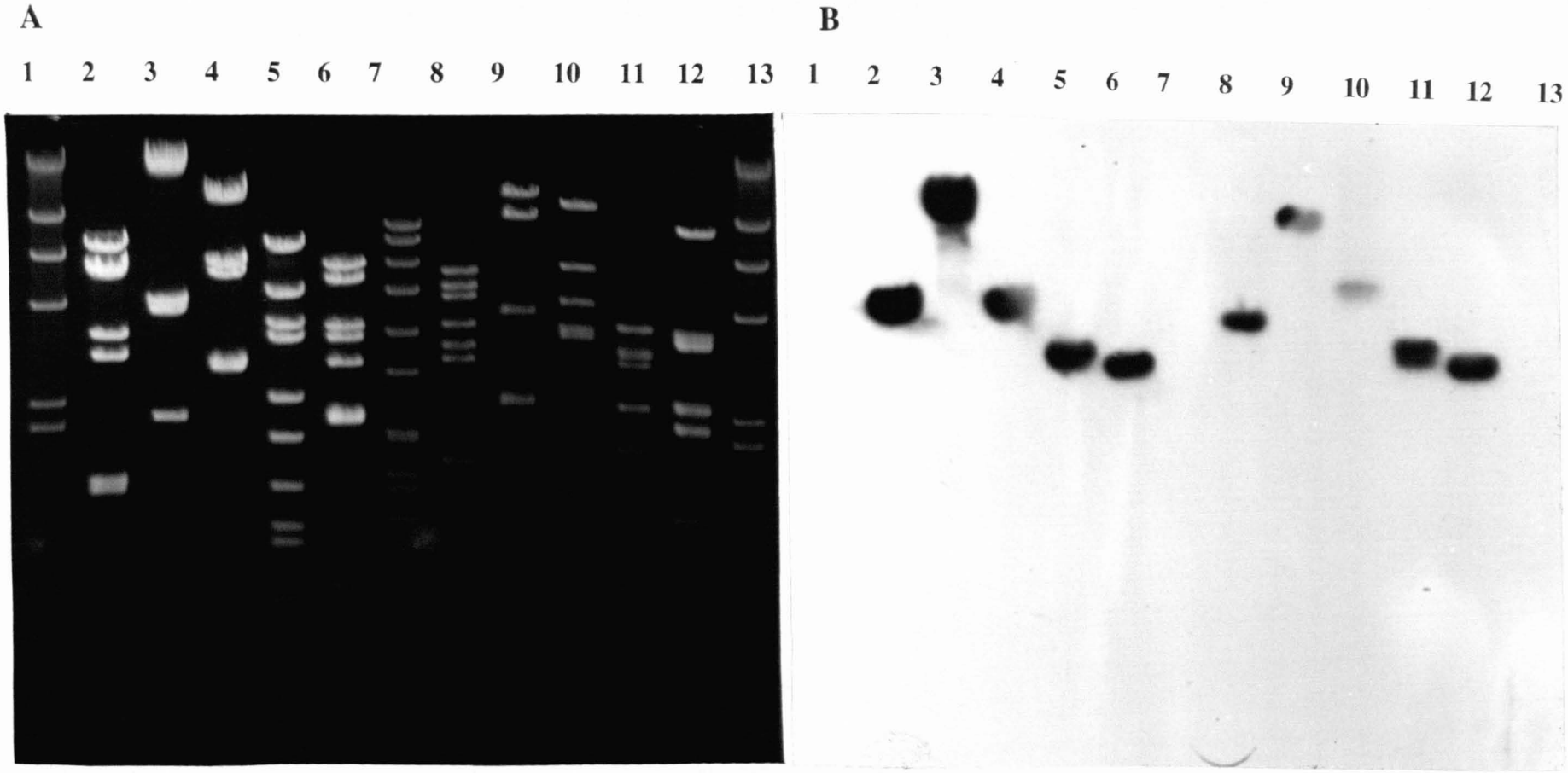


Figure 4.14. **A**. Agarose gel electrophoresis of recombinant plasmids. pACTN12 digested with *Bam*HI (2), *Eco*RI (3), *Sal*I (4), *Bam*HI+*Eco*RI (5), *Eco*RI+*Sal*I (6); pACTN18 digested with *Bam*HI (8), *Eco*RI (9), *Sal*I (10), *Bam*HI+*Eco*RI (11), *Eco*RI+*Sal*I (12), λ DNA *Hin*dIII digest. SPPI DNA *Eco*RI digest (7). **B**. Southern hybridisation analysis using 0.92 kb *Pst*I internal fragment of Tn*pho*A as a probe.

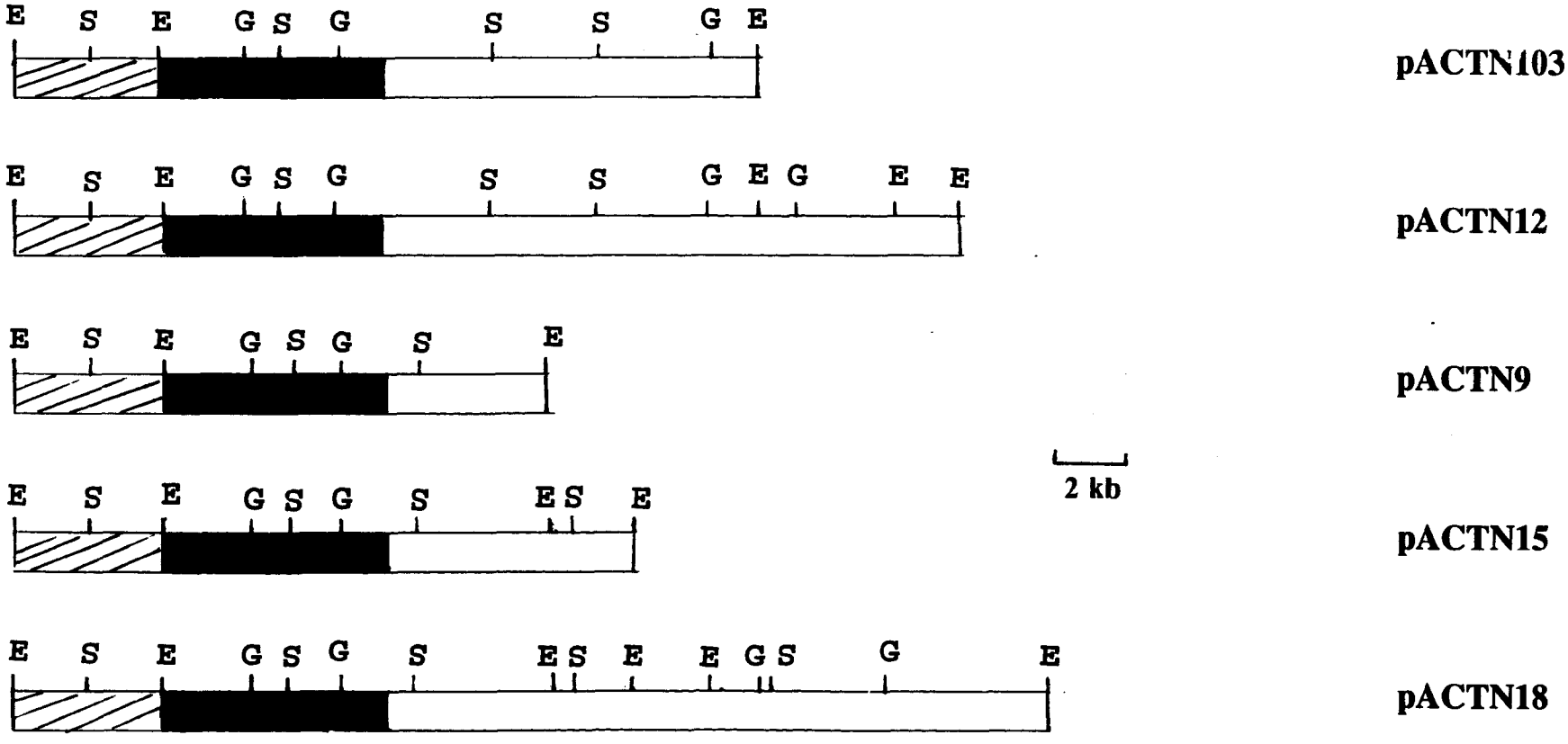


Figure 4.15. Partial restriction map of recombinant plasmids: pACTN103, pACTN12, pACTN9, pACTN15, and pACTN18. E= *Eco*RI, G= *Bgl*II, S= *Sal*I. ▨ pACYC18; ■ Tn*phoA*; □ insert DNA.

4.3.6. Complementation analysis

All *E. coli* clones containing recombinant pACTN12, pACTN15, pACTN18, pACTN9, or pACTN103 failed to express *Klebsiella* K1-specific antigen when screened by double immunodiffusion. In an attempt to check the effect of *rcs* genes, pCOS474A or pLV592 depending on the compatibility of the recombinant plasmid, were transformed into *E. coli* clones containing pACTN12, pACTN15, pACTN18, pACTN9, or pACTN103. Although all the transformants were mucoid as a result of the induction of colanic acid synthesis by *rcs* genes, there was no evidence of *Klebsiella* K1 specific capsule production. This could either the inserted DNA might not be sufficient to direct the expression of the capsular antigen of *Klebsiella* or may require additional genes that are not necessarily closely linked together. It is also possible that transposition of Tn*phoA* may be within the ORF of the structural genes so that the genes may not be sequentially transcribed.

In an attempt to complement capsule gene mutations, each of the recombinant plasmids was also transformed into various noncapsulated mutants of *Klebsiella* strains that were sensitive to Tc. The presence of the recombinant plasmids and their stabilities was confirmed by a series of miniplasmid preparations. However, neither the restoration of capsule nor the expression of K1 antigen was obtained.

4.4. Attempted transfer of capsule biosynthetic genes by transformation

4.4.1. Introduction

Transformation of chromosomal DNA into recipient cells is used for the characterisation of certain genes. One of the classical examples is the transformation of noncapsulated mutants of *S. pneumoniae.* The incorporation of DNA from the capsulated strain into the recipient enabled the expression of the capsule and enhanced virulence. Transformation has also been used for mapping of genes in certain bacteria. Mapping of genes is based on the principle that two genes can be transformed together if they are closely linked (Freifelder *et al,* 1987).

Transformation of covalently closed circular plasmid DNA into competent bacteria is extensively used in bacterial genetic studies. Linear DNA is susceptible to exonuclease V encoded by *recB* or *recC* gene. *E coli* strains that lack *recB recC* function and also mutants in the *sbcB* gene (suppression of *rcB recC* mutations) can be transformed at high frequency. In this case the linear DNA is not degraded because of the absence of exonuclease V and the functional activity of the *recF* pathway (Cosloy and Oishi, 1973).

4.4.2. Transformation of chromosomal DNA

In an attempt to co-transfer closely linked genes, a multiple auxotrophic *E. coli* JC7623 strain, defective in exonuclease activity, was used to transform large fragments of chromosomal DNA of *Klebsiella.* The chromosomal DNA was either partially digested with *Sau*3A or sonicated to give ≥40 kb and transformed into competent *E. coli.* Transformants were grown at 30°C for 48 h in minimal media

supplemented with required amino acids. A total of 50 His^+ transformants were isolated. All the clones were screened for the expression of K antigen by dot blotting and/or ELISA method using anti-K1 antiserum, however all the transformants failed to produce K1 specific capsular antigen.

Two of the His^+ transformants (*E. coli* JC7623A and JC7623B) appeared mucoid when grown in minimal media. It was assumed that they might have acquired *his*-linked genes that might induce production of polysaccharide. The *rcsA* gene which induces the expression of capsule has been reported to be linked to the *his* region (McCallum and Whitfield, 1991). Analysis of chromosomal DNA from the mucoid transformants by Southern hybridisation using *rcsA* and *rcsB* of *Klebsiella* K1 as probes indicated the absence of either of these genes. The conversion of nonmucoid to mucoid phenotype could be either due to spontaneous genetic mutation or an induction by an unidentified gene linked to the *his* region.

This study showed that chromosomal markers from *Klebsiella* could be transformed. However, the co-transformation of linked genes may also depend on the size of the genes that can be transferred in a single step and expressed in the recipient cells. Even if *his*-linked genes are transferred, there could be other genes that might not be necessarily close to this region and might also be crucial in regulating the production of *Klebsiella* capsule.

4.5. Mobilisation of chromosomal DNA using pULB113

4.5.1. Introduction

In many Gram-negative bacteria conjugative plasmids could be responsible for the transfer of particular genes between individual cells. In addition to their ability to transfer copies of themselves, many conjugative plasmids mediate the transfer of genes carried on other plasmids or chromosomal DNA from a donor to a recipient strain. Such a mechanism of transfer is thought to involve covalent linkage of the plasmid with the chromosome as in the form of high frequency of recombination (Hfr) donor or R-prime plasmid (Holloway, 1979).

The sex factor F of *E. coli* can promote the transfer of the chromosome and the stable formation of F-plasmids in the recipient strain (Jacob and Wollman, 1961). The excision or integration of F-plasmid into the chromosome is mediated by insertion elements present in the plasmid. The broad host range conjugative plasmids that can be transferred to many related bacteria such as *E. coli*, *Klebsiella*, *Salmonella*, and *Shigella*, have been used for genetic analysis. These plasmids are able to mobilise chromosomal DNA at very low frequencies. In recent years variants of these plasmids have been constructed to enhance the mobilisation of chromosome.

4.5.2. Mobilisation of chromosomal DNA

Plasmid pULB113 (RP4::min-Mu) constructed from a conjugative RP4 plasmid and Mu3A (min-Mu) phage has been previously used to transfer different chromosomal markers into different bacterial strains. The conjugative plasmid pULB113 contains Mu3A where the viral function lethal to the host is deleted, whilst the functional transposase is retained. Mu3A introduced to the broad host range

plasmid RP4 mediates random integration with the chromosome and excision of the plasmid to form an R-prime plasmid (van Gijsegem and Toussaint, 1982).

Plasmid pULB113 was transferred from *S. typhi* SA2876 into *Klebsiella* K1 (3L103). *Klebsiella* (pULB113) was selected on minimal media containing Tc and Km. *Klebsiella* (pULB113) was mated with auxotrophic *E. coli* PA360. Selections were made using minimal media supplemented with appropriate amino acids together with Ap, Km, Tc, and Sm. The *his*$^+$ marker was transferred from *Klebsiella* to *E. coli* PA360 at a frequency of 10^{-6}. The transfer of chromosomal markers to multiple auxotrophic *E. coli* PA360 recipients was similar to that previously reported (van Gijsegem and Toussaint, 1982).

A total of 60 His$^+$ phenotype and Ap^{r}Km^{r}Tc^{r}Sm^r transconjugants were screened for the production of capsule in the donor strain. Antigen extracted from whole cell lysate was analyzed by dot blot/ELISA using *E. coli* PA360 absorbed anti-*Klebsiella* K1 antiserum. None of the His$^+$ transconjugants showed a positive reaction or became mucoid.

The formation of R-prime plasmid was also confirmed by isolation of plasmid from randomly chosen His$^+$ transconjugants. As seen in Fig 4.16, lane 2 and 3, the plasmid carried by *E. coli* PA360A and PA360B appeared to be similar to the parental plasmid pULB113 (lane 4). Miniplasmid preparations from another 10 His$^+$ transconjugants were of similar size. Since the donor and recipient strains are not homologous, recombination could not occur, thus the emergence of prototrophic transconjugants must be the result of R-prime plasmid formation.

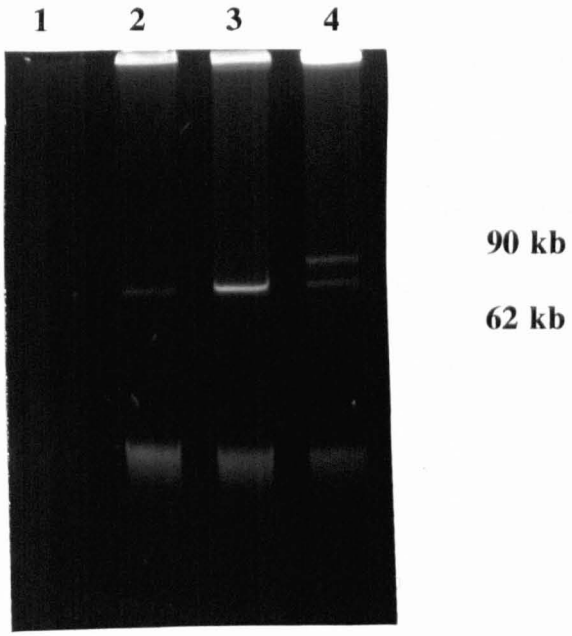


Figure 4.16. Agarose gel electrophoresis of plasmid isolated from His^+ *E. coli* PA360 transconjugants. *E. coli* PA360 (1); *E. coli* PA360A (2); *E. coli* PA360B (3); pULB113 (lower band) and resident plasmid pSLT (upper band) isolated from *S. typhi* SA2876 (4).

The R-prime plasmid usually contains 20 to 40 kb chromosomal DNA in addition to the plasmid and would appear to be larger than the parent plasmid (van Gijsegem and Toussaint (1982). The apparent reduction rather than increase in the size of the R-prime plasmid isolated from the transconjugants remains to be explained.

Despite the transfer of the *his*$^+$ marker to either *recA*$^+$ or *recA*$^-$ strains of *E. coli* K12, transfer of *his*-linked genes that could direct the synthesis of *Klebsiella* K1 antigen proved unsuccessful. Laakso *et al* (1988) have reported the transfer of *his*-linked genes that determined the expression of the *Klebsiella* K20 or *E. coli* K30 K antigen in *E. coli* K12 using pULB113. There could be a difference in the nature of the capsule biosynthesis genes of *Klebsiella* K1 or pULB113 may not be appropriate for mobilisation of these genes.

4.6. Attempted cloning of a regulator gene from pKLS1

4.6.1. Introduction

In pathogenic *Klebsiella* K1 and K2 serotypes, a 180 kb resident plasmid that encodes the hydroxamate siderophore aerobactin is known to contribute to virulence (Nassif *et al,* 1989). Two genes (*rmpA* and *rmpB*) that are located in the resident plasmid have also been reported to be involved in the regulation of colanic acid synthesis in *E coli* K12 (Nassif *et al,* 1989). The role of these genes in *Klebsiella* is not clear.

4.6.2. Construction of gene library

In *Klebsiella* serotype K1 strains studied in this work a 65 kb resident plasmid (pKLS1) was isolated. This plasmid was cloned to determine whether it contains gene(s) that might be involved in the regulation of capsule synthesis. *Sau*3A digested plasmid DNA (Fig 4.17) was ligated into the *Bam*HI site of pHC79 and transformed into *E. coli* HB101. Miniplasmid analysis of 10 randomly selected clones revealed 10 to 25 kb DNA insert. 200 Ap^r clones which contained insert DNA were constructed. The library was assumed to represent with a probability of 99% that any of the plasmid genes would be present.

In addition, a *Bam*HI partial digest of the plasmid was cloned into the *Bam*HI site of pACYC184. Random selection and miniplasmid analysis of the resulting recombinants showed the presence of 1-8 kb DNA insert. Southern hybridisation analysis of pKLS1 using the insert DNA of recombinant DNA (pKC50) confirmed the localisation of the DNA in the resident plasmid (Fig 4.18).

From all the *E. coli* clones (cosmid or plasmid clones) screened, no mucoid phenotype was isolated when grown at 30°C or 37°C for 24-48 h. Based on this study, plasmid pKLS1 does not appear to be involved in regulation of the mucoid phenotype or polysaccharide expression. Whether this plasmid is involved in other functions such as in encoding the hydroxamate siderophore aerobactin or any other virulence determinant, remains to be elucidated.

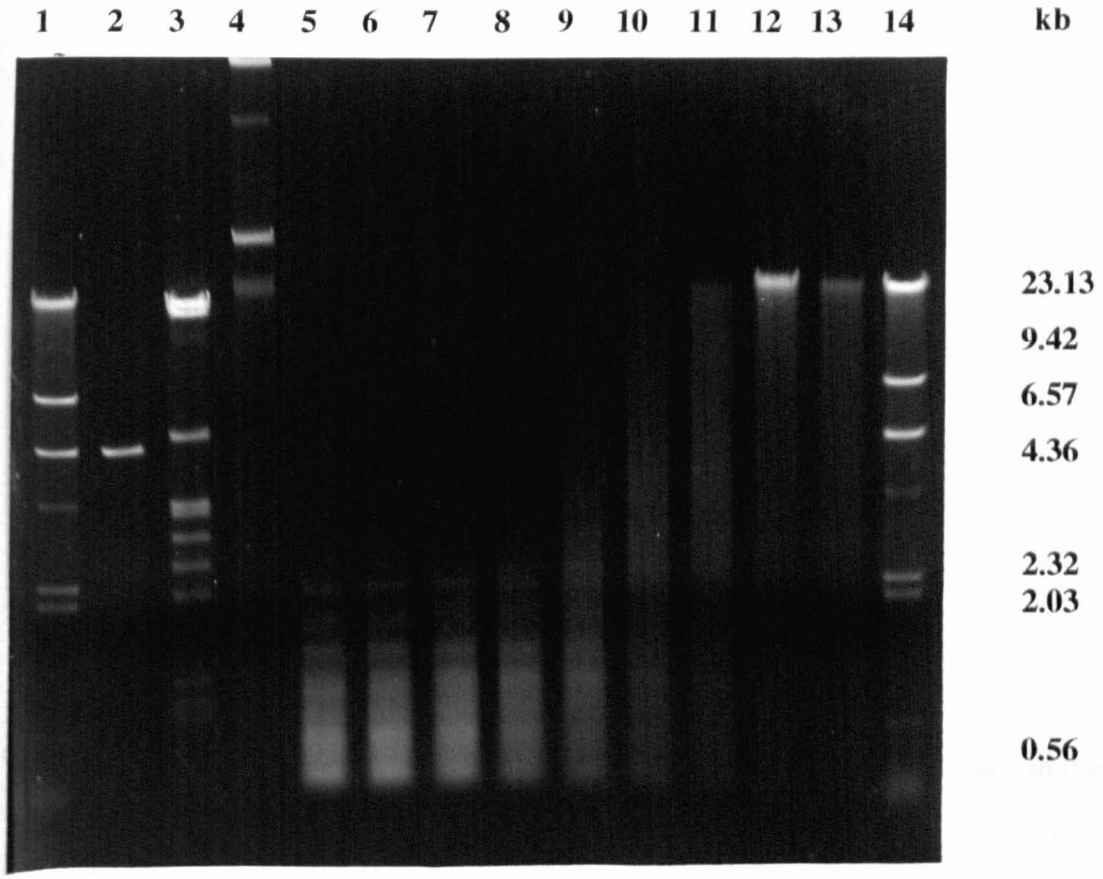


Figure 4.17. Agarose gel electrophoresis of a *Sau*3A partial digest of plasmid pKLS1. pHC79 *Bam*HI digest (2); pKLS1 *Bam*HI digest (3);) undigested pKLS1 ((4); partial digests of pKLS1 with 2 fold dilutions of *Sau*3A (5-13); λ DNA *Hin*dIII digest (1, 14).

4.6.3. Conjugal transfer of pKLS12.

A Km^r *Klebsiella* K1 (3L101) containing pKLS1 was mated with *E. coli* HB101 or PA360 and the resulting transconjugants were selected by growing on Sm and Km plates. Km^r transconjugants that were grown at 30°C were visually screened for a mucoid phenotype. None of the transconjugants showed any change of colonial morphology. Plasmid isolation and analysis from Km^r *E. coli* transconjugants showed the presence of a plasmid with an approximate molecular weight of 90 kb. The Km^r encoding plasmid was designated as pKLS12. Restriction digestion (Fig 4.19) and Southern hybridisation analysis (Fig 4.18) demonstrated the absence of any homology between pKLS1 and pKLS12. Since pKLS12 was not present as a separate plasmid in the donor strain, it must have been integrated into the chromosome as in the form of Hfr. Curing of pKLS12 from either the donor or transconjugant resulted in the loss of Km^r. Because it is mobilisable and encodes an antibiotic resistance, it is likely to be a conjugative plasmid.

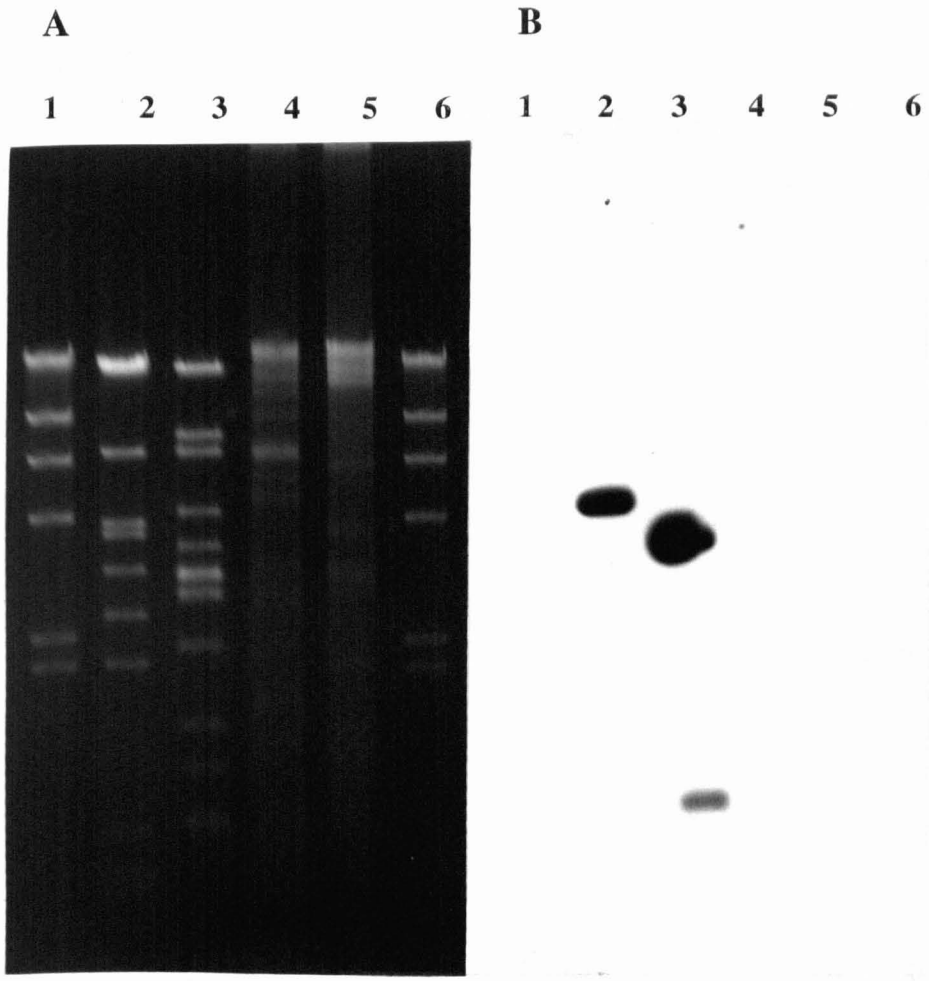


Figure 4.18. A. Agarose gel electrophoresis of pKLS1 digested with *Bam*HI (2) and *Eco*RI (3); pKLS12 digested with *Bam*HI (4) and *Eco*RI (5). λ DNA *Hin*dIII digest (1, 6). **B.** Southern hybridisation analysis using 4 kb *Bam*HI fragment pKC50.

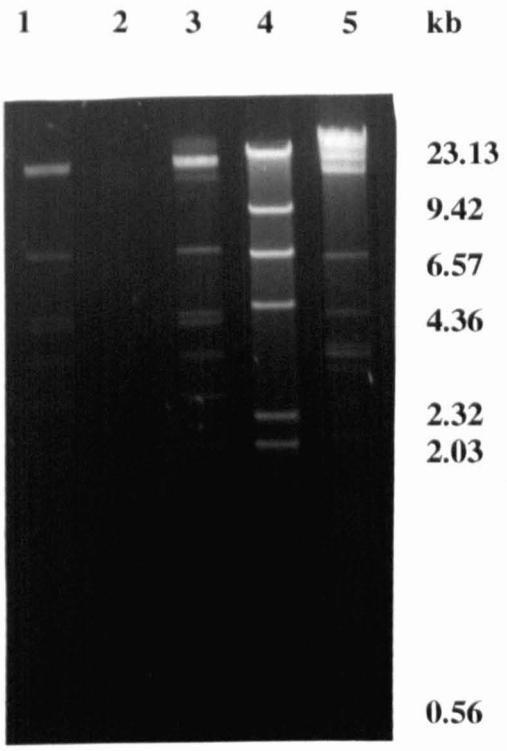


Figure 4.19. Agarose gel electrophoresis of *Eco*RI digests of plasmid pKLS1 and pKLS12. pKLS1 isolated from 3L101 (1) and 3L102 (3); pKLS12 isolated from *E. coli* HB101 transconjugant (5); *E. coli* HB101 (2); λ DNA *Hin*dIII digest (4).

4.7. Discussion

Direct cloning methods (plasmid or cosmid) have been successfully used for genetic analyses of capsule biosynthesis in many Gram-negative bacteria including group II capsule genes of *E. coli*. *Klebsiella* and group I of *E. coli* capsules are thought to be more complex and are predicted to involve more genes for expression. Application of direct cloning methods for the genetic analyses of capsules of these groups has proved unsuccessful so far. Although cloning of large fragments of *Klebsiella* DNA using cosmids or plasmids was partly successful, attempts to clone capsule biosynthetic or major regulator genes in *E. coli* K12 was not successful.

Alternative approaches including transposon mutagenesis methods (Arakawa *et al*, 1991) and chromosome mobilisation (Laakso *et al*, 1988) have been reported to be partly successful. Transposon mutagenesis requires a *Klebsiella* strain that can stably maintain the transposon delivery plasmid and is susceptible to the selective markers. This work has also shown the use of transposon delivery plasmids pHSG415::Tn*10* or pRT733 (pJM703.1::Tn*phoA*) for transposition and generation of noncapsulated mutants in some *Klebsiella* strains. This work was the first report on generating noncapsulated mutants of *Klebsiella* serotype K1 by transposon mutagenesis. The stable inheritance of the transposon marker and the irreversible loss of capsule production suggests the generation of insertion mutations in the capsule biosynthesis genes.

The use of Tn*phoA* for insertion mutation of *Klebsiella* capsule genes appeared to be simple and can be accomplished in a single step. Although cointegrate formation between the conjugative plasmid and the chromosome might occur at a low frequency, it does not pose a major problem and mutants can be selected following

Southern hybridisation. Tn*phoA* plus capsular genes were stable when cloned in multicopy plasmids. Although large fragments of DNA were cloned, they neither complemented the noncapsulated mutants nor expressed *Klebsiella* K1-specific antigen. The reason for the failure of the expression of the cloned genes could be that the entire gene cluster (if linked) might not be contained in a single cloning system or might require additional non-linked genes.

Previous reports (Schmidt *et al*, 1977, Laakso *et al*, 1988) have shown the presence of *his*-linked genes that are involved in the synthesis of group I K-antigen. The work described in this thesis demonstrated that the *his* region of *Klebsiella* can be directly transferred or mobilised by the plasmid pULB113. Although the transfer of genes which express *Klebsiella* K1 antigen in *E. coli* K12 was not successful, it is possible that part of the capsule gene cluster could be co-transferred with the *his* operon. The entire gene cluster may also occupy a large area and may not be transferred in a single step. Some of the genes that may participate in the regulation of capsule synthesis may not be expressed.

High molecular weight resident plasmids of *Klebsiella* serotypes are known to be involved in encoding the hydroxamate siderophore aerobactin and contribute to virulence (Nassif and Sansonetti, 1986). Plasmid-encoded mucoid phenotype regulator genes have also been characterised in some pathogenic strains of *K. pneumoniae* K1 and K2 and also contribute to virulence (Nassif *et al*, 1989). However, the high molecular weight plasmid (pKLS1) that was isolated in this study from *K. pneumoniae* serotype K1 is not involved in regulation of the mucoid phenotype or polysaccharide production. At present, the role of this plasmid is not known.

Most clinical isolates of *K. pneumoniae* are multiply resistant to antibiotics because of the acquisition of R-plasmids. These plasmids not only mediate antibiotic resistance (Casewell and Phillips, 1981) some may also encode for adhesive factors that might be involved in enhancing virulence (Darfeuille-Michaud *et al*, 1992). The isolation of a Km^r R-plasmid from *K. pneumoniae* 3L101 corroborates previous reports.

Although the reasons are far from understood, the expression of *Klebsiella* capsular polysaccharides may be complex and might require several sets of genes that might be located far apart or occupy a larger chromosomal DNA segment than originally thought. The structural genes that determine the biosynthesis of capsule might also not be stable or expressed when cloned.

CHAPTER V

MOLECULAR CLONING AND SEQUENCING OF *RCS* GENES FROM *KLEBSIELLA PNEUMONIAE* K1

5.1. Introduction

Several Gram-negative bacteria can also produce nonspecific capsular polysaccharide commonly known as colanic acid (M antigen) under appropriate growth conditions. Unlike other capsular polysaccharides, the regulation of biosynthesis of colanic acid in *E. coli* K12 has been extensively studied. The transcription of capsule polysaccharide synthesis (*cps*) genes is regulated by *lon* (Trisler and Gottesman, 1984 and three other genes, *rcsA*, *rcsB*, and *rcsC* (Gottesman et al, 1985; Gottesman and Stout, 1991). The *rcsA* gene product is an unstable positive regulator that is degraded by Lon protease and is normally limiting for colanic acid formation (Torres-Cabassa and Gottesman, 1987). The *rcsB* and *rcsC* constitute a two component regulator system in which the *rcsC* product acts as a sensor of environmental stimuli and the *rcsB* product acts as an effector to activate colanic acid synthesis (Stout and Gottesman, 1990).

Homologous RcsA proteins that can also activate colanic acid production in *E. coli* K12 have been characterised from *Klebsiella* (Allen *et al*, 1987; McCallum and Whitfield, 1991), *E. coli* K30 (Keenleyside *et al*, 1992) and *Erwinia* (Torres-Cabassa *et al*, 1987; Coleman *et al*, 1990). This chapter describes cloning, expression and nucleotide sequences of two *rcs* genes from *Klebsiella* K1. This work also shows that these genes share homology with other characterised genes.

5.2. Cloning of *Klebsiella* K1 chromosomal DNA

Plasmid pLV59, a positive selection cloning vector (O'Connor and Humphreys, 1982) and pACYC184 (Chang and Cohen, 1978) were used to clone the chromosomal DNA of *Klebsiella* K1 (3L101) into *E. coli* HB101. Plasmid pLV59 contains a temperature sensitive *Eco*RI methylase gene that is functional at 28°C. The vector is lethal to its host when it is grown at 37°C. Insertion of a DNA fragment within the region inactivates the *Eco*RI restriction gene and allows the hybrid plasmid to grow at 37°C.

*Sau*3A partial digest of chromosomal DNA was ligated into the *Bgl*II site of pLV59. The library was constructed in *E. coli* HB101. 1500 clones were visually screened for the mucoid phenotype. Two mucoid *E. coli* clones were isolated. Analysis of the recombinant plasmids (pLV590 and pLV591) isolated from the clones showed that they were identical and each contained a 12 kb DNA insert. They are possibly siblings that derive from a single transformation event during the construction of the library. The recombinant pLV591 was used for further study.

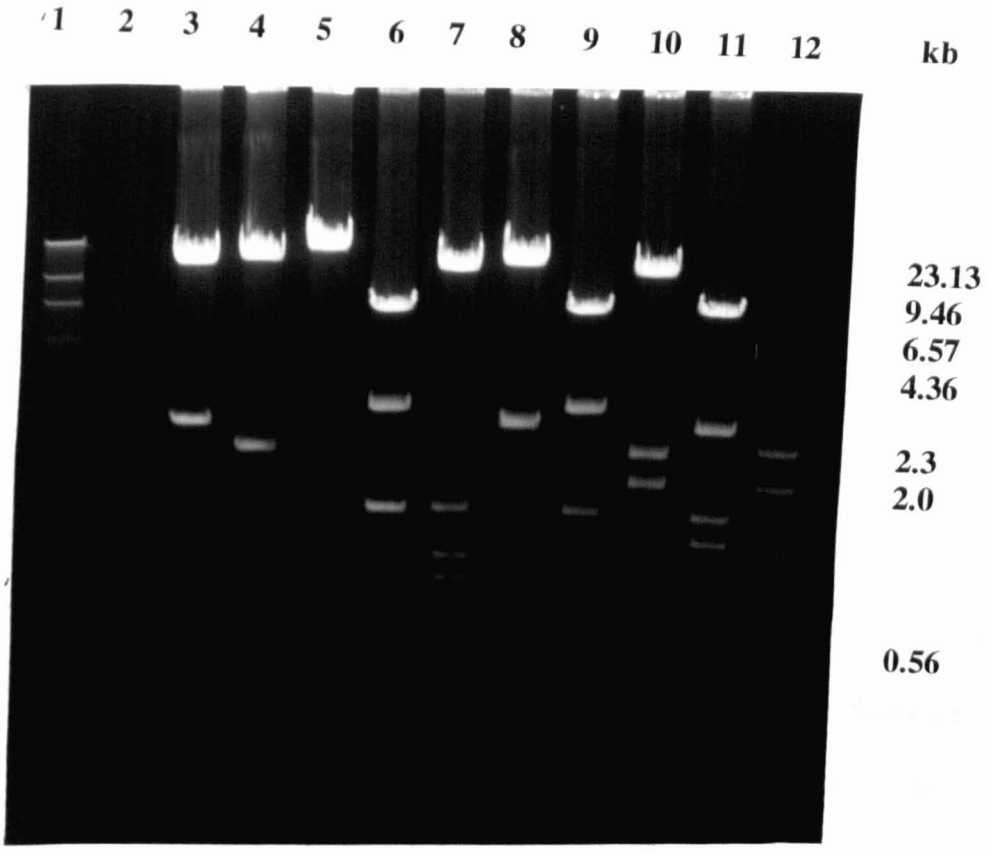


Figure 5.1. Agarose gel electrophoresis of endonuclease digests of recombinant pLV591. *Bgl*II (3); *Cla*I (4); *Hin*dIII (5); *Pst*I (6); *Bgl*II+*Cla*I (7); *Bgl*II+*Hin*dIII (8); *Bgl*II+*Pst*I (9); *Cla*I+ *Hin*dIII (10); *Cla*I+*Pst*I (11). pLV59 *Bgl*II digest (2); λ DNA *Hin*dIII digest (1); Molecular weight marker VI (12).

5.3. Subcloning of pLV591

Plasmid pLV591 was purified and a partial restriction map was constructed (Fig 5.1 and 5.2). Different fragments generated by different enzymes were subcloned. Only the 2.5 kb *Bgl*II fragment that was subcloned into the *Bam*HI site of pACYC184 (pLV592) induced a mucoid phenotype in *E. coli* K12. The minimum insert DNA size that could induce this phenotype was also determined by subcloning the *Sau*3A digest of the 2.5 kb *Bgl*II fragment into the *Bam*HI site of pACYC184. Two recombinant subclones (pLV5962 and pLV5963) that induced a mucoid phenotype in *E. coli* DH1 at 30°C were isolated. Since restriction digestion analysis of these plasmids suggested that both were identical and each contained 1.4 kb DNA insert, only pLV5962 was analyzed further. When pLV5962 was transformed into different strains of *E.coli* K12, it induced a mucoid phenotype at both 30 and 37°C. Subcloned pLV5964 containing 1 kb *Bgl*II/*Cla*I fragment of pLV5962 did not activate colanic acid formation in *E. coli*, suggesting that part of the gene sequence was deleted (Table 5.1).

Another subclone designated pLV593 (the 1.5 kb *Pst*I fragment of pLV592 cloned into the *Pst*I site of pBR322) induced the mucoid phenotype when it was co-transformed with pLV213 into *E. coli* K12. This indicated that pLV593 complemented the requirement for *rcsB* of *Klebsiella* K21b in increasing the level of polysaccharide synthesis at 37°C in *E. coli* clones containing pLV213 (Allen *et al*, 1987). The recombinant plasmids had no effect on nonmucoid *Klebsiella* strains (Table 5.1).

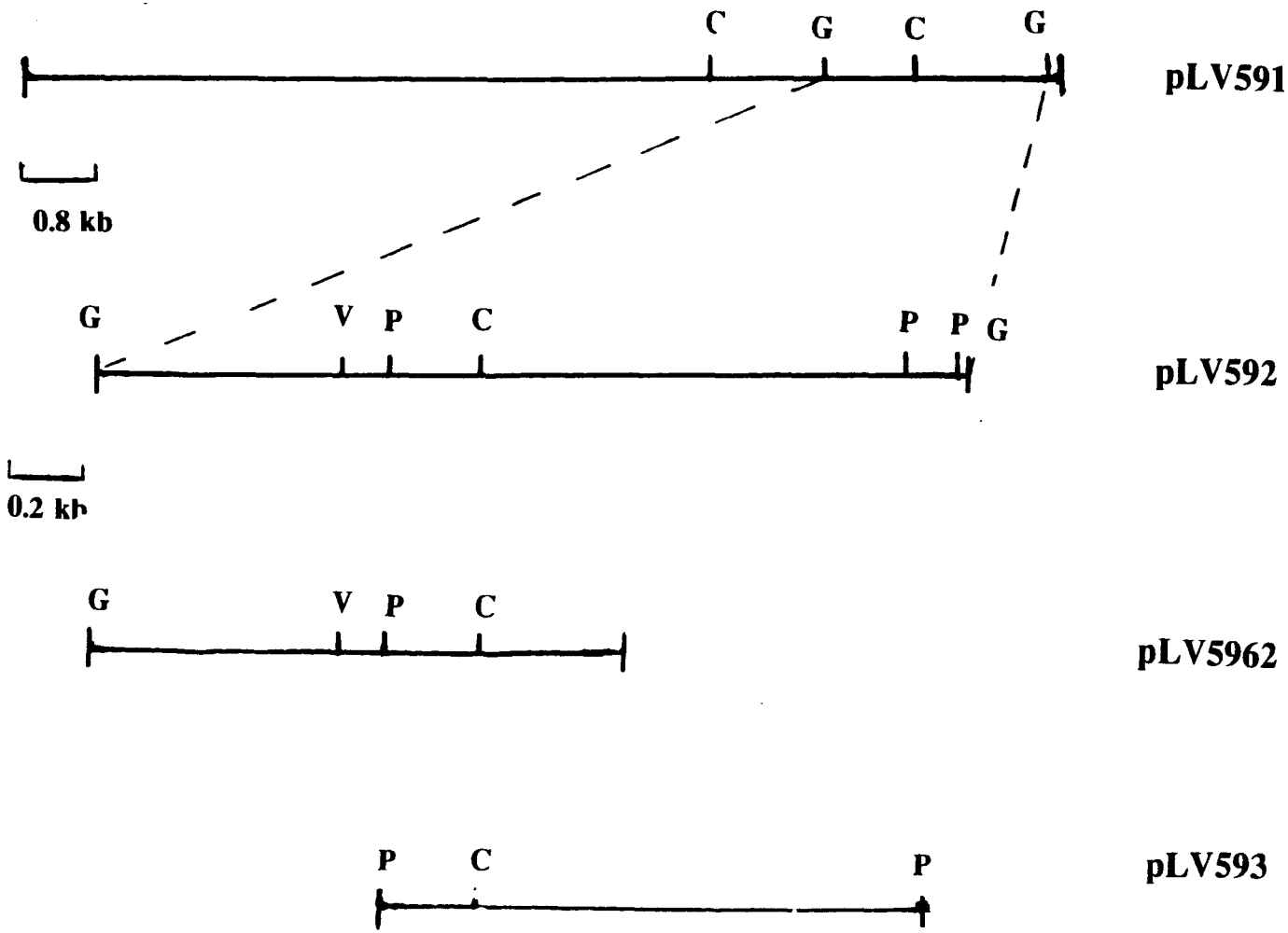


Figure 5.2. Partial restriction map of recombinant plasmid pLV591 and subclones. Letters on top of vertical lines indicate endonuclease restriction site. C= *Cla*I, G= *Bgl*II, P= *Pst*I, V= *Eco*RV. Only the insert DNA is shown.

Table 5.1. Transformation of subclones of pLV591 into *E. coli* or nonmucoid *Klebsiella* strains.

Strain	Plasmid	Mucoid phenotype at	
		30°C	37°C
3L103	pLV5962	-	-
M10	"	-	-
DH1	"	++	+
HB101	"	++	+
LE392	"	++	+
AB1157	"	++	+
CSHΔF26	"	++	+
All *E. coli* strains	pLV213	++	-
"	pLV593	-	-
"	pLV5964	-	-
"	pLV5964 + pLV593	-	-
"	pLV213 + pLV593	++	+

++: Highly mucoid + : mucoid - : nonmucoid

5.4. Southern hybridisation analysis

The recombinant pLV591 and its derivatives were also analyzed by Southern hybridisation using the *ClaI/PstI* fragment of pLV213 as a probe. Fig 5.3 shows that all the plasmid clones contain a gene homologous to the *rcsA* gene of *Klebsiella* K21b ($rcsA_{K21}$). The presence of a second regulator gene was also analyzed by Southern hybridisation using a labelled synthetic oligonucleotide homologous to the *rcsB* gene of *Klebsiella* K21b ($rcsB_{K21}$). pLV591, pLV592 and the subclones pACC6 and pACC7 of the recombinant cosmid pCOS596 contained a gene homologous to $rcsB_{K21}$, whereas there was no hybridisation with pLV5962 (Fig 5.4). The genes that were homologous to the $rcsA_{K21}$ and $rcsB_{K21}$ were designated as $rcsA_{K1}$ and $rcsB_{K1}$ respectively.

Chromosomal DNA isolated from different serotypes of *Klebsiella* and *E. coli* K12 was analyzed by Southern hybridisation. Chromosomal DNA from *Klebsiella* hybridised with labelled $rcsA_{K1}$ and $rcsB_{K1}$ genes and the hybridisation appeared to be localised at the same site in all the serotypes tested. Both $rcsA_{K1}$ and $rcsB_{K1}$ genes were located in an 18 kb *Bam*HI fragment of the chromosome of *Klebsiella* strains (Fig 5.5). No hybridisation was detected between $rcsA/B_{K1}$ and *E. coli* chromosomal DNA. These genes were not found in the resident plasmid pKLS1 of *Klebsiella* K1 (3L101).

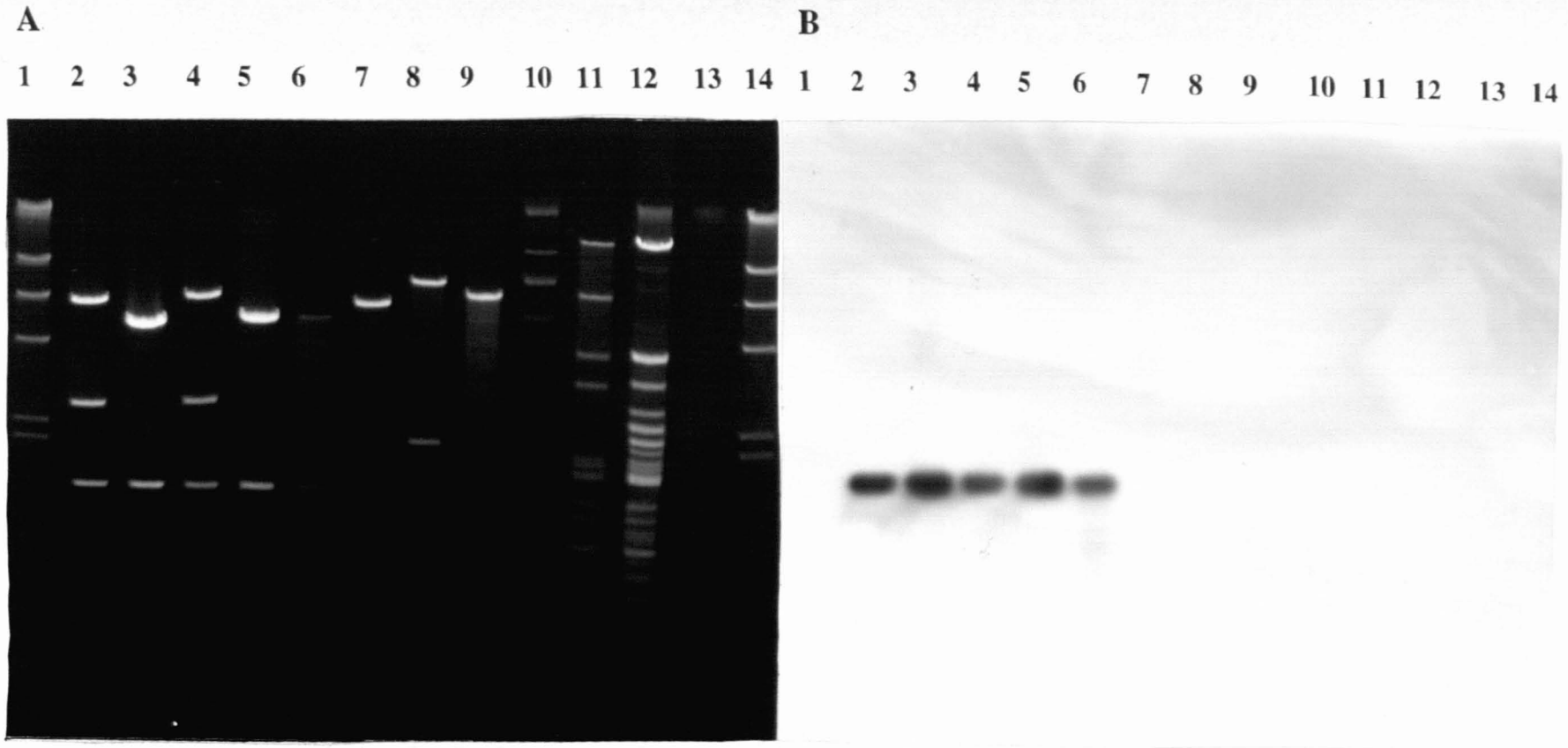


Figure 5.3. **A**. Agarose gel electrophoresis of *Pst*I digests of recombinant plasmid. pLV591 (2); pLV592 (3); pACC6 (4); pACC7 (5); pACC6 (6); recombinant plasmid containing insert DNA of pKLS1 (7-10); pCOS596 (11); pKLS1 (12); uncut pKLS1 (13). λ DNA *Hin*dIII digest 1 and 14). **B**. Southern hybridisation analysis using the *Cla*I/*Pst*I fragment of pLV213 ($rcsA_{K21}$) as a probe.

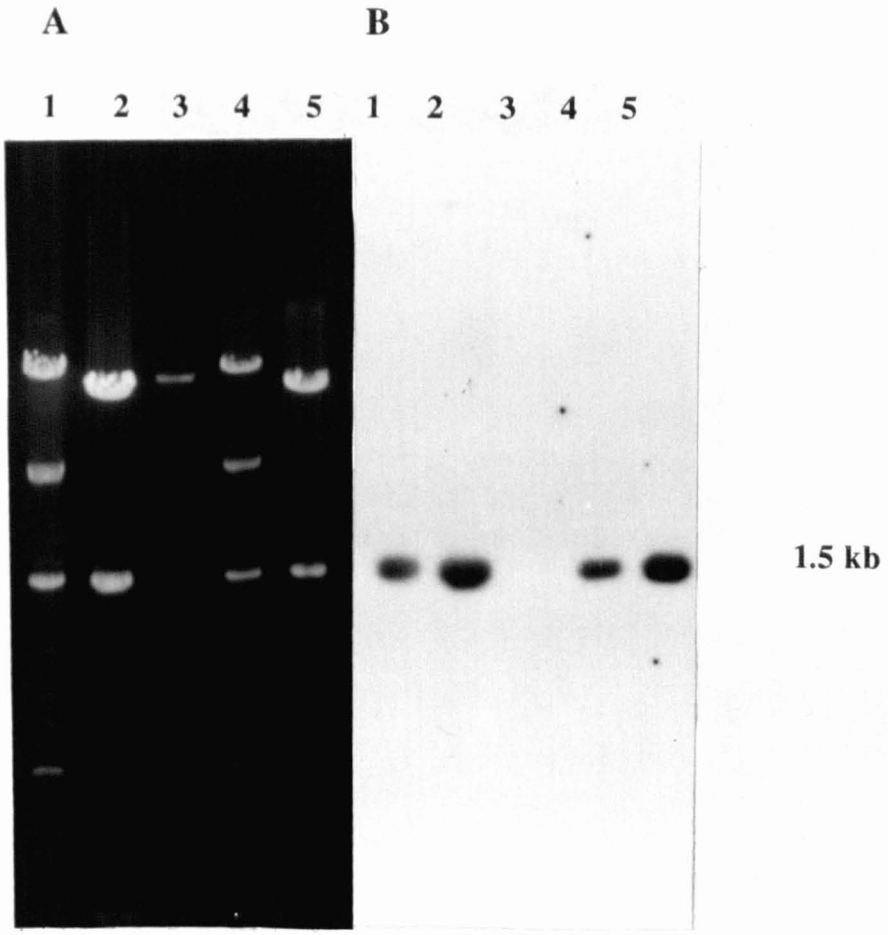


Figure 5.4. **A**. Agarose gel electrophoresis of *Pst*I digests of recombinant plasmids. pLV591 (1); pLV592 (2); pVL5962 (3); pACC6 (4); pACC7 (5).

B. Southern hybridisation analysis using an $rcsB_{K21}$-specific oligonucleotide (5'ATGTTTCAACGCATCGGATTTCCCG3') as a probe.

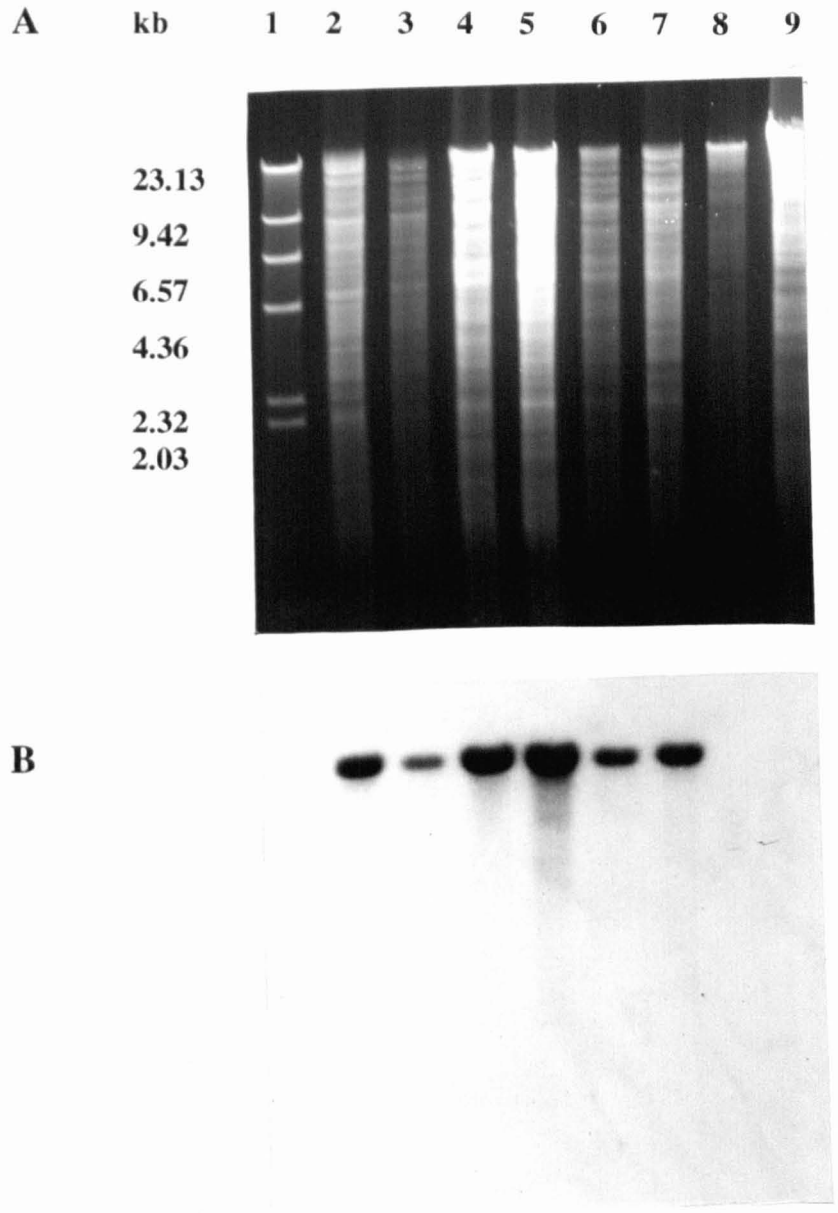


Figure 5.5. **A**. Agarose gel electrophoresis of chromosomal DNA digested with *Bam*HI: *Klebsiella* 3L101 (2), 3L102 (2), 3L103 (3), 3L104 (4), 3L105 (5), 3L106 (6), *E. coli* DH1 (8), HB101 (9). λ *Hin*dIII digest (1) **B**. Southern hybridisation analysis using 1.5 kb *Pst*I fragment of pLV592 as a probe.

5.5. Screening of cosmid library

E. coli cosmid clones that contained chromosomal DNA from *Klebsiella* K1 were screened by *in situ* hybridisation using the 1.5 kb insert DNA of pLV592 (*rcsA/B*$_{K1}$). Out of the 800 clones screened, one gave positive hybridisation. The recombinant cosmid isolated from this clone contained an insert of about 35 kb of *Klebsiella* DNA and was designated pCOS596. The *E. coli* clone containing recombinant pCOS596 neither induced a mucoid phenotype (at 30 or 37°C) nor expressed *Klebsiella* K1 specific antigen (Table 5.2). However, deletion and subcloning of the recombinant cosmid resulted in induction of a mucoid phenotype in *E. coli* K12. Restriction digestion and Southern hybridisation analyses showed that the insert DNA was the same as *rcsA*$_{K1}$ and *rcsB*$_{K1}$ genes (Fig 5.3).

The inability of the *rcsA*$_{K1}$ gene to express or induce a mucoid phenotype when cloned as a large fragment might be due the presence of an unidentified repressor gene. In order to check whether it had an effect in suppressing expression of the cloned *rcsA*$_{K1}$ gene, pLV592 was transformed into *E. coli* containing pCOS596. The presence of pLV592 induced a mucoid phenotype which was not suppressed by the unidentified gene present in the recombinant cosmid pCOS596. Initial attempts to clone the controlling gene(s) were not successful. Subsequent growth and analysis of the *E. coli* cosmid clone (pCOS596) has also shown deletion of part of the insert DNA.

Table 5.2. Serotyping of mucoid *E. coli* clones using anti-*Klebsiella* K1 antiserum.

Strain	Phenotype	ID*	CIE*
K. pneumoniae K1	MC	+++	+++
E. coli HB101	NM	_	_
E. coli HB101 (pLV590)	MC	_	_
E. coli HB101 (pLV591)	MC	_	_
E. coli HB101 (pLV592)	MC	_	_
E. coli HB101 (pLV593)	NM	-	-
E. coli HB101 (pCOS596)	MN	_	_
E. coli HB101 (pACC6)	MC	_	-

*: capsular antigen was extracted from whole cell lysate.

MC: Mucoid phenotype **NM**: Nonmucoid phenotype

ID: Immunodiffusion **CIE**: Counter-current immunoelectrophoresis

-: not typable +++: Strongly positive reaction

In an attempt to clone structural genes from *Klebsiella* K1, more than 2000 cosmid clones were screened (discussed in Chapter IV). Although mucoid *E. coli* cosmid clones were not detected, it is most probable that all *Klebsiella* genes including *rcs* genes would be represented in the library. Since analysis of representative recombinant cosmids revealed the presence of DNA insert ≥30 kb, it is most likely that the activity of $rcsA_{K1}$ gene is suppressed by the gene(s) that is closely linked to it.

5.6. Analysis of polysaccharide

The CPS isolated from the mucoid *E. coli* clone was serotyped using anti-*Klebsiella* K1 antiserum. The polysaccharide produced by the mucoid clone was not *Klebsiella* specific capsule (Table 5.2).

The purified polysaccharide was hydrolysed with acid and the sugar components were analyzed by paper chromatography. The sugar components of polysaccharide synthesised by the mucoid clone consisted of glucose, galactose, glucuronic acid and fucose. The sugar components and the mobility of these sugars were the same as the sugar units of the polysaccharide produced by the *E. coli* mucoid clone containing pLV213. The polysaccharide of *Klebsiella* consisted of glucose, fucose, and glucuronic acid but not galactose (Fig 5.6). The polysaccharide produced in *E. coli* was not serotypeable and contained an additional sugar component, suggesting that it was colanic acid.

5.7. Identification of pLV5962-encoded protein

For the detection of the gene products expressed by the recombinant plasmid, the maxicell system was used. The plasmids were introduced into an *E. coli* CSH▲26F strain defective in DNA repair. The strain containing the plasmid was irradiated with UV light to prevent synthesis of chromosomally encoded proteins. Plasmid encoded proteins were labelled with [^{35}S] methionine. The labelled proteins were analyzed by SDS-PAGE and autoradiography.

As shown in Fig 5.7, the chloramphenicol acetyl transferase (CAT) that was encoded by the *cm* gene was common to pACYC184, pLV5962 and pLV5964, and has a molecular weight of 25,000 Dal. The Tc^r protein (34,000 Dal) encoded by pACYC184 was absent in the recombinant plasmids, confirming that the gene had been inactivated as a result of insertion of DNA. An additional protein with a molecular mass of about 23,000 Dal was encoded by the recombinant plasmid pLV5962 (Fig 5.7, lane 4). This protein product was absent in the vector pACYC184 suggesting that it was an RcsA protein. The RcsA protein was not expressed by the recombinant pLV5964. The *rcsB* gene of *Klebsiella* might encode an RcsB protein of 7,000 Dal. It did not however specify any detectable protein in maxicells. Previous studies by (Allen *et al*, 1987) did not find the RcsB protein in minicells.

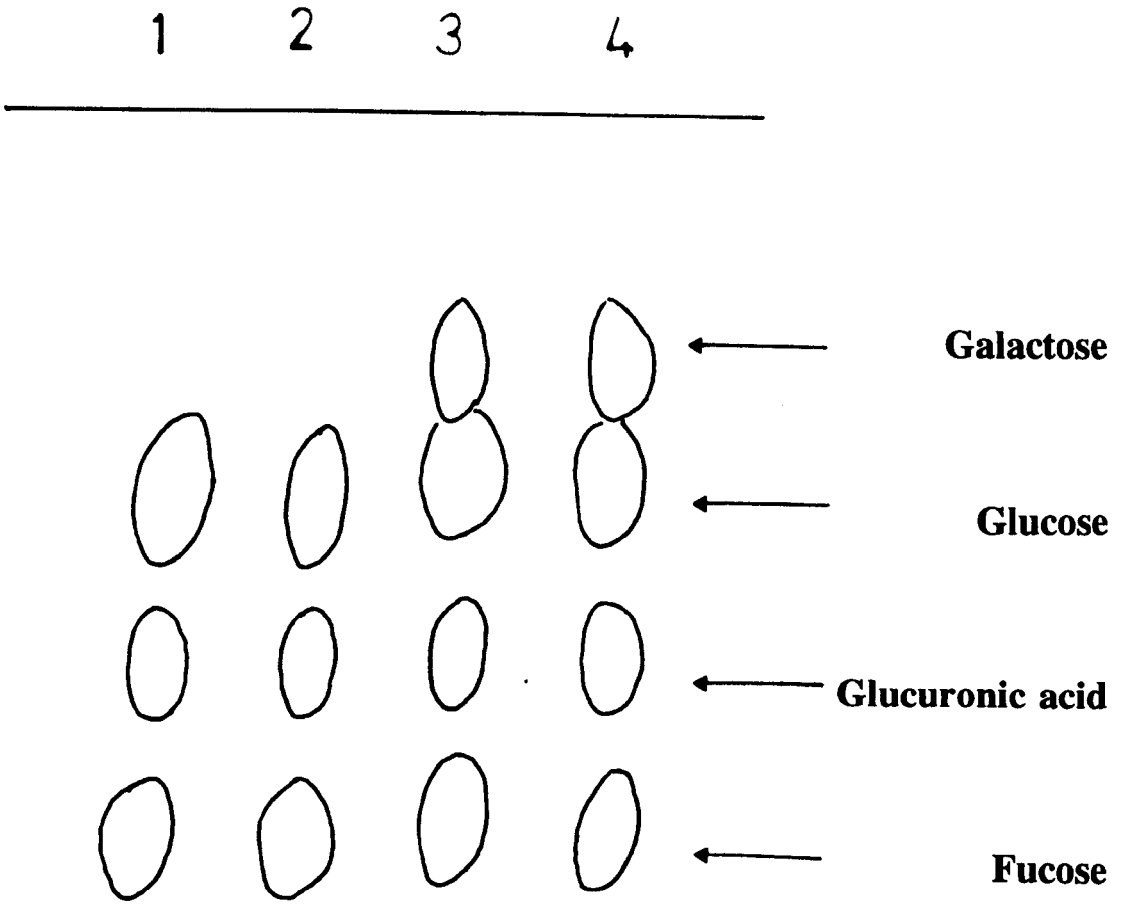


Figure 5.6. Analysis of sugar components by paper chromatography. Capsular polysaccharide isolated from *Klebsiella* 3L100 (1), *Klebsiella* 3L101 (2), Mucoid *E. coli* HB101 (pLV213) (3), mucoid *E. coli* (pLV5962). The figure represents the actual size of the sugar components detected on the paper.

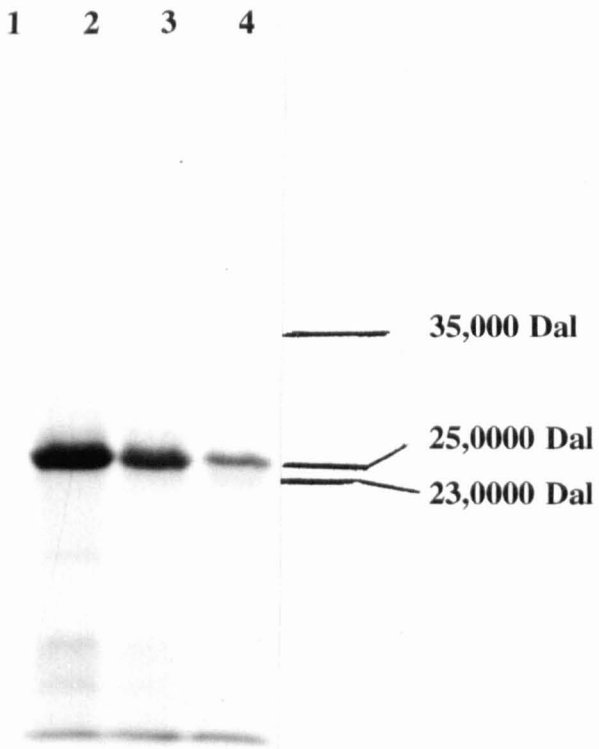


Figure 5.7. Polyacrylamide gel electrophoresis of [^{35}S] methionine-labelled protein in maxicells: *E. coli* CSH▲26 (1);pACYC184 (2); pLV5964 (3); and pLV5962 (4)

5.8. Nucleotide sequences of rcs_{K1} genes

The DNA sequence of the 2.5 kb insert that contained both $rcsA_{K1}$ and $rcsB_{K1}$ in pLV592 was determined by direct chain termination sequencing of plasmid DNA. Sequencing was done in both directions on the same template using the pACYC184 *Bam*HI site and newly sequenced nucleotides as oligonucleotide primers. Initially the subclone pLV5962 which contained the *rcsA* gene was sequenced. Then part of the insert DNA of pLV592 that contained *rcsB* was sequenced. A diagrammatic representation of the sequencing strategy is presented in Fig 5.8.

Analysis of the complete 2,450 bp of pLV592 showed two open reading frames of 621 and 180 bp. The 621 bp region represented the $rcsA_{K1}$ ORF and encoded a protein of 207 amino acids with a predicted molecular mass of 23,000 Dal, similar to the RcsA protein determined by maxicell analysis. The suggested ribosome binding site, GAGGA is located 6 bases upstream (at -11 to -7 bp) of the ORF and a possible promoter containing a -10 consensus sequence of TATTAT is located at -75 to -70 bp (Fig 5.9).

The $rcsB_{K1}$ ORF contains a 180 bp region on the complementary strand of pLV592. The ribosome binding site, GAGGA is located at -7 to -3 and the possible -10 promoter sequence TATTTT is located at -47 to -41 bp (Fig 5.10). The $rcsA_{K1}$ and $rcsB_{K1}$ are closely linked and the distance between the translational stop codons is 444 bases, and appeared to be transcribed towards each other (Fig 5.8).

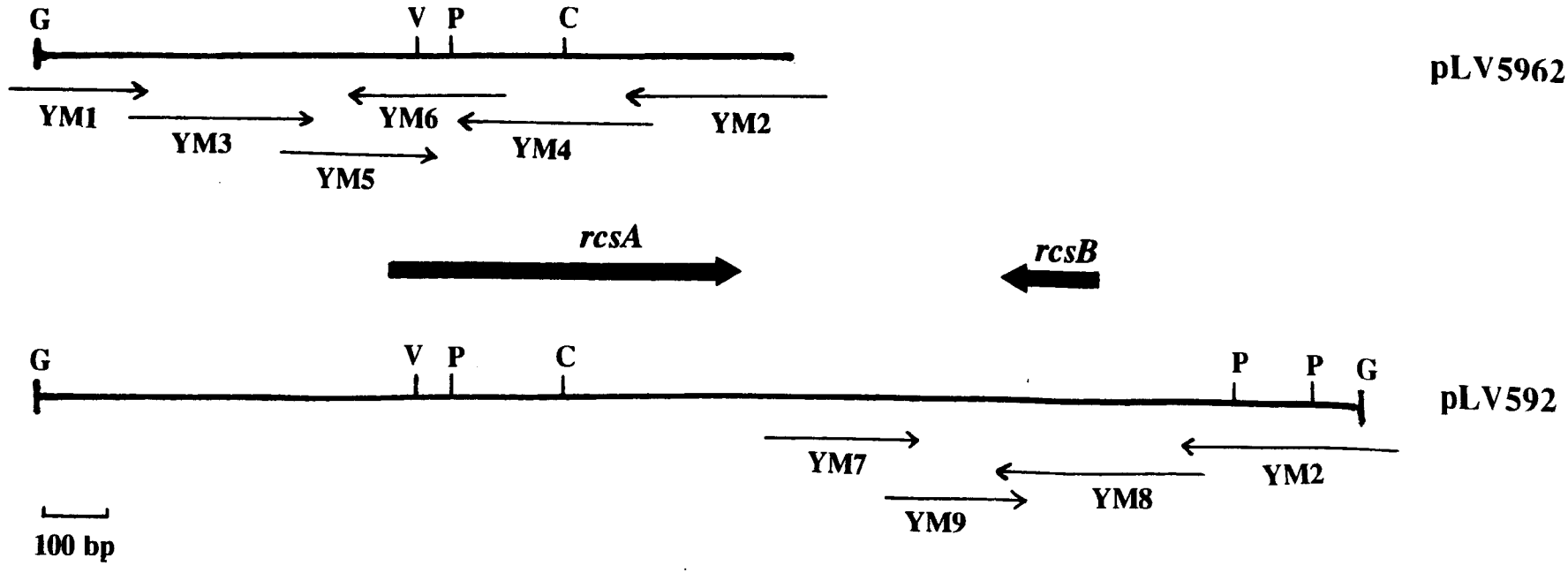


Figure 5.8. Partial map and sequencing strategy of *rcs* genes using oligonucleotide primers. The bold arrows show the direction of the transcription of *rcsA* and *rcsB* genes. The sequencing strategy shown in the lower portion indicates the direction and length of each fragment that was sequenced. YM1 and YM2 are pACYC184 *Bam*HI site primers. YM3 to YM9 are synthetic oligonucleotide primers from the insert DNA. C= *Cla*I, G= *Bgl*II P= *Pst*I, V= *Eco*RV.

Figure 5.9. Nucleotide sequence of *rcsA*$_{K1}$ and alignment with rcsA$_{K21}$. The ribosome binding site is underlined at -11 bp and the -10 promoter is at -75 bp. The predicted amino acid sequence of RcsA is indicated in three letters below the appropriate nucleotide bases. :indicates identical *rcsA*$_{K21}$ nucleotides.

```
                                                GATCTCACTGTCACGATTCATCAG    24
TTTCCCCGGCTGCGCAATCACGCGCTGCCACTGGCGGCAGTCGAAGGTGTC                        75
CCCTTCTTTGGTGACAATCAGGCTGGCAATGGCGTCAGGACTCATCATCGC                       126
GCTCTGCGGCCCTTTCGTTTGCCAGAAACCTGCAAGGCCAGCCGGCGCCGG                       177

                                  :::::::::::::::::::::::::::
CGCTTTAACGACGTCCTCATAACGATCAACCTGTACGCAACCGGCCAGCGC                       228
:::::::::::::::::::::::::::::::::::::::::::::::::::
CACCACCATGCCAGCAAAAAATAACTTTTTCATCATTCATCCCACAAGCCG                       289

:::::::::::::::::::::::::::::::::::::::::::::::::::
ATAAAAAAGTATTGTGGCATTAAAGCGTTAAGCCTTCCACCCCCACCGCAA                      330

:::::::::::::::::::::::::::::::::::::::::::::::::::
AGGGATAAGCGAGCAAGCCAGCAACCGCTAACGTGGGTTTCATTTGAAGTA                      381

:::::::::::::::::::::::::::::::::::::::::::::::::::
AGGAAATTCTGAAAGTAAAAGAATACTGGGCGCGTAACCATAGCATCTATG                       432

:::::::::::::::::::::::::::::::::::::::::::::::::::
GGCACTTTTTGTTTTTAATTCGGTCACACTACCGGTTCTTGACTTTACTTT                       483

:::::::::::::::::::::::::::::::::::::::::::::::::::
AAGAGTTTTCCTGGCAAATTATATGCATAGATGCGGAATAGTTTAATGGAG                       534

:::::::::::::::::::::::::::::::::::::::::::::::::::
CTAATGGGTTCTTTCTAAACCTACTATTATTATCGCCCGCAAGGACTGCTT                       585

:::::::::::::::::::::::::::::::::::::::::::::::::::
CGCACAGCCAGTGCGAAGTGTATATCGTTACGTGTTGATTGAGGATGGGTC                       636

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
ATG TCA ACG ATG ATT ATG GAT TTG TGC AGC TAT ACC CGG                         675
Met Ser Thr Met Ile Met Asp Leu Cys Ser Tyr Thr Arg

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
TTG GGA TTG ACG GGA TAT CTG ACC AGT CGG GGA ATT AAA                        714
Leu Gly Leu thr Gly Tyr Leu Thr Ser Arg Gly Ile Lys

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
AAA CAG GAA ATC GTT GAG GTC AAC AGT GCT GCG GAT CTG                        753
Lys Gln glu Ile Val Glu Val Asn Ser Ala Ala Asp leu

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CAG AAA CAC TGT ACG TCG TGT TGC CCG GCG GTG GTG TTT                        792
Gln Lys His Cys Thr Ser Cys Cys Pro Ala Val Val Phe
```

Figure 5.9-continued

```
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CTG AAT GAA GAC TGT TTC GTG CAT GAT GAT GAA AGT AAT      831
Leu Asn Glu Asp Cys Phe Val His asp Asp Glu Ser Asn

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
GGC ATT ATT CGC CAG ATC ATT ACG CAA AAC CCG GCG ACG      870
Gly Ile Ile Arg Gln Ile Ile Thr Gln Asn pro Ala Thr

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CTG TTT GTT ATC TTT ATG TCG CTG GCG AAC ATC CAT TTT      909
Leu Phe Val Ile Phe Met Ser Leu Ala Asn Ile His Phe

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
GAC CGC TAT TTG CGG GTA CGG AAG AAT CTG CTA ATC AGT      948
Asp Arg Tyr Leu Arg Val Arg Lys asn Leu Leu Ile Ser

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
TCA AAA TCG ATA ACC CCA AAA GAC CTT GAT GTT ATT CTG      987
Ser Lys Ser Ile Thr Pro Lys Asp Leu Asp Val Ile Leu

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::C :::
GTT AAT TAT CTT AAA TAC AAA AAC ACC AGT GTA GGG CAG     1026
VAl Asn Tyr Leu Lys Tyr Lys Asn Thr Ser Val Gly Gln

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
TTA ACT TTA CCG ACA TTG TCA CTG AGT AAA ACA GAA TCA     1065
Leu Thr Leu Pro Thr Leu Ser Leu Ser Lys Thr Glu Ser

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
AAT ATG CTG CAA ATG TGG ATG GCC GGG CAT GGT ACT TCG     1104
Asn Met Leu Gln Met Trp Met Ala Gly His Gly Thr Ser

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CAA ATC TCA ACG CAA ATG AAC ATC AAA GCG AAG ACG GTA     1143
Gln Ile Ser Thr Gln Met Asn Ile Lys Ala Lys Thr Val

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
TCG TCG CAT AAA GGC AAT ATT AAA AAG AAA ATA CAA ACG     1182
Ser Ser His Lys Gly Asn Ile Lys Lys Lys Ile Gln Thr

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CAT AAT AAG CAG GTG ATT TAT CAT ATC GTT CGG CTG ACC     1221
His Asn Lys Gln Val Ile Tyr His Ile Val Arg Leu Thr

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
GAA AAC ATC ACC TCC GGT ATT CAG GTA AAT ATG CGC TGA     1260
Glu Asn Ile Thr Ser Gly Ile Gln Val Asn Met Arg *

:::::::::::::::::::::::::::::::::::::::::::::::::::
AACAAACTGGCGGCGCTCCCGCCAGTTCAGTTTTACGAGACCTCTTTTTCC     1311

::G:::::::::::::::::::::::::::::::::::::::::
ACAAAAATCCCATCCGGGTGGCCTATTTCATTAAAAAACCAGATC          1356
```

Figure 5.9-continued

```
                                                GATCTGCTGGGCAGAGTG      18

GGCGGAGAAGAGTTTTGTATCGTGATGCCGAATAACACCCTGCAGGAAGCG                     69

GCGGCGGTGGCGGACGGATTGCGCCACGGTATCCAGGGACGGGAAGTCTTT                     120

TTGCATAACAACGTGACGCTGCGGGTTAGCGCCTCGTAAGGGGTGAGCGCC                     171

ATAGAGAGCGGGGGGAGTATCAGTTTGAAGCGCTGCAGTCGGTGGCCGATG                     222

GGCGACTCTATCTGGCCAAGCAAAATGGGCGTAACCAGGTCTGTTTTCGCA                     273

GCGCAGCTGAGTAAAACGTTTCGATGCCGATTGGCAAAATGAGAATTATCT                     324

          :::::::::::::::::::::::::::A:::::::::::::::::
TAAAGTGATCGCAGGCGATCGCGCTATCGAATGGAACCGGTTTGCGGCTAA                     375

:::::::::::::::::::::::::::::::::::::::::::::::::::::
GCCTTCTCACATCAGCCTTGCAGCCCGATAAGTCATCAAGCGCCAACATTC                     426

:::::::::::::::::::::::::::::::::::::::::::::::::::::
TCAATATTTTGCGTCATGAATATCTCTTGTCATGCAAAATAATTGAGGATA                     477

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
ATG TTT CAA CGC ATC GGA TTT CCC GGT GTA ACG AAT TTT                      516
Met Phe Gln Arg Ile Gly Phe Pro Gly Val Thr Asn Phe

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
CAA GTG CTT CTT GCA TTA GCA AGT TTG ATC CCG ACT CCT                      555
Gln Val Leu Leu Ala Leu Ala Ser Leu Ile Pro Thr Pro

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
GCG AGT CGG GAT TTT TTT CGC CTG CCG CTA ACG GTT GGC                      594
Ala Ser Arg Asp Phe Phe Arg Leu Pro Leu Thr Val Gly

::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
TTC ACT GGC GCT GAA ATC GTG AAT ATC GAG TTC GAA TGC                      635
Phe Thr Gly Ala Glu Ile Val Asn Ile Glu Phe Glu Cys

::: ::: ::: ::: ::: ::: ::: ::: ::: ::::::::::::G::
CTC CGC CAG CTC ACG CCA GGT ATG TGA CCCGCGGCCATCAAT                      675
Leu Arg Gln Leu Thr Pro Gly Met *

::::::::::::
GCTGGCGCGCTGCGGGTGATCTCATTAATTGCCGCCAGCAGGGCATCTACG                      726
```

Figure 5.10-continued

```
TTGACGTCAAGGTCGTCCCCGGTCTGGCCTGTTAATAGGTGCTTCATCATT   777

ACCCCCTCGCTGAGCGTCGCTCAAGCCTCTCTAAGTATAGTCGGTCTAAAA   828

AAGCGCCAGTCACGTGCCGCGACGCTGCGAGCTTGCGTCGTTTTGTTCTAC   879

ACTGGCTGAAAATATAGAGGAGATGATTATGCAGGTGAACGATCGTGTCAC   930

GGTGAAGACCGACGGTGGCCCACGTCGATCGGGAGGAACGATGTATCTGGT   981

TTCACTACTGGAAGACTATCCTCTGGGGATC                       1012
```

Figure 5.10. Nucleotide sequence of $rcsB_{K1}$ and alignment with $rcsB_{K21}$. The ribosome binding site is underlined at -7 bp and the -10 promoter is at -47 bp. The predicted amino acid sequence of RcsB is indicated in three letters below the appropriate nucleotide bases. : indicates identical $rcsB_{K21}$ nucleotides.

5.9 DNA homology analysis

The sequence nucleotides containing $rcsA_{K1}$ and $rcsB_{K1}$ genes were screened for homology with sequences in the EMBL and GenBank databases. Comparison of $rcsA_{K1}$ with $rcsA_{K21}$ shows almost identical homology. Within the ORF of *rcsA*, the only difference was a single nucleotide substitution from the ORF of $rcsA_{K21}$ (Fig 5.9), while the predicted amino acid sequence was conserved.

The *rcsA* gene of *Klebsiella* shares more than 60% identity in 755 bp overlap with the *rcsA* of *E. coli*. It also shares about 62% identity in 646 bp overlap with *rcsA* of *Erwinia* sp. Comparison of the predicted amino acid sequences of the RcsA protein of *Klebsiella* and *E. coli* or *Erwinia* spp shows a high degree of homology (Fig 5.11). RcsA proteins of *K. pneumoniae* and *E. coli* are identical in size and share 68% exact amino acid identity. A 56% exact amino acid identity between the RcsA protein of *K. pneumoniae* and *Erwinia* was also found (Fig 5.11).

The ORF of $rcsB_{K1}$ sequence was identical with the ORF of $rcsB_{K21}$ (Fig 5.10). A computer search of nucleotide sequence failed to identify any gene homologous with $rcsB_{K1}$. The RcsB protein of *Klebsiella* which consists of 60 amino acids had no similarity with any other proteins including the RcsB protein of *E. coli*.

```
KP1   MSTMIMDLCSYTRLGLTGYLTSRGIKKQEIVEVNSAADLQKHCTSCCP   48
KP21  :::::::::::::::::::::::::::::::::::::::::::::::::   48
EC12  :::I:::::::::::::::L:::V::R::NDIETVD::AIA:D:QR:   48
EC30  :::I:::::::::::::::L:::V::R::NDIEFVD::AIA:D:QR:   48
EAV   :P:I:::S:N:::::::E:M:VK:V::KN:SLI:DI:Q::NK:QQLK:   48
EST   :P:I:::S:N::::::SD:MS:K:V::KN:TS:SDIEQ::QR:EQLK:   48

KP1   AVVFLNEDCFVHDDESNGIIRQIITQNPATLFVIFMSLANIHFDRYLR   96
KP21  :::::::::::::::::::::::::::::::::::::::::::::::::   96
EC12  S:::I:::::I::ASNSQR:KL::N:H:N:::IV::AI::V:::E::L   96
EC30  S:::I:::::I::ASNSQH:KH::N:H:N:::IV::AI::V:::E::L   96
EAV   G::LI:::::I:ESDASER::K::L:H:D:::F:::AIS::::EE::Y   96
EST   G:::I:::::I:ETD:SER::S::N:H:E:::F:::AIS::::EE::Y   96

KP1   VRKNLLISSKSITPKDLDVILVNY----LKYKNTSVGQLTLPTLSLSK  140
KP21  :::::::::::::::::::::::::----:::::::::::::::::::  140
EC12  ::::::::::::K:ES::D::GDI----::KET:ITSF:NM:::::R  140
EC30  ::::::::::::K:ES::D::GDI----::KET:ITSF:NM:::::R  140
EAV   ::N::I:T::A:KIST::SL:NG:FQKK:NLSVRHGTHSEVHP:T::Q  144
EST   :::::I:T::A:K:ST::SL:ST:LQKK:NMSPRISSG:DVHP:T::Q  144

KP1   TESNMLQMWMAGHGTSQISTQMNIKAKTVSSHKGNIKKKIQTHNKQVI  188
KP21  :::::::::::::::::::::::::::::::::::::::::::::::::  188
EC12  :::S::R:::::Q::I:::D:::::::::::::::::R::K:::::::  188
EC30  :::S::R:::::Q::I:::D:::::::::::::::::R::K:::::::  188
EAV   ::::::K:::S::D:I:::DK:Q:::::::::::::::R::K:::::::  192
EST   ::::::K:::S::D:I:::DK:Q:::::::::::::::R::K:::::::  192

KP1   YHIVRLTENITSGIQVNMR   207
KP21  ::::::::::::::::::   207
EC12  ::V::::D:V:N::F::::   207
EC30  ::::::::::::::::::   207
EAV   ::V::::D:V::::Y::E:   211
EST   ::V::::D:V::::Y::V:   211
```

Figure 5.11. Alignment of the predicted amino acid sequence of RcsA protein from *K. pneumoniae* K1 with other amino acid sequences of RcsA proteins. KP1: *Klebsiella* K1. KPN21: *Klebsiella* K21b (Allen *et al*, 1987). EC12: *E. coli* K12 (Stout *et al*, 1991). EC30: *E. coli* K30 (Keenleyside *et al*, 1992). EAV: *Erwinia amylovora* (Coleman *et al*, 1990). EST: *E. stewartii* (Poetter and Coplin, 1991). : indicates identical amino acid. The dashed lines inserted in the *Klebsiella* and *E. coli* RcsA proteins are used to accommodate four additional amino acids of the *Erwinia* RcsA.

5.10. Discussion

Using the positive selection vector, pLV59, a genomic library of *K. pneumoniae* K1 DNA was constructed in *E. coli*. The recombinant plasmid that induced a mucoid phenotype was isolated. Deletion and subcloning analyses have shown that a 2.4 kb *Bgl*II fragment of the chromosomal DNA of *Klebsiella* which contained $rcsA_{K1}$ and $rcsB_{k1}$ genes, was responsible for induction of the mucoid phenotype. Chemical and immunological analyses of the polysaccharide produced by the mucoid clone suggested that the polysaccharide was colanic acid rather than *Klebsiella* K1 capsule.

rcs genes have also been characterised from *Klebsiella* K21b (Allen *et al*, 1987) and were identical with the *rcs* genes of *Klebsiella* K1 described here. However the *rcsA* gene from *Klebsiella* K21b required cloned multicopy *rcsB* to confer mucoidy in *E. coli* K12 at 37°C, whereas the *rcsA* of *Klebsiella* K1 described in this thesis activated independently of *rcsB* and growth temperature. An *rcsA* allele of *Klebsiella* K20 which does not require *rcsB* has also been reported previously (McCallum and Whitfield, 1991) and was in agreement with this work. The genetic background of *E. coli* strains or the growth media had little effect. Although the nucleotide sequences of both $rcsA_{K1}$ and $rcsA_{K21}$ genes were the same, it is not clear why such subtle differences were observed.

The *rcsA* or *rcsB* gene cloned in a multicopy plasmid increase the CPS production in both lon^+ and lon^- *E. coli* K12 cells. Although the cloned *rcsA* gene in multicopy increases the expression of RcsA protein, it still requires an intact $rcsB^+$ chromosomal gene for activating polysaccharide synthesis (Brill *et al*, 1988). This work has also shown that cloned $rcsA_{K1}$ activates colanic acid synthesis with or

without the presence of the cloned $rcsB_{K1}$ gene in *E. coli* K12 (possibly containing an *rcsB* gene in its chromosome). Whether, $rcsA_{K1}$ is also sensitive to Lon protease and is limiting for polysaccharide synthesis has not been investigated. Since almost all clinical isolates of *Klebsiella* constitutively produce capsule and are possibly lon^+, the $rcsA_{K1}$ gene might be stable or there could be a difference in the way the $rcsA_{K1}$ gene acts in this genus.

The introduction of a multicopy *rcsB* gene into rcs^+ or rcs^- *E. coli* K12 can activate *cps* gene expression (Brill *et al*, 1988). This suggested that *rcsB* may bypass the normal requirement for *rcsA* and may be crucial for capsule synthesis. Although multicopy $rcsB_{K1}$ gene increased the level of capsule synthesis when present along with cloned $rcsA_{K1}$ gene, it did not bypass the requirement of multicopy $rcsA_{K1}$ gene in this study. The $rcsB_{K1}$ gene has been shown to be involved in increasing *rcsA* gene transcription in *E coli*. Preliminary observation also suggested that multicopy $rcsB_{K1}$ gene makes *E. coli* strain temperature sensitive (>32°C) and might be lethal to some strains (D. Sledjeski, personal communication). Multicopy *rcsB* gene from *E. coli* K12 has also been shown to be lethal when introduced into *rcsC137* mutant *E. coli* strain (Brill *et al*, 1988). This could suggest that $rcsB_{K1}$ may have functional similarities with *rcsB* of *E. coli*, although both do not share any homology including their protein products.

Comparison of the predicted amino acid sequences of RcsA proteins has shown extensive homology and all RcsA proteins can activate colanic acid synthesis in *E. coli* K12, although *Klebsiella* and *Erwinia* spp do not appear to synthesize colanic acid polysaccharide. It is increasingly evident that the normal role of the RcsA protein in the respective species is in regulating the synthesis of species-specific capsule. Studies

have shown that RcsA regulates expression of species specific capsular antigen in *E. coli* K30 (Keenleyside *et al*, 1992), *K. pneumoniae* K20 (McCallum and Whitfield, 1991), and *Erwinia* (Coleman, *et al*, 1990).

Recently, the *rcsB* gene of *E. coli* has been shown to regulate the transcription of cps_K for the synthesis of *Klebsiella* K2 specific capsule in the presence of a second regulator of mucoid phenotype, the *rmpA* gene (Wacharotayankun *et al*, 1992). Since the *rmpA* gene has been shown to act as an alternative to *rcsA* and activate colanic acid biosynthesis at 30°C (Nassif *et al*, 1989), it might have also complemented the requirement of *rcsA* in the regulation of biosynthesis of *Klebsiella* K2 capsular antigen in *E. coli* K12. Both *rcsA* and *rcsB* genes of *Klebsiella* may be critical in the regulation of capsule production. Although the *rmpA* gene is required for the mucoid phenotype, it may not be necessarily required as many capsulated *Klebsiella* do not appear to possess plasmids that carry *rmp* genes.

A regulator gene similar to *rcsC* of *E. coli* K12, an environmental sensor that acts on *rcsB*, has also been reported from *E. amylovora* (Roberts and Coleman, 1991). It is not yet known whether an *rcsC*-like gene is also present and which might be involved in the regulation of capsule production in *Klebsiella*. Although not confirmed yet, preliminary study described in this thesis suggests the presence of an unidentified gene that might negatively regulate the expression of $rcsA_{K1}$.

It is obvious that the *rcs* regulatory network is wide spread and conserved among the different species of the family *Enterobacteriaceae*. The *rcs* genes may function as common regulatory system for the expression of structurally divergent capsular polysaccharides including *Klebsiella* specific capsule.

CHAPTER VI

GENERAL DISCUSSION

General discussion

K. pneumoniae is one of the commonest opportunistic pathogen and causes a number of infections. Almost all clinical isolates are capsulated. Although the capsule is not essential for life, it does however play an important role in the life-style of these bacteria. Considering the involvement of capsule in the pathogenicity of *Klebsiella*, understanding of the environmental and nutritional conditions that influence its production and the regulation of its expression at molecular levels is certainly important.

The results of both batch and chemostat culture studies showed that large quantities of polysaccharide were produced in nutrient-limited culture conditions. The synthesis of high levels of polysaccharide was also influenced by the growth rate of the organism, growth temperature, and pH. The inverse relationship between growth rate and polysaccharide production could be the result of limitation of the availability of lipid carrier (C_{55}) for both cell wall and capsule. When conditions are favourable for rapid growth, the synthesis of polysaccharide decreases while synthesis is directed towards essential cell wall polymers.

Nutrient-limited conditions can affect the physiological status of an organism including intracellular energy metabolism. Growth rates would decrease and the organism would need to become more efficient in utilising scarce nutrients. New proteins involved in scavenging specific nutrients and conferring a more stress-resistant phenotype for prolonged survival would be synthesised (Matin *et al*, 1989). Another event that occurs during nutrient limited conditions is the synthesis of exopolysaccharides (Allison and Sutherland, 1985; Wrangstadh *et al*, 1987). The general consequence of nutrient limitations other than the carbon source may allow

uptake and further metabolism of the excess carbon source. Thus, polysaccharide polymer products that are not directly involved in cell growth are produced. The polysaccharide produced may thus alter the surface of bacteria, and also protects against host clearance mechanisms (Simoons-Smit *et al*, 1986; Williams *et al*, 1986; Merino *et al*, 1992).

The capsule may provide a means of binding certain complexing cations because of its negative charge. Terry and co-workers (1992) have also suggested that the capsule may give growth advantage by increasing binding or transport proteins, synthesis or modification of porins and may also increase the activity of the ATP synthase. The mucoid form might have a growth advantage by conservation of cellular energy through recycling by end product excretion of organic acid thereby creating an electrochemical gradient (Verdoni *et al*, 1990). It is also possible that the capsule of *Klebsiella* might have similar role.

The increase in the production of CPS in nutrient limited condition or at low growth rate *in vitro* culture, may also be true *in vivo* where the potential growth substrates might not be readily available. The physiological alterations readily monitored *in vitro* may provide an explanation for the survival and persistence of mucoid *Klebsiella* under growth conditions likely to exist in the hosts environment.

It is clear the biosynthesis of *Klebsiella* capsule is complex and the expression of the genes involved are possibly regulated by genes that might respond directly or indirectly to the environmental conditions. The regulation of polysaccharide synthesis presents a challenging problem to our understanding at the molecular level.

An attempt has been made to clone the capsule gene cluster directly using cosmid vectors. It was possible to clone genes for an antigenic component that was

specific to *Klebsiella*. The gene product might be a surface protein that could be periplasmic or located in the outer membrane.

This work was the first to be able to generate mutants of *K. pneumoniae* K1 using Tn*10* or Tn*phoA*. The noncapsulated mutants of *Klebsiella* are thus the result of insertional inactivation of structural genes or major regulator genes for capsule production. Transposon-fusion joints from the chromosome of noncapsulated mutants have also been cloned. Unfortunately, the cloned genes containing major structural genes for capsular expression failed to express *Klebsiella* K1 antigen in *E. coli* or complement various noncapsulated mutants of *Klebsiella*.

One of the explanations for the failure of expression of the *Klebsiella* capsule genes could be that they may not be clustered and thus could not be contained in a single plasmid cloning vector system. Generation of noncapsulated mutants of *K. pneumoniae* K1 with the insertion of transposons at different sites is an indication of the possible involvement of several genes either consisting of a large segment of DNA or dispersed at different loci within the chromosome. The involvement of more than one gene locus including *his*-linked determinants and a possible polymerase *rfc*-linked determinant has been proposed by Schmidt *et al* (1977). Another possible reason might be due the instability of clones carrying the capsule biosynthetic genes. The incomplete expression of capsule on the surface of the host cell could result in the accumulation of gene products in the cytoplasm. This may interfere with bacterial cell metabolism and might be lethal to the host cell. Spontaneous deletion of cloned fragments of the *cps* gene cluster that determines the expression of *E. amylovora* exopolysaccharide (Belleman and Geider, 1992) might also reflect an analogous situation with *Klebsiella* capsule biosynthetic genes.

Group I *E. coli* capsule, colanic acid and *Klebsiella* capsules share common features; they generally are of high molecular weight and are expressed at 20°C (Jann and Jann, 1990). In addition, the major structural genes for these capsules are located at the *his* region and are also under the control of *rcs* genes (Gottesman and Stout, 1991). The genetic organisation may therefore be different from the better-characterized group II *E. coli kps* gene clusters.

It is possible that the *Klebsiella* capsular genes could be organised into at least two components: the structural genes containing unknown lengths of coding DNA and the regulatory components, *rcsA* and *rcsB*. The requirement of additional regulator genes (*rmpA* and *rmpB*) for cps_K (Arakawa, 1991 and 1992) in the expression of *Klebsiella* K2 antigen in *E. coli* would suggest such a model.

The two regulator genes, $rcsA_{K1}$ and $rcsB_{K1}$ characterised from *K. pneumoniae* K1 appeared to regulate expression of colanic acid positively in *E. coli* K12. The present study together with the earlier studies (*Allen et al*, 1987; McCallum and Whitfield, 1991) confirm that *Klebsiella* serotypes contain two genes which are highly conserved. The $rcsA_{K1}$ shared extensive homology with *rcsA* genes from other bacteria. The similarity in size and extensive homology among various RcsA proteins and their capabilities for positive regulation of transcription of *cps* genes directly or indirectly in heterologous species of bacteria, may be an indication of the presence of a conserved system for the genetic regulation of capsule synthesis among enteric bacteria, not least in *Klebsiella*.

K. pneumoniae produces CPS independent of growth temperature. The expression of large quantities of capsular material by *K. pneumoniae* at low temperature does not give an advantage in mammalian hosts. However, the results of

batch and chemostat cultures have shown the increase in the level of polysaccharide synthesis at lower temperature rather than 37°C. It is plausible to say that *Klebsiella* may also contain an unidentified temperature sensing *rcsC*-like gene that may respond to shifts in temperature and possibly transmit signals to the positive regulator gene, $rcsB_{K1}$. There is also evidence that *rcsB* and *rcsC* genes from *Erwinia* sp can complement *rcsB* and *rcsC* mutants in *E. coli* (Roberts and Coleman, 1991). It would be interesting if *rcsB* of *K. pneumoniae*, which encodes a relatively small putative polypeptide as compared to *rcsB* of *E. coli* or *Erwinia* can complement *rcsB* mutants of *E. coli* K12.

Rcs proteins of *E. coli* are thought to belong to a subfamily of regulator proteins. RcsB and RcsC belong to a family of two-component regulatory pairs that respond to environmental stimuli (Stout and Gottesman, 1990). Interestingly, the *algR*, positive regulator of mucoidy in *Pseudomonas aeruginosa* has also been found to be homologous to sequences of environmentally responsive regulator genes (Deretic *et al*, 1989). Regulators of the subfamily consisting of NtrC and DctC proteins are involved in the cellular response to low external levels of nitrogen and act with RpoN (σ^{54}) to activate their target genes. The *rcsB* gene expression in *E. coli* has also been reported to be dependent on RpoN indicating that another factor affects *rcsB*. RpoN is a sigma factor that is required for the transcription of physiologically diverse genes (Kutsu *et al*, 1989). The 300-fold reduction in the transcription of the positive regulator *rcsB* gene in an rpoN mutant supports this concept (Stout and Gottesman, 1990).

RcsA and RcsB proteins belong to a family of transcriptional activator proteins defined as the LuxR group by Henikoff *et al* (1990) and redefined as UhpA-LuxR

family by Stout and his colleagues (1991). UhpA-LuxR family of regulator proteins have a conserved region. The domain conserved in this family is found at the C-terminal region of the protein. Protein sequence analysis by Stout *et al* (1991) suggested that the C-terminal region contained a possible helix-turn-helix DNA binding. Since the RcsA protein from *Klebsiella* shared extensive homology with other RcsA proteins, it also belongs to this family. The second regulatory protein from *Klebsiella*, RcsB did not share any homology with UhpA-LuxR family suggesting that it does not belong to this group.

Both RcsA and RcsB proteins may interact to promote *cps* transcription in *E. coli* K12. As proposed by Stout *et al* (1991), RcsA protein may facilitate phosphorylation of RcsB protein or its interaction with RcsB might also promote DNA binding by RcsB. Whether such interactions occur between the RcsA and RcsB proteins of *Klebsiella* to promote transcription of *Klebsiella* capsule biosynthetic genes, it remains to be seen.

In conclusion, the expression of capsule in *K. pneumoniae* K1 is quantitatively influenced by the availability of nutrient and environmental conditions. Such an expression of low or high levels of CPS could be partly explained by the activity of regulatory elements that are directly or indirectly responding to environmental stimuli. Perhaps the most important future development would be to determine how the *rcs* genes respond to conditions *in vivo*, possibly by use of gene fusion techniques.

This study suggests that genetic regulation of capsule biosynthesis in *K. pneumoniae* is probably more complex than originally thought. Not only may a gene cluster consisting of a large fragment of chromosomal DNA be involved, but also additional genes which may be located at different loci may be required for complete

expression of the capsule. The two genes characterised from *K. pneumoniae K1* are thus likely to be part of the regulator genes. Their gene products, RcsA and RcsB might interact to positively regulate the transcription of capsule biosynthetic genes. We still do no know the genetic organisation of the biosynthetic genes for *Klebsiella* capsules. As more genes are characterised and sequenced, and their proteins determined, a clearer picture should emerge in the near future. Molecular cloning and analysis of capsule biosynthesis should also enable a better understanding of the elusive role of capsular material as a virulence determinant in these bacteria.

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LIST OF REFERENCES

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