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

NEIL GERARD JOHNSTON

Department of Genetics and Microbiology

March 1992

SUMMARY

Various transposons were used in conjunction with plasmid, bacteriophage and prophage delivery strategies in an attempt to generate non-capsular, but otherwise isogenic mutants in Klebsiella pneumoniae. No single system proved to be entirely suitable for all strains of Klebsiella pneumoniae, although the majority enjoyed a limited degree of success amongst a narrow host range.

Stable non-capsular mutants of K.pneumoniae 5055 were finally generated using the suicide vector pRT733 and its constituent transposon, TnphoA. Mutants failing to express capsular polysaccharide were identified using a battery of immunological and electron microscopy techniques.

TnphoA fusion joints were cloned from the non-capsular mutants into pUC19 and preliminary restriction maps of the resultant recombinant plasmids were produced. Transposon-induced mutations leading to the production of a non-capsular phenotype could not be complemented either by recombinant plasmids from other non-capsular mutants or by plasmids containing the rcsA gene which has been shown to be involved in Klebsiella capsule biosynthesis.

A major role was confirmed for K.pneumoniae K2 capsular polysaccharide in resistance to phagocytosis by polymorphonuclear leucocytes. However, the capsule does not appear to function as a barrier to the bactericidal effects of serum.

ACKNOWLEDGEMENTS

I would firstly like to express my gratitude to my supervisor Dr. Peter G. G. Miller for his invaluable help and advice during the course of this research project. I also wish to thank the various academics in the Department of Genetics and Microbiology especially Professor J. R. Saunders and members of his research group for their assistance.

I am indebted to Dr. E. Roe and Dr. R. Jones for their help in the early stages of this work. Thanks are also due to Dr. J. M. Pratt and others for their gifts of strains and DNA (as detailed in this work) and for their many helpful suggestions.

I am particularly grateful to Dr. Tyrone Pitt and Beverley Ayling-Smith for their assistance with Klebsiella capsular serotyping and phage typing and to Dr. Steven Edwards and members of his research team for help with phagocytosis assays.

In addition, I would like to thank Maureen Wilde for photography work and Gill Ollerhead and Brian Getty for electron microscopy services.

I would especially like to thank all the members of the department for their help, friendship and humour over three and a half years.

I also wish to take this opportunity to thank my family, especially my mum and dad for their support and sound advice over the years.

Finally, I am eternally grateful to Karen for her endless encouragement, sense of humour and for her superb help in typing this thesis.

The work described in this thesis was supported by a project grant from the Medical Research Council.

ABBREVIATIONS

Ap^R	- ampicillin resistance
Ap^S	- ampicillin sensitivity
BSA	- bovine serum albumin
^oC	- degree(s) centigrade
c.f.u.	- colony forming unit
μCi	- microCurie(s)
CPS	- capsular polysaccharide
Cm^R	- chloramphenicol resistance
Cm^S	- chloramphenicol sensitivity
cm^{-3}	- cubic centimetre(s) (10^{-3})
DNA	- deoxyribonucleic acid
EDTA	- ethylenediaminetetraacetic acid
g	- gram(s)
μg	- microgram(s) (10^{-6})
h	- hour(s)
kb	- kilobase(s)
kDa	- kilodalton(s)
Km^R	- kanamycin resistance
Km^S	- kanamycin sensitivity
λ	- lambda
lbs sq in^{-1}	- pounds per square inch
LPS	- lipopolysaccharide
M	- molar(s)
mA	- milliampere(s)
min	- minute(s)
ml	- millilitre(s) (10^{-3}l)
μM	- micromolar(s) (10^{-6}M)

mM	- millimolar(s) (10^{-3}M)
ng	- nanogram(s) (10^{-9}g)
nm	- nanometre(s) (10^{-9}m)
OD	- optical density
RNA	- ribonucleic acid
s	- second(s)
SDS	- sodium dodecyl sulphate
Sm^R	- streptomycin resistance
TES	- TRIS-EDTA-saline
Tc^R	- tetracycline resistance
UV	- ultra violet
U/µg	- units per microgram
V	- volt(s)

CONTENTS

Summary

Acknowledgements

Abbreviations

Chapter 1: General Introduction

1.1 Introduction 2

1.2 Clinical Significance of Klebsiella 2

1.3 Seroepidemiology of Klebsiella 4

1.4 Virulence Factors in Klebsiella - An Overview 5

1.4.1 Capsular polysaccharide 5

1.4.1.1 Serum resistance 6

1.4.1.2 Phagocytosis 7

1.4.1.3 Role of capsule size 10

1.4.1.4 Role of capsular serotype 10

1.4.1.5 Interference with opsonisation by capsular polysaccharide 11

1.4.2 Lipopolysaccharide 13

1.4.3 Extracellular toxic complex 15

1.4.4 Pili and mannose-inhibitable adhesins 16

1.4.5 Iron-sequestering mechanisms 17

1.4.6 Enterotoxins and haemolysin 18

1.5 The Structure of Klebsiella Capsules 18

1.6 Biosynthesis of Capsular Polysaccharide in Klebsiella 21

1.7 Genetic Analysis of Capsule Production 23

1.7.1 Genetic analysis of capsule production in E.coli 23

1.7.1.1 Region 1 23

1.7.1.2 Region 2 24

1.7.1.3 Region 3 25

1.7.2 Genetic comparison of capsule production in other species 25
1.7.3 Genetics of capsule production in Klebsiella 26
1.8 Alternative Strategies to Isolate Genes Involved in Klebsiella Capsular Polysaccharide Biosynthesis 28
1.9 Objectives of This Work 31

Chapter 2: Materials and Methods

2.1 Materials 33
2.1.1 Antibiotics 33
2.1.2 Chemicals 33
2.1.3 Enzymes 33
2.1.4 Miscellaneous 33
2.1.5 Growth media 34
2.1.6 Buffers and solutions 36
2.1.7 Bacterial strains, plasmids and bacteriophages 39
2.2 Methods 45
2.2.1 Cultivation and storage of bacteria 45
2.2.2 Isolation of DNA 45
2.2.3 Purification, concentration and storage of DNA 46
2.2.4 Agarose gel electrophoresis 46
2.2.5 Restriction enzyme digests 46
2.2.6 Dephosphorylation and ligation of DNA 47
2.2.7 Transformation 47
2.2.8 In vitro packaging and transfection of cosmid DNA 47
2.2.9 Conjugations 48
2.2.10 DNA:DNA hybridisations 48
2.2.11 Preparation of radiolabelled DNA probes 49
2.2.12 Bacteriophage λ manipulations 50

2.2.13 Assessment of urease production 50
2.2.14 Bacteriophage P1 manipulations 50
2.2.15 Purification of outer membranes 50
2.2.16 Purification of lipopolysaccharide 51
2.2.17 Polyacrylamide gel electrophoresis 51
2.2.18 Capsular serotyping 51
2.2.19 Phage typing 52
2.2.20 Immuno-gold labelling 52
2.2.21 Visualisation of capsular material 52
2.2.22 Preparation of bacterial strains for serum and phagocytic assays 52
2.2.23 Serum bactericidal assays 53
2.2.24 Preparation of phagocytes for phagocytosis assays 53
2.2.25 Pre-opsonisation of bacteria 53
2.2.26 Chemiluminesence (CL) assay 53

Chapter 3: Attempts to Generate Random Transposon Insertions in the K.pneumoniae Genome

3.1 Introduction 56
3.2 The Rts1-Based Transposon Delivery System 56
3.2.1 Introduction 56
3.2.2 Introduction of pRU669 and pRU670 into K.pneumoniae 57
3.2.3 Elimination of donor plasmid 60
3.2.4 Screening of prospective transposon mutants 64
3.3 Attempts to Use λ-Based Delivery Systems 69
3.3.1 Introduction 69
3.3.2 Transfer of pLB8000 and pTROY9 to K.pneumoniae 70
3.3.3 Isolation of λ sensitive mutants 73
3.4 Attempts to Use the pHSG415 Delivery System 77

3.4.1 Introduction 77

3.4.2 Attempts to transfer pHSG415::Tn10 to K.pneumoniae 77

3.4.3 Stability of inheritance of pHSG415::Tn10 in K.pneumoniae at different temperatures 80

3.4.4 Screening of $Tc^R Km^R$ colonies 84

3.5 Use of Bacteriophage P1-Based Delivery Systems 84

3.5.1 Introduction 84

3.5.2 Attempts to isolate P1-sensitive derivatives of K.pneumoniae 85

3.5.3 Screening of prospective transposon mutants 88

3.6 Transposon Mutagenesis Using the pRT733 Delivery System 90

3.6.1 Introduction 90

3.6.2 Isolation of transposon mutants of K.pneumoniae 90

3.7 Discussion 96

Chapter 4: Isolation and Characterisation of Transposon Mutants Deficient in Capsular Polysaccharide Biosynthesis

4.1 Introduction 100

4.2 Isolation of Non-Capsular Mutants of K.pneumoniae K2 5055 100

4.3 Immunological Analysis of Prospective Mutants 101

4.3.1 Double immunodiffusion analysis 101

4.3.2 Counter-current immunoelectrophoresis and quellung analysis 101

4.3.3 Immune electron microscopy analysis 104

4.4 Electron Microscopy Analysis 104

4.5 Phage Sensitivity Analysis 112

4.6 Southern Hybridisation Analysis 114

4.7 Examination of the Outer Membrane 118

4.8 Preliminary Analysis of Location of Transposon Insertions 118

4.9 Discussion 121

Chapter 5: Genetic Analysis of Non-Capsular Mutants

5.1 Introduction 124

5.2 Attempts to Clone TnphoA plus Klebsiella DNA Using the Cosmid Vector pHC79 124

5.3 Attempts to Clone TnphoA plus Klebsiella DNA Using Plasmid Vectors 125

5.4 Characterisation of TnphoA Recombinant Plasmids 127

5.5 Complementation of Non-Capsular Mutants 131

5.6 Discussion 131

Chapter 6: Influence of the Klebsiella K-Antigen on Serum Killing and Phagocytosis

6.1 Introduction 134

6.2 Influence of the K.pneumoniae K-Antigen on Serum Killing 134

6.3 Influence of the K.pneumoniae K-Antigen on Phagocytosis 135

6.4 Discussion 140

Chapter 7: Closing Discussion **142**

Bibliography **147**

CHAPTER 1: General Introduction

1.1 Introduction

Among the members of the family Enterobacteriaceae is the genus Klebsiella. Klebsiellae are non-motile, aerobic and facultatively anaerobic Gram-negative rods. Both Klebsiella pneumoniae and Klebsiella oxytoca species are known to be pathogenic for man. Most strains of Klebsiella are surrounded by an extracellular polysaccharide material which is organised to form a discrete capsule. This capsule can be readily demonstated by the exclusion of India ink in microscopic preparations (Duguid, 1951). The capsule is immunogenic and can elicit the production of specific anti-capsular antibodies in mammals. Currently, at least 77 different capsular serotypes have been recognised (Orskov and Fife-Astbury, 1977).

1.2 Clinical Significance of Klebsiella

In healthy patients, Klebsiella may exist as commensal micro-organisms either in the upper respiratory tract or, less commonly, in the intestine. Infections due to klebsiellae may be community-acquired (Haddy et al, 1987), but are usually nosocomial (Montgomerie and Ota, 1980). Indeed, where a hospitalized patient has an underlying defect, or has been subjected to an invasive procedure or chemotherapy, the potential for Klebsiella infection is dramatically increased. Factors predisposing to Klebsiella infection include age, cancer (Bodey et al, 1989), surgical incision, catheterisation (Montgomerie and Ota, 1980), low birth weight and ankylosing spondylitis. During the past forty years, klebsiellae have become increasingly important as causes of nosocomial infections, accounting for some 20 infections per 10000 patient discharges (Jarvis et al, 1985). Klebsiella species may produce severe disease including urinary and wound infections, meningitis, peritonitis, osteomyelitis and even emphysematous septic arthritis (Broom and Beebe,1988). Additionally, K.pneumoniae can be

a rare, but devastating primary lung pathogen (Silverblatt and Weinstein, 1979).

Klebsiellae have also played a major role in the general increase in Gram-negative bacteraemias during recent decades, ranking second only to Escherichia coli in this respect (Bryan et al, 1983; McGowan et al, 1985). Mortality rates for patients with Klebsiella infections have been reported to be as high as 12% (Steinhauer et al, 1966) and between 20 and 50% for Klebsiella bacteraemias (Garcia de la Torre et al, 1985; Montgomerie and Ota, 1980; Terman et al, 1972). Although in many cases associated with the patient's underlying condition, mortality rates have also reportedly increased where the strain of Klebsiella was resistant to antibiotics used in treatment (Ma et al,1983; Jarvis et al,1985).

One of the major problems in treating bacterial infections today is the rapid evolution and subsequent spread of plasmids encoding resistances to the most modern of antibiotics. Brun-Buisson and co-workers (1987) have identified a plasmid in Klebsiella responsible for combined resistance to a cephalosporin and an aminoglycoside which has been shown to be transmissible to other Enterobacteriaceae in the hospital environment. This report is especially disturbing in light of the fact that this is often the treatment of choice for Klebsiella infections.

These factors have led an upsurge of interest in klebsiellae. Much work has centered on the development of potential vaccines for the control of Klebsiella infections (Cryz, 1990) and major studies have been conducted in order to improve typing methods and to facilitate a greater understanding of the epidemiology of the species (Simoons-Smit, Ph.D Thesis, 1987).

1.3 Seroepidemiology of Klebsiella

Four different methods have generally been used in order to type Klebsiella spp.:-

1. Serotyping (Kauffmann, 1949)
2. Bacteriophage typing (Slopek et al, 1967)
3. Bacteriocin typing (Bauernfeind, 1984)
4. Biochemical typing (Simoons-Smit et al,1987)

Serotyping, in particular capsular serotyping, has generally become the most popular due to the existence of a large number of capsular types (K-antigens) as well as its high reproducibility. Several methods may be used in this respect including, capsular swelling (quellung reaction, Kauffman, 1949), immunofluoresence (Riser et al, 1978) and counter-current immunoelectrophoresis (Palfreyman, 1978).

Numerous seroepidemiological studies have been undertaken in order to assess any correlation between capsule type and both virulence and site of isolation within the body (Riser and Noone, 1981). Many of these studies have implicated serotype K2 as one of those most frequently isolated (Cryz et al, 1986; Casewell amd Talsania, 1979). In addition, it has also been shown in one study of urinary tract infections that K2 isolates clearly predominated, possibly due to some, as yet, unidentified advantage (Martin et al, 1971). It has been suggested that strains of K.pneumoniae whose capsular antigen contains mannose may be more susceptible to lectin-mediated phagocytosis and thus less virulent than other isolates where the K-antigen does not contain mannose (Athana et al, 1989). However, the fact that both the K2 and K21 (another frequently isolated serotype) capsules contain mannose may call this argument into question.

1.4 Virulence Factors In Klebsiella - An Overview

Amongst the numerous putative virulence determinants so far described in Gram negative organisms, the following have all been shown to play a role in the capacity of klebsiellae to cause disease:-

i) Capsular polysaccharide (CPS; K antigen).

ii) Lipopolysaccharide (LPS; O antigen).

iii) Extracellular toxic complex.

iv) Pili.

v) Iron-sequestering mechanisms.

vi) Enterotoxin and haemolysin.

1.4.1 Capsular polysaccharide

The presence of an extracellular capsular polysaccharide is a virulence factor of critical importance in klebsiellae and clinical isolates are, almost without exception, encapsulated.

A number of groups have demonstrated that the degree of virulence is directly related to the presence, the size and rate of synthesis of the capsule in mouse skin (Simoons- Smit et al, 1986), burn-wound sepsis (Cryz et al, 1984), intraperitoneal (Ehrenworth and Baer, 1956) and pulmonary (Domenico et al, 1982; Domenico et al, 1985) infection models.

Although the precise function of the capsule in the pathogenesis of klebsiellae infections has yet to be established, its role in enhancing resistance to both serum killing and phagocytosis has received much attention, both in Klebsiella and other bacteria.

1.4.1.1 Serum resistance

The complement system and phagocyte are major components of the mammalian host defence mechanism and their killing effect must therefore be circumvented if the bacteria are to survive and proliferate.

Complement is a series of proteins (C1-C9) which acts in a cascade fashion resulting finally in the death of the bacterial cell. It can be activated either by the classical pathway, requiring the formation of an antigen-antibody complex or by the alternative pathway which can be triggered by, amongst others, microbial polysaccharides. The alternative pathway is particularly important in neonates where the supply of circulating antibodies may be limited (Morrison and Kline, 1977). In spite of their being activated by different stimuli, each pathway leads to the deposition of the protein C3b on the activating cell surface and the cell is killed by subsequent insertion of the terminal C5-C9 attack complex into the bacterial cell membrane and concomitant leakage of cellular fluids. The C3b component of complement and specific antibody can also serve as opsonins, rendering bacteria highly susceptible to phagocytosis. Opsonisation is mediated through phagocyte receptors for C3b and the Fc portion of immunoglobulin G.

A number of reports usually concerning E.coli have implicated capsular polysaccharide in bacterial resistance to serum killing (Glynn and Howard, 1970; McCabe et al, 1975; Leying et al, 1990).

Work by different groups has suggested that the K-antigen does have some role to play in the conferment of serum resistence upon some strains of Klebsiella. Simoons-Smit et al (1986), using serum-resistant K.pneumoniae strains of different serotypes found that some remained resistant to the effects of 10% normal human serum when devoid of their capsular antigen. Fresh human serum was also found to be bacteriostatic for a non-capsular smooth

mutant ($K2^{-}01^{+}$) (Williams et al, 1983). These studies were conducted using mutants deficient in capsular polysaccharide synthesis obtained by detection of spontaneous K^{-} colonies on nutrient agar or by mutagenesis with nitrosoguanidine respectively (Poxton and Sutherland, 1976). In each case, the mutants were then assumed to be isogenic with respect to all other outer envelope constituents. Trautmann et al (1988) have also shown using a CPS-specific monoclonal antibody that the capsule can interfere with the deposition of complement onto the cell surface, thus preventing activation of the complement cascade. However, other studies using different strains have demonstrated that capsular polysaccharide does not play an important role in survival in serum (Tomas et al, 1986). This study also used spontaneous non-capsular mutants.

1.4.1.2 Phagocytosis

A more conclusive role for the K-antigen of Klebsiella and other bacteria seems to be in the protection of these organisms from phagocytosis. Williams et al (1983) found that in the absence of specific antisera, encapsulated K.aerogenes strains were far more resistant to phagocytic uptake than non-capsulated mutants. Work by Allen et al (1987) has confirmed these observations in a K.aerogenes K21 clinical isolate. This effect has also been demonstrated in Pseudomonas aeruginosa (Meshulam et al, 1984), Bacteriodes fragilis (Connolly et al, 1984; Reid and Patrick, 1984) and in E.coli (Verweij-Van Vught et al, 1984). Hence, loss of K-antigen may render an organism suseptible to phagocytosis following opsonisation by complement (Williams et al, 1983, 1986; Simoons-Smit et al, 1986). This resistance effect of encapsulated bacteria in non-immune serum appears to be the result of the capsule interfering with the ability of complement to opsonize either by blocking activation and subsequent binding of C3b to

surface components or by burying bound and activated complement within the capsular material (Joiner et al, 1984), thereby denying its exposure to polymorphonuclear leucocytes.

Bacterial cell interaction with phagocytes and opsonins is known to be affected by the physiochemical properties of the bacterial cell surface and, in particular by its degree of hydrophilicity (Stendahl, 1983). Loss of either the K or O antigen may influence these properties in Klebsiella, although only the K-antigen protected the organism from phagocytosis in the presence of complement (Ciurana et al, 1987).

Surface charge has been postulated, in a number of bacteria, to inhibit contact between the bacterial cell and phagocyte (Stendahl, 1983). Both the capsule of Klebsiella type K21b and the polysaccharide colanic acid have been shown by partitioning experiments in aqueous two-phase polymer systems, to confer a strong negative charge on the surface of the cell. However, only the Klebsiella capsule was shown to provide almost complete resistance to phagocytic uptake (Allen et al, 1987) although this may also be a function of the form of attachment of the capsule, the colanic acid being more loosely fixed (Sutherland, 1977). Indeed, it has also been observed that different capsules may confer different anti-phagocytic properties (Glynn and Howard, 1970).

Van Oss and Gilman (1972) measured the angle of contact between flat layers of bacteria and drops of saline and, using this technique, found a correlation between the surface properties of bacteria and their degree of phagocytosis by polymorphonuclear leucocytes. Pathogenic bacteria tended to have a lower contact angle than that of the polymorphonuclear leucocyte. The ability of bacterial capsules to lower this contact angle may be responsible for their anti-phagocytic properties.

Upon contact with the polymorphonuclear leucocyte, the bacterium becomes engulfed by it, and comes to lie within a phagosome. Fusion of the phagosome with a lysosomal granule forms a phagolysosome and bacteria are subjected to a range of oxygen-dependent and independent destructive mechanisms. Since the oxygen-dependent processes are dominant, there is an associated dramatic increase in oxygen consumption and stimulation of the hexose-monophosphate shunt. Chemiluminescence-based detection (Allen et al, 1977) takes advantage of this shift in oxygen use and is often used to indirectly quantitate phagocytosis using luminol to enhance the reaction.

The Klebsiella K antigen has also been shown to play a role in resisting phagocytic killing (Allen et al, 1987). The non-capsular derivative also showed some level of resistance to intracellular degradation and was far less susceptible than the E.coli DH1 (a rough strain) which may emphasize an involvement for lipopolysaccharide in this type of resistance.

Specific antibody is generally necessary for effective opsonisation to occur (Robbins et al, 1980). The specific antibody need not be anti-capsular since anti-LPS antibodies have also been shown to be effective in opsonizing encapsulated klebsiellae of serotype K2 (Williams et al, 1983). This suggests that the K-antigen does not completely prevent access of antibody directed against the O-antigen. This has subsequently been confirmed in other studies, notably by Meno and Amako (1990) and by Williams et al (1988). This latter group has also shown however, in an earlier study, that the K1 capsular antigen may exert a potential masking effect over the anti-O antibodies. They described, using immunoabsorption studies, how anti-O sera failed to opsonize a clinical isolate ($K1^{+}$ $O1^{+}$) possessing lipopolysaccharide immunologically identical to that of the K2 strain, unless the amount of capsule was physically reduced. The anti-O antibodies were bound, but not situated at the outermost point of the cell surface, thus failing to promote

phagocytosis. This line of thought is also supported by Kadurugamawa et al (1988) who have shown that klebsiellae have the capacity to be agglutinated by anti-LPS antisera, but only in the presence of subinhibitory amounts of cephalosporins which serve to reduce capsule formation. When taken together, this would suggest a possible role for capsule size, specific capsule type, or both, as important virulence determinants.

1.4.1.3 Role of capsule size

Ehrenworth and Baer (1956) demonstrated that capsule size and its rate of production were important factors in determining the pathogenicity in mice of a K.pneumoniae K2 type strain. Cryz et al (1984) showed that in a mouse burn wound model, a highly encapsulated strain of K.pneumoniae was far more virulent than colonial variants possessing smaller capsules. A distinct correlation between capsule size and pathogenicity has also been observed in a rat lobar pneumonia model (Domenico et al, 1982). Heavily encapsulated strains of K.pneumoniae capsular serotype K1 have also been shown to cause severe metritis in horses. Interestingly a less heavily encapsulated strain caused only a very mild metritis and a non-capsular strain none at all (Kikuchi et al, 1987). No attempt was made however, to analyse other cell surface components with regard to their consistency between strains.

1.4.1.4 Role of capsular serotype

A number of reports have also described a correlation between Klebsiella capsular serotype and virulence as observed in a variety of animal models. Cryz et al have found that only serotypes K1 and K2 are virulent in burn-wound sepsis (Cryz et al, 1984), lobar pneumonia (Cryz et al, 1986) and immunosuppression models (Cryz et al, 1983). These serotypes, together

with strains representative of serotypes K4 and K5 were also found to be far more virulent in a murine model (Simoons-Smit et al, 1984) than all other serotypes tested. This particular model defined a virulent strain as having the ability to persist and multiply in experimentally induced skin lesions in mice. In a further study, eighty-two strains of varying origins, all belonging to the O1 group but to different K groups, were tested for virulence for mice by intraperitoneal inoculation (Mizuta et al, 1983). Of these, only members of the K2 serotype proved to be highly virulent, that is the 50% lethal dose was less than 10 colony-forming units. However, two strains were judged avirulent in spite of the fact that they possessed K2 capsules as revealed by the quellung reaction, indicating that other factors were necessary for full expression of virulence. Of the K1:O1 type strains tested, a small number were shown to be moderately virulent, with the remainder being of low or no virulence. All strains exhibiting K antigens 3 to 82 were found to be of very low virulence or were avirulent. In each of the latter two studies, no correlation was observed between virulence in mice and the clinical site of isolation in human patients.

1.4.1.5 Interference with opsonisation by capsular polysaccharide

Although highly opsonic in vitro, the protective effect of anti-Klebsiella CPS antibody can sometimes be negated. It has been proposed that Klebsiella capsular polysaccharide interferes with opsonisation mediated by anti-CPS antibody at several different levels (Cryz, 1990). Firstly a proportion of the CPS may become physically detached from the cell when anti-CPS antibody is bound. Because this CPS is no longer cell-bound, the cell cannot be opsonized or subsequently phagocytosed. Virulent strains release large quantities of cell-free extracellular capsular polysaccharide into the surrounding culture medium (Domenico and Straus, 1985). This

extracellular CPS may bind anti-CPS antibody at a distance far removed from the cell, thereby nullifying its effect. This role for CPS could conceivably be supported by the study of Pollack (1976) who demonstrated that high levels of circulating capsular antigen (antigenemia) in patients seemed to correlate well with the severity of infection, with persistence of active infection areas and with a poorer prognosis than in patients without antigaenemia. Reductions in the amount of capsular polysaccharide produced in vitro can be accomplished via the addition of sodium salicylate (Domenico et al, 1989). The concentrations required are apparently physiologically achievable in humans and within the therapeutic range of aspirin, thus providing a possible means to reduce the drastic effect of Klebsiella CPS.

Other effects of Klebsiella capsular polysaccharide include:-

i) Interference with the protective immune response

Orskov (1956) found that the more heavily encapsulated strains were, the lower the level of homologous antibodies produced during an infection. This phenomenon was also apparent when crude preparations of CPS were injected into mice (Batshon et al, 1963; Baer et al, 1954) and was thought to be caused either by inhibition of antibody production itself or by neutralisation of tissue-fixed polysaccharide.

ii) Macrophage development and function

The generation of macrophages from precursor cells in an in vitro cultivation system was markedly suppressed by the addition of this polysaccharide (Yokochi et al, 1977; 1979), although the precise mechanism of action was not completely clear. The CPS preparation was not cytotoxic for any major spleen cell population.

iii) Adjuvant effect

Purified CPS from klebsiellae is believed to have a strong adjuvant effect on antibody responses to various antigens in mice (Ohta et al, 1979)

and can act as a polyclonal B-cell activator (Nakashima and Kato, 1974). Furthermore, CPS has interferon-inducing (Kato et al, 1975) and infection-promoting effects (Kato et al, 1976). This latter action is believed to be caused by an inhibition of phagocyte intracellular anti-bacterial activity.

iv) Biofilm formation

The CPS may be instrumental in promoting the formation of biofilms, an important factor in the later stages of colonisation of mucosal surfaces (Williams and Tomas, 1990).

v) Barrier to water loss

During passage between hosts, the capsule prevents dessication of the cell. Indeed, encapsulated klebsiellae seem to survive on skin for longer periods than non-capsulated strains (Hart et al, 1981).

Recently, Nassif et al (1989), described a 180-kilobase plasmid which was believed to be responsible for the observed virulence of K.pneumoniae K1 and K2 isolates. The plasmid directs the synthesis and expression of a mucoid phenotype in klebsiellae which was observed to correlate well with virulence. The substance responsible for the mucoidy was shown to be neither the Klebsiella capsular polysaccharide nor colanic acid.

1.4.2 Lipopolysaccharide

Lipopolysaccharide is a major constituent of the outer membrane and is unique to Gram-negative bacteria. It is composed of three regions: a lipophilic region (lipid A) covalently attached to a rough core oligosaccharide which is, in turn, covalently bound to a polysaccharide side chain, the O-antigen. This is the portion of the molecule which determines serotype specificity. Some eight chemically distinct antigens have so far been recognised in Klebsiella species (Mizuta et al, 1983) with the O1 serotype being the most common in clinical isolates (Orskov and Orskov, 1978).

Bacteria whose LPS contains the O-antigenic side-chain are referred to as smooth, whereas those lacking this component are known as rough, owing to their colonial morphology.

While there is still some degree of controversy concerning the function of the K-antigen in resistance of klebsiellae to the effects of complement-mediated serum killing, there seems to be little doubt that the lipopolysaccharide plays a crucial role. Williams et al (1983) found that serum, via both the classical and alternative pathways, was bacteriostatic for a smooth, encapsulated mutant ($O1^{+}K2^{-}$) and rapidly bactericidal for a rough encapsulated mutant ($O1^{-}K2^{-}$), both of which were derived from the same smooth encapsulated parent ($O1^{+}K2^{+}$). They suggest, therefore, that the O-antigen confers a high level of protection against the killing effect of serum.

In contrast, the results of other studies show that LPS and, in particular, the O-antigenic side chain is the sole mediating factor of serum resistance in strains of this species (Tomas et al, 1986; Ciurana and Tomas, 1987). Specifically, they discovered that, unlike low-molecular weight LPS, high-molecular weight LPS from serum-resistant strains of Klebsiella could completely inhibit the serum bactericidal activity. These findings have been corroborated by McCallum et al (1991) who also suggested that the O-antigen chain length and its ability to bind complement (C3b) at a distance away from the outer membrane, as in Salmonella montevideo (Joiner et al, 1986) may be the crucial factor. McCallum et al (1991) do not however, dismiss a structural alteration as being the source of the extra protection endowed by the high molecular weight LPS, as has been shown in other salmonellae (Grossman and Leive, 1984; Liang-Takasaki et al, 1983). Although LPS is not thought to interfere with phagocytic uptake, it may, as mentioned previously, allow longer intracellular survival subsequent to phagocytosis (Allen et al,1987)

Absorption of sodium ions in tracheal epithelium may also be affected by LPS from K.pneumoniae (Tamaoki et al,1991). It has been postulated that when a bacterial infection causes damage to the airway epithelia, LPS may interact with the sub-mucosal membrane and lead to inhibition of sodium ion absorption, possibly resulting in hypersecretion of airway surface fluid.

1.4.3 Extracellular toxic complex

K.pneumoniae is capable of causing a lobar pneumoniae in normal animals (Domenico et al, 1982), characterised by extensive pulmonary tissue destruction. Mizuta et al (1983) suggest that something other than cell-associated material may be involved in virulence and Straus and co-workers have implicated an "extracellular toxic complex" (ETC) as an important factor in klebsiellae infections (Straus et al, 1985; Straus, 1987). Isolates of both serotypes K1 and K2 were shown to produce the material which is composed of capsular polysaccharide (56-79%), lipopolysaccharide (14-33%) and protein (4-10%) during all phases of growth. When infected intraperitoneally, the purified ETC was lethal for mice and was capable of producing the same extensive pulmonary pathology as seen in an active Klebsiella pneumonia. The toxic effect of the complex was attributed to its LPS fraction, since the toxicity was not destroyed by boiling or exposure to proteolytic enzymes, but was completely removed after saponification. Immunisation with sub-lethal quantities of ETC was able to afford complete protection against homologous and heterologous challenge, possibly due to antibody directed toward the O1 LPS antigen which was common to all strains used. Further, Straus (1987) goes on to envisage the normal course of a K.pneumoniae infection as being determined initially by the size of the capsule and the immunological status of the animal. If early infection is established, release of extracellular LPS may lead to a depression of the

reticulo-endothelial system and the organism will multiply freely. Once sufficient numbers of bacteria exist, they will produce large quantities of the ETC which in turn kills the host.

1.4.4 Pili and mannose-inhibitable adhesins

As in many other bacterial species, the existence of pili in Klebsiella would seem to be a major factor in mediating bacterial attachment to mucosal surfaces, a necessary step if an infection is to become established. Fader and Davis (1980; 1982) have shown that type 1 pili (mannose-sensitive), found on the surface of more than 80% of clinical isolates (Przondo-Hessek and Pulverer, 1983), promote adherence to the surface epithelium of rat bladders and that piliated strains cause a far greater degree of renal damage than do non-piliated strains in a rat pyelitis model. Furthermore, Maayan et al (1985) demonstrated that fimbriate klebsiellae were positively selected for from a mixed population of fimbriate and non-fimbriate phenotypes following intravesicular inoculation of mice. Genetic analysis has shown that two antigenically distinct forms of type 1 fimbriae exist (Gerlach and Clegg, 1988a), although no attempt has so far been made to correlate these forms with virulence.

Most strains of K.pneumoniae also produce a mannose-resistant type 3 pilus (Przondo-Hessek and Pulverer, 1983) which has been reported to facilitate tanned erythrocyte agglutination as well as bacterial adherence to yeast cells and plant roots (Duguid, 1959; Korhonen et al, 1983). Both type 1 and type 3 pili are expressed in encapsulated strains; indeed, type 3 pili may actually be anchored into position by the capsular matrix (Gerlach and Clegg, 1988b)

Pruzzo and colleagues (1980) have discovered that outer membrane proteins acting as receptors for coliphage T3 and T7 can also serve as a

mannose-inhibitable adherence (MIAT) mechanism bringing about binding to epithelial cells. This seems to protect the bacteria from phagocytosis and intracellular killing by polymorphonuclear leucocytes, thus enhancing their virulence. Moreover, MIAT-negative derivatives of MIAT-positive strains were approximately 60-fold less pathogenic, had lost their ability to colonize epithelia and were more susceptible to phagocytosis (Pruzzo et al, 1982). Passive immunisation with antibody against MIAT-positive bacteria was also found to be more protective than antisera derived from immunization against MIAT-negative cells. However, the MIAT system has so far only been found in unencapsulated strains and hence the extent to which it is involved in human Klebsiella disease, where almost all isolates are encapsulated, is unclear at the present time. One recent report has characterised the protective capacity of outer membrane proteins from a non-capsular derivative of an encapsulated parent strain (Serushago et al, 1989). The involvement of the MIAT proteins was not determined.

1.4.5 Iron-sequestering mechanisms

Some strains remain avirulent in spite of possessing the K1 or K2 capsular serotype and the O1 or other O-antigens, suggesting that other factors may be involved in virulence. Work by Nassif et al (1986) has correlated the virulence of some K1 and K2 isolates with the presence of a 180-kilobase plasmid which encodes the production of the hydroxymate siderophore aerobactin and its receptor protein. Aerobactin and its associated receptor form an iron-scavenging system which is produced by a number of enterobacteria. Only strains which carried this plasmid were virulent. When the aerobactin genes were introduced into avirulent isolates, their virulence was increased 100-fold as judged by a mouse lethality test.

K.pneumoniae has also been reported to produce enterochelin (a phenolate siderophore) as well as a number of iron-repressible outer membrane proteins (Williams *et al*, 1984; 1987). These systems can be used to overcome the condition of iron deprivation that is generally found in infected animal tissues (Bullen and Griffiths, 1987).

1.4.6 Enterotoxins and haemolysin

Klebsiellae are known to produce heat-labile (LT) and heat-stable (ST) enterotoxins (Klipstein *et al*, 1983).

Albesa (1989) has identified a thiol-activated haemolysin produced by a number of *K.pneumoniae* strains which behaved similarly in some ways to streptolysin O. Both of these extracellular proteins may be involved in the pathogenicity of klebsiellae.

1.5 The Structure of *Klebsiella* Capsules

Klebsiella capsules are mainly composed of water (99%) which hydrates a heteropolymeric polysaccharide (Sutherland, 1972), thus expanding the space that would be occupied by the dehydrated cell.

The fine structure of *Klebsiella* capsular polysaccharide has been demonstrated using electron microscopy and was initially shown to consist of a layer of thin filaments extending from the outer membrane and surrounding the cell (Springer and Roth, 1973). Further analysis has revealed two distinct layers of capsule (Amako et al, 1988). In the inner layer the filaments extend outward, lining up almost parallel to one another, while in the outer layer the fibres intertwine with each other to form the capsular matrix.

Detailed structural studies have been carried out, using a wide variety of chemical techniques, on the capsular material of most of the 77 *Klebsiella* K serotypes (Sutherland et al, 1977; Kenne and Linberg, 1983). They have

been found to be acidic polysaccharides composed of a repeating oligosaccharide unit, the vast majority of which contain glucuronic acid linked to hexose and 6-deoxyhexose sugars. Some serotypes are also known to contain pyruvate, acetyl or formyl groups. The repeating units of different serotypes are usually similar in that they contain a three (K2; Gahan et al, 1967), four (K21; Choy and Dutton, 1973) or five unit sugar backbone (K44; Dutton and Folkman, 1980) with a single or double sugar side chain. However, serotype K5 contains only a three sugar repeat unit with no side chain (Dutton and Yang, 1972). Despite the fact that only a relatively small number of individual monosaccharides are involved, different arrangements of these components allow for a large number of alternative structures to be constructed. Inevitably though, some epitopes are similar, leading to immunological cross-reactions between serotypes such as K2 and K13.

As has been mentioned previously, the K2 capsule is one the most frequently encountered serotypes. This particular serotype contains a 3 sugar unit backbone consisting of 2 units of glucose on either side of, and linked by 1-β-4 bonds to a single mannose unit. Branching from the mannose, and linked through a 1-α -3 bond is a glucuronic acid molecule (Figure 1.1).

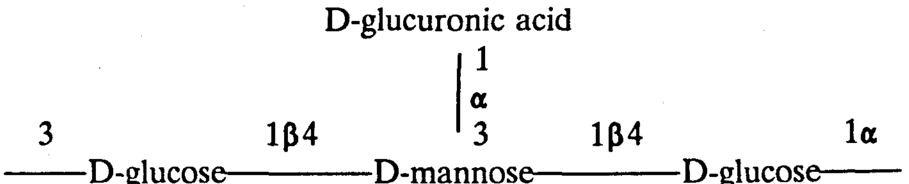


Figure 1.1: Structure of the repeating unit of Klebsiella pneumoniae type 2 capsular polysaccharide.

1.6 Biosynthesis of capsular polysaccharide in Klebsiella

The central role of undecaprenyl phosphate as the phospholipid carrier in the biosynthesis of capsular polysaccharide in K.aerogenes was discovered by Troy et al (1971). In the strain investigated the repeating unit was found to be a tetrasaccharide. A particulate enzyme complex catalyses the incorporation of the constituent sugar nucleotide derivatives into the oligosaccharide unit and subsequently into the lipid carrier fraction (Figure 1.2). Undecaprenyl phosphate initially takes up a single galactosyl phosphate molecule and then mannose, glucuronic acid and a second galactose moiety are added in turn to form the lipid-linked tetrasaccharide. This is then polymerised to form the native capsular polysaccharide. Work by Sutherland and Norval (1970), using mutants blocked at different stages of polysaccharide production has confirmed that the lipid-linked sugars are involved in capsular polysaccharide biosynthesis in K.aerogenes. The lipid carrier is also involved in other areas where a particular unit structure is repeated throughout a macromolecular product, notably in lipopolysaccharide (Wright et al, 1967) and peptidoglycan (Higashi et al, 1967).

Undecaprenyl phosphate was also postulated to play an important role in the biosynthesis of the E.coli K1 capsular polysaccharide (Troy, 1979). Recent studies on the E.coli K5 polysaccharide, however, have suggested that a lipid intermediate is not involved (Boulnois and Jann, 1989).

Figure 1.2 Steps involved in the biosynthesis of capsular polysaccharde from <u>K.aerogenes</u> (Troy et al, 1971).

(i) UDP-Gal + P-lipid $\rightleftharpoons$ Gal-P-P-lipid + UMP

(ii) Gal-P-P-lipid + GDP-Man $\longrightarrow$ Man-Gal-P-P-lipid + (GDP)

(iii) Man-Gal-P-P-lipid + UDP-GlcA $\longrightarrow$ GlcA-Man-Gal-P-P-lipid + (UDP)

(iv) GlcA-Man-Gal-P-P-lipid + UDP-Gal $\longrightarrow$ Gal-(GlcA)-Man-Gal-P-P-lipid + (UDP)

1.7 Genetic Analysis of Capsule Production

The genes responsible for the production of the polysaccharide capsule and exopolysaccharide have been cloned from a number of different bacterial species including E.coli serotypes K1 (Silver et al, 1981; Echarti et al, 1983), K4 (Drake et al, 1990), K5, K7, K12, and K92 (Roberts et al, 1986); Neisseria meningitidis group B (Frosch et al, 1989) and Haemophilus influenzae type b. In addition, a great deal of work has been done in attempting to analyse the genetics of capsule biosynthesis in type III group B streptococci and Xanthamonas campestris pv. campestris (Hotte et al, 1990) while somewhat less emphasis seems to have been placed upon klebsiellae.

1.7.1 Genetics of capsule production in E.coli

The genes encoding the E.coli K1 capsular polysaccharide are to be found within a 17kb fragment and are arranged into three distinct functional regions (Boulnois et al, 1987).

1.7.1.1 Region 1

Region 1 (8kb) is believed to encode functions responsible for transport of the fully formed polysaccharide across the outer membrane and its subsequent expression on the outer surface of the cell (Boulnois and Roberts, 1990; Kronke et al, 1990). Mutations in this region will result in the intracellular (periplasmic) accumulation of the mature polysaccharide.

Minicell analysis has revealed that five proteins, ranging in size between 38kD and 74kD are determined by region 1 (Boulnois and Roberts, 1990). It

has been speculated that the 38kD protein may be responsible for dispersal of newly exported polysaccharide and its organisation into a capsule (Boulnois and Jann, 1989).

This region is also thought to be important during the onset of polymer biosynthesis via the production of CMP-KDO synthetase. Studies have indicated that the gene for CMP-KDO synthetase, the enzyme forming CMP-KDO, lies in region 1 (Finke et al, 1989). It has been postulated that the transfer of KDO from CMP-KDO to an acceptor initiates synthesis of group II polysaccharides (Finke et al, 1989). If the CMP-KDO gene is co-regulated along with export functions, then polysaccharide biosynthesis may be coupled to the export process (Boulnois and Jann, 1989).

Much of the information concerning the role of region 1 has been gleaned from studies on the genetics of E.coli K5 and K7 polysaccharide production, although the conserved nature of this region in all serotypes so far

tested implies that the same should hold true for the K1 capsule. Indeed, complementation studies have revealed that these transport functions may be transferred between different serotypes with no loss of activity (Roberts et al, 1986), thus suggesting that a common export system is in place for each of the E.coli group II antigens.

Deletion analysis has revealed that Bayer junctions, sites of contact between the outer and inner membranes, may represent the points of transport of the capsular material from the cytoplasm to the outside of the cell (Kröncke et al, 1990).

1.7.1.2 Region 2

This region is believed to be specific for each serotype and is thought to encode the genes responsible for biosynthesis and polymerisation of the polysaccharide (Boulnois et al, 1987). Mutations in this region result in the absence, both intracellularly and extracellularly, of capsular polysaccharide. The size of respective region 2 determinants varies according to the degree of complexity of the final polysaccharide. For example the K4 polysaccharide, a fructose substituted chondroitin, has a relatively complicated structure and indeed has a far larger region 2 than other serotypes thus far investigated (Drake et al, 1990). The degree of relatedness between the repeating units of different serotypes is also reflected in the nucleotide sequences of this region. In particular K1 and K92 serotypes are highly related and their region 2 determinants share a number of restriction sites (Roberts et al, 1986) whereas the region 2 of K1 and K4 serotypes bear little, if any, resemblance to one another (Roberts et al, 1986; Drake et al, 1990).

1.7.1.3 Region 3

Region 3, in common with region 1, also seems to be conserved in all serotypes so far tested. Mutations in this region will also lead to the build-up of intracellular capsular polysaccharide, although this material was found to be shorter than the mature polymer, at least in the K5 serotype (Boulnois et al, 1987; Roberts et al, 1988; Kröncke et al, 1990). Initially, there was some confusion concerning the function(s) of region 3, except that it was probably involved in post-translational modification of the polysaccharide. However, recent work has identified two genes, kpsM and kpsT, the products of which have been proposed to act in the translocation of the growing polysaccharide chain across the cytoplasmic membrane in an energy-requiring process (Smith et al, 1990; Boulnois and Jann, 1990).

1.7.2 Genetic comparison of capsule production in other species

The complete repertoire of genes necessary for the production and expression of the N.meningitidis group B polysaccharide capsule have been cloned in E.coli GC6 and have been found to lie within a 24 kb DNA fragment (Frosch et al, 1989). In spite of the fact that the E.coli K1 serotype and N.meningitidis group B capsules are chemically and immunologically identical, there appears to be no sequence homology between them (Echarti et al, 1983).

Despite differing DNA sequences, the organisation of the gene complexes for capsule synthesis in N.meningitidis, E.coli and furthermore, in H.influenzae (Kroll et al, 1988) seems to be very similar, with each consisting of a number of distinct regions, as judged by insertion analysis, possibly suggesting common origins and, or gene mobility (Boulnois and Jann, 1990).

In particular, it appears that a conserved mechanism exists for the energy-dependent export of capsular polysaccharide to the surface of H.influenzae and E.coli with BexA/BexB and KpsT/KpsM playing equivalent roles (Kroll et al, 1990; Smith et al, 1990).

Two other open reading frames have been described within the H.influenzae bex region neither of which presently has an analogue in E.coli. The bexC gene product, BexC, is believed to be a periplasmically orientated component of a capsular polysaccharide exporter, perhaps contributing to a pore or carrier mechanism involved in transferring growing polysaccharide from cytosol to outer membrane (Kroll et al, 1990). A possible role for the bexD gene product, BexD, remains to be defined.

1.7.3 Genetics of capsule production in Klebsiella

There is generally a paucity of information on the genetic mechanisms which underpin the biosynthesis of capsular polysaccharide in Klebsiella and the gene system responsible has yet to be fully characterised.

It is usually held that the genetic determinants of K antigen production are chromosomally encoded (Laakso et al, 1988). This group used an RP4::mini-Mu chromosome-mobilising system to transfer the full complement of genes necessary for Klebsiella K20 capsular antigen production to an unencapsulated E.coli K12 strain. The capsule expressed by the E.coli was identified as being chemically and immunologically identical to the Klebsiella capsular polysaccharide using a specific monoclonal antibody and a capsule depolymerase enzyme.

However, Barr (1981) found that some K21 isolates spontaneously lost the ability to elicit a capsule, a phenomenon which was enhanced by treatment with acridine orange, a plasmid curing agent. Moreover it was discovered that the ability to synthesize this antigen could in fact be transferred, by conjugation, to E.coli thus adding weight to the argument that the capsule genes were actually plasmid encoded.

Other observations by Moore, Hart and Saunders (unpublished) suggest that a chromosomally encoded mucoid phenotype could be mobilised via a Tn7 element and transferred from a Klebsiella K36 isolate to E.coli although the mucoidy was not immunologically or chemically identifiable as an authentic Klebsiella capsule.

Lysogeny of some strains of Klebsiella with certain bacteriophage has been shown to bring about dramatic alteration of capsular phenotypes (Pruzzo

and Satto, 1988). Lysogens of a K59 serotype were found to lack the original capsular antigen and to express new, non-typeable capsules.

Allen et al (1987) cloned two genes from the chromosome of a K.aerogenes K21 clinical isolate, designated rcsA and rcsB, which were capable of activating the synthesis and expression of colanic acid in E.coli K12 (DH1). The gene rcsB was not capable of inducing the mucoidy on its own, but acted in conjunction with rcsA to allow production of the colanic acid at temperatures between $30^{0}$and 37^{0}C. The rcsA gene only induced the mucoidy at 30^{0}C. These results were explained by hypothesising that the rcsA gene product (a polypeptide of 23kDa) may function as a positive regulator of colanic acid biosynthesis which is subject to the activity of the Lon protease, which may itself be regulated by the rcsB gene product. However, it was not known whether either of these genes were actually involved in the expression of the Klebsiella polysaccharide.

Work by Nassif et al (1989) uncovered the existence of two other genes which seemed to play a role in regulating the production of the chromosomally encoded mucoid structure, a phenotype apparently distinct from the polysaccharide capsule. These genes, rmpA and rmpB, were actually plasmid encoded and were isolated from a K2 serotype strain. The rmpA and rmpB genes seemed to exhibit a number of functional similarities to rcsA and rcsB, although differences do exist in protein product size and respective amino acid sequences. It has been proposed by Nassif and co-workers that the capsular polysaccharide production in Klebsiella is regulated by the rcs system while the mucoid phenotype is controlled by the rmp genes.

Recently it has been demonstrated that the rcsA gene is indeed involved in the expression of capsular polysaccharide in Klebsiella (McCallum and Whitfield, 1991). The rcsA gene was cloned using a cosmid vector from a K20 serotype isolate and was found to restore fully the capsular

antigen producing capabilities of a mutant which would normally only synthesize minute amounts of the polysaccharide. Unfortunately however, mutants which were fully deficient in the production of capsular polysaccharide could not be complemented.

E.coli also carries the rcsA gene group which acts in the regulation of expression of extracellular polysaccharide (Gottesman et al, 1985; Brill et al, 1988) as do Erwinia amylovora and Erwinia stewartii (Coleman et al, 1990; Torres-Cabassa et al, 1987). There also seems to be extensive homology between the rcsA genes of these different bacteria and it may be that the proteins produced by these genes have a common function and perhaps, a common origin. There did not however, appear to be any homology between the predicted rcsA gene and other DNA-binding activator proteins, suggesting that the regulatory effect may be exerted through other intermediate regulatory proteins.

The transcription of genes necessary for the synthesis of colanic acid in E.coli is regulated by at least two other genes, rcsB and rcsC in addition to rcsA (Brill et al, 1988). Both gene products RcsA and RcsB are believed to act as positive regulators. RcsA is normally limiting for capsule expression, its availability being limited by Lon protease. RcsB activity or availability may be directly controlled by RcsC, a possible environmental sensor.

1.8 Alternative Strategies to Isolate Genes Involved in Klebsiella Capsular Polysaccharide Biosynthesis

The direct cloning of the complete gene repertoire necessary for the full expression of serospecific Klebsiella capsules in E.coli has been attempted in these laboratories. However, despite the use of numerous different strategies, it has so far proved unsuccessful (Jones and Roe, pers. comm.; Allen, pers. comm.).

It has been suggested that the genes required for the biosynthesis of the Klebsiella capsule may be scattered on the chromosome therefore preventing the direct cloning of these genetic determinants (Allen Ph.D Thesis, 1987). If this is the case then an alternative approach would be to use the technique of transposon mutagenesis.

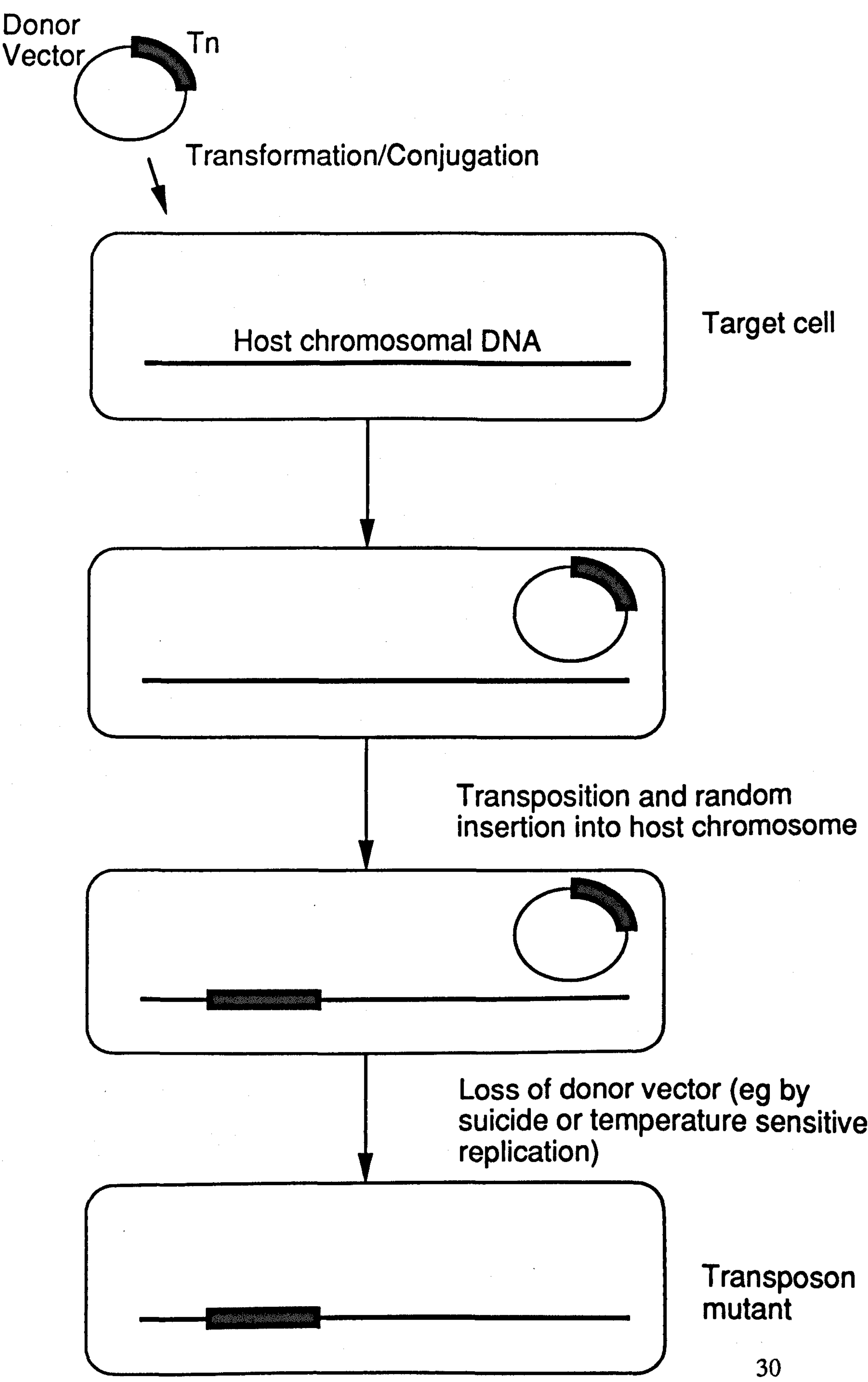
The basic principles of this procedure are presented in Figure 1.3. There are a number of characteristics which contribute to the usefulness of transposon mutagenesis, including:-

i) Insertion of the transposon into a structural gene will generally lead to a specific inactivation of that particular gene.
ii) Transposon DNA usually carries antibiotic resistance genes which can allow mapping, transduction and cloning of the mutated gene.
iii) Such insertions are usually non-leaky and only revert at very low frequencies.

Transposon mutagenesis has been used to help overcome a wide range of genetic problems by virtue of its ability to introduce precise, mappable mutations into a large number of different bacterial species (Bopp et al, 1991; Lam et al,1987; Goldberg et al; 1990). In Klebsiella, the technique has been widely used in the genetic analysis of nitrogen fixation (Merrick et al, 1978), histidine utilization (Schwacha et al, 1990) and alginate lyase production (Gacesa et al, 1987) while Rubens et al, (1987) have successfully exploited the regime in studies of capsule expression in type III group B streptococci.

During the final stages of this work, Arakawa et al (1991) have published details of the successful cloning of the structural genes for the biosynthesis of K2 capsular polysaccharide. The recombinant plasmid involved, pCPS7B06, contains a sufficient portion of the Klebsiella cps gene cluster to allow the restoration of K2 capsular polysaccharide production in non-capsular mutants of a K2 serotype parental strain. It also encodes the genes responsible for the expression of K2 capsular polysaccharide on the cell surface of non-capsular Klebsiella mutants of other capsular serotypes. However, E.coli HB101 (pCPS7B06) required the presence of the rmpA gene for the production of Klebsiella K2 capsular polysaccharide.

Figure 1.3: Basic principles of transposon mutagenesis (using chromosome as target DNA).



The 23kb chromosomal DNA fragment which carried the required genetic information was actually isolated from a non-capsular mutant (NM7) of K.pneumoniae strain Chedid, a K2 serotype isolate. Thus, those non-capsular mutants complemented by pCPS7B06 must themselves contain intact copies of the gene (designated gene X by Arakawa et al,1991) disrupted in K.pneumoniae NM7.

This work also demonstrated, through hybridisation analysis, the relatively conserved nature of the organisation of the K2 cps gene cluster among a number of K.pneumoniae strains.

1.9 Objectives of this work

Despite recent advances, the genetic mechanisms by which the capsular polysaccharide of K.pneumoniae is synthesized and expressed remain unresolved. The objectives of this project were firstly to develop a transposon mutagenesis system for use with Klebsiella clinical isolates and to use it to generate mutants defective in capsular synthesis and expression. Secondly it was proposed to use the non-capsular mutants to facilitate a greater understanding of this complex system using molecular genetic and immunological techniques.

CHAPTER 2: Materials and Methods

2.1 Materials

2.1.1 Antibiotics

Antibiotics were obtained from Sigma Chemical Company Ltd. Stock solutions of antibiotics were filter sterilized and stored at -20°C.

2.1.2 Chemicals

All chemicals were supplied by BDH Ltd. or Sigma Chemical Company Ltd unless stated otherwise. Chemicals were of analytical or reagent grade.

2.1.3 Enzymes

Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase and random hexanucleotide DNA labelling kits were obtained from Boehringer Mannheim GmbH. Lysozyme, RNase A and proteinase K were supplied by Sigma Chemical Company Ltd.

2.1.4 Miscellaneous

Agarose (medium electroendosmosis grade) and low melting point agarose were supplied by FMC Corporation and Bethesda Research Laboratories respectively. Nitrocellulose filters for DNA hybridisations were supplied by Schleicher and Schull GmbH (BA 85, 0.45 μm pore size). Polaroid type 665 positive/negative films were used for photography of agarose gels. Kodak X-Omat S or Fuji Nif Rx X-ray film was used for autoradiography of radioactive filters. X-ray film developer (Ilford PQ Universal Developer) and fixer (Ilford Hypam Fixer) were obtained from Ilford Ltd.

Radiolabelled compounds and the λ DNA in vitro packaging system were supplied by Amersham International.

Visking dialysis tubing was supplied by Gallenkamp.

Standard chromatography and 3MM papers were obtained from Whatman Ltd.

2.1.5 Growth media

Growth media were supplied by London Analytical and Bacterial Media (Lab M) Ltd. or Difco Laboratories. All salts in the media were anhydrous unless stated.

(a) Liquid Media

(i) Nutrient Broth

Lab M Nutrient Broth No. 2	25 g
Distilled water to	1000 ml

(ii) Luria broth

Difco Bacto tryptone	10 g
Difco Yeast extract	5 g
NaCl	5 g
Distilled water to	1000 ml

(iii) Minimal media

Na_2HPO_4	6 g
KH_2PO_4	30 g
NaCl	0.5 g
NH_4Cl	1 g
Distilled water to	1000 ml

After autoclaving $MgSO_4.7H_2O$ was added to 10 mM and $CaCl_2$ to 1mM.

(b) Solid media

(i) Nutrient agar

Lab M Nutrient broth No.2	25 g
Lab M Agar No.2	12 g
Distilled water to	1000 ml

(ii) MacConkey agar

MacConkey agar	51.5 g
Distilled water to	1000 ml

(iii) Worfel Ferguson agar

Bacto Yeast Extract	2 g
$MgSO_4$	0.25 g
K_2SO_4	1 g
NaCl	2 g
Sucrose	20 g
Distilled water to	1000 ml

(iv) Luria agar

Difco Bacto tryptone	10 g
Difco Yeast extract	5 g
NaCl	5 g
Agar	15 g

(v) Minimal salts agar

Na_2HPO_4	6 g
KH_2PO_4	30 g
NaCl	0.5 g

NH_4Cl	1 g
Difco Bacto agar	15 g
Distilled water to	1000 ml

After autoclaving $MgSO_4$.7H2O was added to 10mM and $CaCl_2$ to 1mM.

(c) Supplements

Glucose was added to minimal media to give a final concentration of 0.2% (w/v). Amino acids were added, when required, to minimal media to give a final concentration of 0.0025%. Antibiotic supplements were added to media when required at the following concentrations : Ampicillin (100 μg ml^{-1}), chloramphenicol (40 μg ml^{-1}), kanamycin (100 μg ml^{-1}), tetracycline (12.5 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and rifampicin (100 μg ml^{-1}) unless stated otherwise.

The chromogenic indicator 5-bromo-4-chloro-3-indolyl-phosphate (XP) was incorporated into agar at a final concentration of 100 μg ml^{-1}.

(d) Sterilization of Media

Media was sterilized by autoclaving at 121°C and 15lbs $sq.in^{-1}$ pressure for 15 min. Supplements were sterilized seperately by passing through a 0.45 μm filter.

2.1.6 Buffers and solutions

(i) Tris-borate buffer

Tris	89 mM
Boric acid	89 mM
EDTA	2 mM

(ii) TE buffer

Tris-HCl	0.01 M
EDTA	0.001 M
	pH 8.0

(iii) Loading buffer (agarose gels)

EDTA	0.05 M
Urea	4.0 M
Ficoll	8.0% (w/v)
Bromophenol blue	0.1% (w/v)

(iv) 20x SSC solution

Tri-sodium citrate	0.3 M
NaCl	3.0 M
	pH 7.0

(v) Denaturation solution

NaOH	0.5 M
NaCl	1.5 M

(vi) Neutralising solution

Tris-HCl	1.0 M
NaCl	1.5 M
	pH 8.0

(vii) 50x Denhardt's solution

Ficoll	1.0% (w/v)

Polyvinylpyrrollidone	1.0% (w/v)
Bovine serum albumin	1.0% (w/v)

(viii) SM buffer

Tris-HCl	10 mM
NaCl	0.4% (w/v)
$MgSO_4$	10 mM
Gelatin	0.2% (w/v)
	pH 7.5

(ix) Phosphate buffered saline

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water to	100 ml
	pH 7.4

(x) CIE buffer

Tris	0.04 M
NaCl	0.4 M
EDTA	0.004 M
	pH 8.6

(xi) Final sample buffer

Glycerol	10% (w/v)
2-mercaptoethanol	5% (w/v)
SDS	3% (w/v)

Tris-HCl (pH 6.8)	0.0625 M
Bromophenol blue	0.01% (w/v)

2.1.7 Bacterial strains, plasmids and bacteriophages

Bacterial strains, plasmids and bacteriophages used in this work are listed in Tables 2.1, 2.2, 2.3 and 2.4.

Table 2.1 K.pneumoniae bacterial strains

Strain	Relevant Phenotype	Source/Reference
5055	K2 reference strain, Ap^S	National Collection of Type Cultures, London.
M10	$K2^-01^+$ derivative of 5055	Poxton and Sutherland, 1976.
M10B	$K2^-01^-$ derivative of 5055	Poxton and Sutherland, 1976.
5055-114	$K2^-$ derivative of 5055(Ap^S, Km^R)	This Work
5055-480	$K2^-$ derivative of 5055(Ap^S, Km^R)	This Work.
5055-797	$K2^-$ derivative of 5055(Ap^S, Km^R)	This Work.
5055-507	Ure^- derivative of 5055(Ap^S,Km^R)	This Work
BAH2819	K2 clinical isolate, Ap^R	Gift from Dr E.A.Roe
1L918	K21 " , Ap^R	Barr, 1989
2L260	K1 " , Ap^R	Gift from Dr E.A.Roe
2L261	K2 reference strain, Ap^S	"
2L262	K5 clinical isolate, Ap^R	"
2L263	K9 " , Ap^R	"
2L264	K11 " , Ap^R	"
2L265	K12 " , Ap^R	"
2L266	K17 " , Ap^R	"
2L267	K22 " , Ap^R	"
2L268	K23 " , Ap^R	"
2L269	K24 " , Ap^R	"
2L270	K25 " , Ap^R	"
2L271	K26 " , Ap^R	"
2L272	K29 " , Ap^R	"
2L273	K30 " , Ap^R	"
2L274	K32 " , Ap^R	"

2L275	K33 " , Ap^R	"
2L276	K34 " , Ap^R	"
2L277	K38 " , Ap^R	"
2L278	K39 " , Ap^R	"
2L279	K42 " , Ap^R	"
2L280	K46 " , Ap^R	"
3118	K2 " , Ap^R	"
24118	K2 " , Ap^R	"
KAY2026	P1^S, λ^R	Sprenger and Lengeler,1984
C604	K2 clinical isolate, Ap^R	Gift from Dr T.Pitt
C694	"	"
C847	"	"
C887	"	"
C1012	"	"
C1300	"	"
C2071	"	"
C2107	"	"
C2445	"	"
C3646	"	"
C3666	"	"

Table 2.2 *E.coli* strains

Strain	Relevant Phenotype	Sources/Reference
MM294	*thi*, *end*A, *hsd*R	Backman *et al*,(1976)
MCR106	*lam*B deletion, λ^-	Gift from Dr J.M.Pratt
C600	K12, F^-, *leu*B6, *thi*-1, *thr*-1,*lac*Y1, *ton*A21, *sup*E44, λ^-	Appleyard, 1954
LE392	F^-, *hsd*R514(r_K^-, m_K^+), *sup*E44, *sup*F58, *lac*, *gal*K2, *gal*T22, *trp*R55, *met*B1	Maniatis, 1982
DH1	K12, *rec*A, *hsd*, r_K^-, m_K^-	Hanahan, 1983
HB101	F^-, *hsd*S20(r_R^-, m_R^-), *rec*A13,*ara*14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20(Sm^r), *xyl*-5, *mtl*-1, *sup*E44, λ^-	Boyer *et al*, 1969
SK1590	*gal*, *thi*, *sbc*B15, *end*A, *hsd*R4, *hsd*M^+	Kushner, 1978
SM10λ*pir*	*thi*, *thr*, *leu*, *ton*A, *lac*Y,*sup*E, *rec*A::RP4-2Tc::Mu::,λ*pir*	Simon *et al*, 1983,Taylor *et al*, 1989.

Table 2.3 Plasmids used in this work

Strain	Relevant Phenotype	Source/Reference
pRU667	Cm^R, Ap^R	Ubben and Schmidt, 1986
pRU669	*ts*, *inc*T, *tra*$^+$, Cm^R, Km^R	"
pRU670	*ts*, Km *inc*T, *tra*$^+$, Tc^R, Km^R	"
pLB8000	$LamB^+$, Ap^R	Gift from Dr J.M.Pratt
pTROY9	pLAFRB *mal*K::IS-3, *lam*B^c,Tc^R	de Vries *et al*, 1984
pRK2013	colE1::pRK2(pRK4), Km^R, *tra*$^+$	Ditta *et al*, 1980
pHSG415	*ts*, Ap^R, Km^R, Cm^R, Tc^R	Gacesa *et al*, 1987
R64*drd*11	*tra*$^+$, Sm^R	Meynell and Datta, 1967
pRT733	*ori*R6K, *tra*$^-$, *mob*$^+$, Ap^R, Km^R	Taylor *et al*, 1989
pLV213	*rcs*A, Cm^R	Allen *et al*, 1987
pBR322	Ap^R, Tc^R	Bolivar *et al*, 1977
pUC19	Ap^R	Yanisch-Perron *et al*, 1985
pLV750	Ap^R, Km^R	This Work
pLV751	Ap^R, Km^R	"
pLV752	Ap^R, Km^R	"
pLV753	Ap^R, Km^R	"
pLV754	Ap^R, Km^R	"
Cosmid pHC79	Ap^R, Tc^R, *cos*λ	Hohn and Collins, 1980

Table 2.4 Bacteriophage used in this work

Bacteriophage	Relevant Properties	Source/Reference
PICM	Cm^R	Rosner, 1972
P1::Tn5	Km^R	Streicher et al, 1975
λ+	Wild-type	Laboratory Collection
λ::Tn5	b221, Oam, Pam, rex::Tn5, c1857	Berg, 1977

2.2 Methods

2.2.1 Cultivation and storage of bacteria

Bacterial strains were routinely cultivated on MacConkey agar or Luria agar plates supplemented with antibiotics where necessary. Plates were stored at $4^{o}C$ for periods of approximately one month before being subcultured. Liquid cultures were grown in nutrient broth or Luria broth to which antibiotics were added as required. Incubation of strains was carried out at 30 or $37^{o}C$ unless stated otherwise.

For long term storage, strains were kept in glycerol broth at $-70^{o}C$.

2.2.2 Isolation of DNA

(i) Chromosomal DNA

Cells were grown in 1ml cultures, harvested, washed in TES and resuspended in 0.5ml of lysozyme mixture (2mg ml^{-1} lysozyme, sucrose 100mg ml^{-1} and 0.5mg ml^{-1} heat-treated RNAase). The mixture was left to stand at room temperature for 20 min before 0.25 ml of a 24 mg ml^{-1} lauryl sarcosine in TES and 0.5ml of TES were added. The preparation was vortexed for 3 min before being adjusted to a density of 1.62g cm^{-3} and run on a caesium chloride gradient at 55000 rpm for 18 h. The DNA was removed with a syringe, extracted with isopropanol to remove ethidium bromide and dialysed against TE to remove caesium chloride.

(ii) Plasmid DNA

Plasmid DNA was prepared in large amounts either by the bulk method of Guerry et al (1973) or by using QIAGEN plasmid DNA extraction kits. Smaller quantities were isolated using the proce dure of Close and

Rodriguez (1982). Plasmid pRU669 DNA was extracted using the method of Yamamoto et al (1981).

2.2.3 Purification, concentration and storage of DNA

DNA was further purified by phenol and chloroform extraction and concentrated by ethanol precipitation, both according to Maniatis et al (1982). DNA was aliquotted into small quantities and stored at -20°C prior to initial use and then at 4°C.

2.2.4 Agarose gel electrophoresis

DNA samples were analysed on 0.4-0.7% (w/v) agarose gels in TBE buffer, containing ethidium bromide (0.1µg ml^{-1}). The agarose gels were subjected to electrophoresis at a constant voltage of 100 V for 2-4 h or 20 V overnight. DNA was visualised on a 302 nm UV transilluminator and the gels were photographed using a Polaroid camera and film. λ DNA cut with Hind III was used as molecular weight markers.

2.2.5 Restriction enzyme digests

Digests were usually carried out in final volumes of 20µl, but these were altered as required. Following the addition of DNA , 1/10 of the final volume of the appropriate restriction buffer (as supplied by Boehringer Mannheim GmbH) was added. The desired volume was reached with the addition of sterile distilled water and restriction endonuclease(s) was added. Incubation was carried out at 37°C for up to 2 h. If the DNA was to be analysed by agarose gel electrophoresis, 1/10 of the final volume of loading buffer was added prior to sample application. If the DNA was to be

manipulated further, the enzyme was inactivated by phenol extraction and the DNA purified by ethanol precipitation.

2.2.6 Dephosphorylation and ligation of DNA

Cleaved pHC79 vector DNA was treated with calf intestinal alkaline phosphatase (CIAP) to prevent recircularisation. 0.3 units of CIAP were added for every µg of cleaved DNA and the reaction mixture was incubated at $37^{o}C$ for 30-60 min. CIAP was inactivated at $65^{o}C$ for 1 h or $75^{o}C$ for 10 min and removed by phenol extraction. Plasmid vector DNA was not treated with CIAP.

DNA fragments were typically ligated at a concentration of 20-50 µg ml^{-1}. However, DNA that was to be packaged in vitro into λ heads were ligated at a concentration of 200-300µg ml^{-1}. Ligations were carried out overnight at $16^{o}C$ in ligation buffer supplied by Boehringer Mannheim GmbH. T4 DNA ligase was added at a concentration of between 0.2 and 1 U/µg DNA.

2.2.7 Transformation

E.coli and K.pneumoniae bacterial cells were transformed using the methods of either Brown et al (1979) or Merrick et al (1987).

2.2.8 In vitro packaging and transfection of cosmid DNA

Packaging extracts, as supplied by Amersham International were gently thawed on ice and the packaging reaction, using no more than 0.5 µg of DNA was subsequently carried out according to the manufacturers instructions. 0.5ml of SM buffer and 10µl of chloroform were added and the mixture was spun briefly to remove cell debris. 0.01ml of packaged DNA

suspension was added to 0.2ml of E.coli DH1 cells from a late exponential phase culture grown in Luria broth containing 0.2% (w/v) maltose. The culture was incubated for 30 min at 37°C to allow phage adsorbtion. 1ml of fresh LB was then added and incubation continued for a further 1 h after which time suitable dilutions were plated out onto Luria or nutrient agar containing the appropriate antibiotics.

2.2.9 Conjugations

Overnight cultures of both donor and recipient strains in Luria broth, including antibiotics, were spun down and resuspended in fresh Luria broth to the same cell concentration. For solid conjugations, 20 μl of donor cells were applied to the surface of a nutrient or Luria agar plate containing no antibiotics and allowed to dry. 20μl of recipient cells were then applied on top of the donor cells and also allowed to dry. 20μl of donor and recipient were also applied seperately to act as controls. The plate was then incubated at 30°C or 37°C overnight. The resultant growth was taken up using a sterile swab and the cells resuspended in 1ml sterile distilled water. Suitable dilutions were then plated out onto selective media.

For liquid conjugations, equal volumes of resuspended cells were mixed and incubated at the required temperature for up to 16 h without shaking, before being plated out.

2.2.10 DNA:DNA hybridisations

i) Southern hybridisation

Following agarose gel electrophoresis, DNA was transferred to nitrocellulose filters by the method of Southern (1975) with the modifications of Maniatis et al (1982) and baked at 80°C for 2 h under vacuum. The filters

were prehybridised for 3 h at 68°C in 6x SSC, 0.5% SDS, 5x Denhardt's solution, and 100μg/ml denatured calf thymus DNA. This solution was exchanged for hybridisation solution (as pre-hybridisation solution + denatured radiolabelled probe + 0.01M EDTA) and the filters were incubated overnight at 68°C. Excess, unbound probe was removed by washing under conditions of high stringency, firstly at room temperature with 2x SSC, 0.5% SDS for 5 min, then with 2x SSC, 0.1% SDS for 15 min and then at 68°C with 0.1x SSC, 0.5% SDS over 2 h with 4 changes. The filter was then air-dried for 15 min. and exposed to X-ray film overnight.

ii) Colony hybridisation

A sterile nitrocellulose filter was placed onto the surface of the agar plate. The colonies were taken up on the filter which was then air-dried for 10 min. The filter was then placed successively on 3 pieces of Whatman 3MM paper soaked in the following solutions, 0.5M NaOH (2 x 5 min); 1M Tris-HCl pH8 (2 x 5 min) and 1M Tris-NaCl neutralising solution (2 x 5 min) before being air-dried and baked as previously. No pre-hybridisation was required and filters were hybridised and washed by the same proceedure as that used for Southern hybridisations.

2.2.11 Preparation of radiolabelled DNA probes

The required DNA fragments were isolated by electrophoresis on low melting point agarose and recovered according to Maniatis et al (1982). 25ng of probe DNA was labelled with α-^{32}P-dCTP (25μCi) by random nucleotide priming using a DNA Random Priming Kit (Boehringer Mannheim GmbH).

2.2.12 Bacteriophage λ manipulations

Bacteriophage λ infections were performed as in Ellard et al, (1989). Preparation of phage lysate and determination of phage titre were performed as described in Gerhardt et al (1981).

2.2.13 Assessment of urease production

Production of urease was determined by the streaking of a single colony of bacteria onto Christensen's Urea media and incubating overnight at 37°C. Production of a vivid red colour was deemed to indicate production of urease.

2.2.14 Bacteriophage P1 manipulations

Preparation of P1 lysates and P1 infections were performed as described in Goldberg et al (1974).

2.2.15 Purification of outer membranes

Overnight bacterial cultures (100ml) were harvested at an OD of 0.5 (450nm), washed with 20ml ice-cold 10mM sodium phosphate buffer (pH 7.2), resuspended in 10ml of the same buffer and sonicated in an ice-water bath for 3 bursts of 10 s. Unlysed cells were removed during a 5 min spin at 7000 rpm. Cell membranes were pelleted by a 30 min spin at 21000 rpm, resuspended in 1ml of 10mM sodium phosphate buffer, re-pelleted and re-suspended in 200μl of 0.5% sarkosyl in sodium phosphate buffer. Following a 30 min incubation at room temperature, outer membranes were recovered by a 60 min spin at 21000 rpm, washed for 30 min with 1ml 0.5% sarkosyl in sodium phosphate buffer, re-spun and finally resuspended in 150μl sodium phosphate buffer. Sample buffer (1/3 volume) was added and samples were

boiled for 5 min prior to SDS-PAGE analysis. SDS-7 (Sigma) protein standards were used as molecular weight markers.

2.2.16 Purification of lipopolysaccharide

Lipopolysaccharide was purified using the proce-dure of Westphal and Jann (1965).

2.2.17 Polyacrylamide gel electrophoresis

SDS-PAGE of proteins was carried out on 10-14% polyacrylamide gels according to the method of Laemmli (1970). Gels were run at 10mA for 1 h and then at 25mA until the tracking dye reached the bottom of the gel. Gels were Coomassie blue stained and subsequently destained using the Solution 1-5 proceedure as detailed in Hancock and Poxton (1988).

The SDS-PAGE system was also used for the analysis of LPS using 14% SDS gels containing 4M urea. LPS was visualised using the modified silver stain proceedure of Tsai and Frasch (1982) as in Hancock and Poxton (1988).

2.2.18 Capsular serotyping

Capsular serotyping was carried out using counter-current immunoelectrophoresis (CIE) as described by Palfreyman (1978), double-immunodiffusion analysis and the quellung reaction (Kauffmann, 1949). Double-immunodiffusion was performed in 1.0% agarose in PBS. Halos were generally observed after 48 h incubation at $4^{o}C$. Strains were also independently serotyped by the Public Health Laboratory Service at Colindale, London, using CIE and the quellung reaction.

2.2.19 Phage typing

Phage typing (a capsular serotype-independent method) was performed according to Gaston et al (1987).

2.2.20 Immuno-gold labelling

A loopful of bacteria were resuspended in 1ml sterile distilled water and 1 drop placed on a small section of parafilm. A Formvar- coated copper grid was placed face down onto the bacterial suspension for 10 min. The grid was recovered, excess solution removed, and transferred to the following solutions in order: PBS (5 s); PBS + 1% BSA (30 min); PBS (5 s); primary antibody (3 h); PBS (5 s); 15nm gold particle-conjugated anti-mouse antibody diluted 1:10 in PBS (1 h); PBS (1 min). Each grid was then stained with 1% phosphotungstic acid (pH 6) for 1 min, air-dried and observed under a Cora M1232 transmission electron microscope. Control grids were treated in the same way except that the primary antibody stage was omitted.

2.2.21 Visualisation of capsular material

Bacteria from the surface of an agar plate were resuspended to approximately 10^8 cfu ml^{-1} in cacodylate buffered glutaraldehyde (2.5% v/v) incorporating ruthenium red (1% w/v). After 2 h at room temperature, the cells were pelleted by centrifugation, embedded, thin sectioned and stained with Reynold's lead citrate and uranyl acetate (1% w/v). Ultrathin sections were examined using a Philips 301 electron microscope.

2.2.22 Preparation of bacterial strains for serum and phagocytic assays

Strains were stored on M9 minimal media agar plates containing 5% heat-inactivated serum at 4^oC and innoculated into M9 minimal media broth

when necessary. After overnight growth, cells were washed and resuspended in PBS or RPMI medium to between 10^6 and 10^8 cells ml^{-1}.

2.2.23 Serum bactericidal assays

Serum was pooled from 2 adults and was stored at -70°C prior to use. Assays were performed as described in Williams et al (1983). Serum was heat-inactivated by incubation at 56°C for 30 min. All experiments were performed in triplicate.

2.2.24 Preparation of phagocytes for phagocytosis assays

Polymorphonuclear leucocytes (PMNL) were isolated from heparinised blood obtained from normal donors who were not receiving antimicrbial chemotherapy. Approximately 8ml of blood was layered carefully onto 7 ml of Mono Poly Resolving Medium and spun at 2000 rpm for 30 min. Neutrophils were removed to a universal, washed in 20ml RPMI medium and re-spun for 3 min. The resulting supernatant was discarded and the pellet resuspended in 9ml distilled water before adding 1ml 9% NaCl. RPMI was added to 20ml and then spun at 2000 rpm for 3 min. Cells were then resuspended in RPMI to typically between 10^6 and 10^7 cells ml^{-1}.

2.2.25 Pre-opsonisation of bacteria

Opsonisation of bacteria prior to chemiluminescence experiments was performed according to Edwards et al (1987).

2.2.26 Chemiluminescence assay (CL)

CL measurements were performed with an LKB 1250 Luminometer as follows: 0.1ml bacteria (10^8 cells ml^{-1}), 1µl luminol (0.1M) and RPMI to 0.9ml were mixed in a polypropylene cuvette prior to the addition of 0.1 ml

PMNL (5×10^6 cells ml^{-1}). The ratio of bacteria:PMNL was approximately 20:1. CL measurements were recorded until peak readings were obtained during which the reaction mixture was maintained at 37°C. Bacteria were omitted from control samples.

CHAPTER 3: Attempts to Generate Random Transposon Insertions in the *Klebsiella pneumoniae* Genome

3.1 Introduction

Transposon mutagenesis has been used successfully for a wide variety of genetic analyses including those involving Klebsiella pneumoniae environmental isolates and laboratory strains (Gacesa et al, 1987; Schwacha et al,1990, Merrick et al,1978).

The polysaccharide capsule of Klebsiella has been shown to be an extremely important virulence factor in clinical isolates, although the genetic mechanisms responsible for its production and expression on the outer cell surface remain unresolved. Since direct cloning of the full complement of genes necessary for Klebsiella capsule production in E.coli has so far proved unsuccessful, the development of a transposon mutagenesis system which will produce non-capsular, but otherwise isogenic mutants of clinical isolates may be a critical step in the search to locate those genes involved in capsular biosynthesis.

The results in this chapter describe the various attempts to develop such a system using plasmid, prophage and bacteriophage-based transposon delivery vehicles.

3.2 The Rts1-based Transposon Delivery System

3.2.1 Introduction

The plasmid Rts1 was originally isolated from a strain of Proteus vulgaris (Terawaki et al, 1967). Rts1 (217kb) carries a kanamycin resistance gene and expresses a number of temperature-sensitive phenotypes including autonomous replication (Terawaki and Rownd, 1972; DiJoseph and Kaji, 1974), conjugative transfer (Terawaki et al, 1967) and host cell growth (Terawaki et al, 1968; DiJoseph et al, 1973).

The transposon Tn1721, a Tn3-derivative, possesses a number of characteristics which have led to its use in transposon mutagenesis systems. Mutations due to the insertion of the transposon can be precisely located and the adjoining DNA sequenced due to the presence of EcoR1 restriction enzyme sites 15 base pairs from each end of the transposon. Tn1721 comprises of a 'minor transposon' Tn1722, which is capable of independent transposition and a tet region. Lack of stability in the latter however, limits the use of Tn1721 in a high quality, reliable transposon mutagenesis system. Ubben and Schmidt (1986) engineered a number of derivatives of Tn1722 which were designed to avoid this major disadvantage and yet retain the significant benefits of the parent, Tn1721. The new transposons carry resistances to chloramphenicol (Tn1725) and tetracycline (Tn1731, Figure 3.1) and were presented on the conjugative plasmid Rts1, thus producing the donor vehicle plasmids pRU669 (Rts1::Tn1725) and pRU670 (Rts1::Tn1731).

3.2.2 Introduction of pRU669 and pRU670 into Klebsiella pneumoniae

Each of the plasmids was transferred to a number of Klebsiella pneumoniae clinical isolates and laboratory reference strains by conjugation. Both solid surface and liquid conjugations were used in order to optimize the conjugation frequency for each combination of plasmid and strain. All conjugations were performed at 30°C.

From these results (Table 3.1) it is clear that the highest frequency obtained was that of plasmid pRU669 into K.pneumoniae BAH2819 in solid conjugations. For this reason it was decided to concentrate on this strain, a serotype K2 clinical isolate.

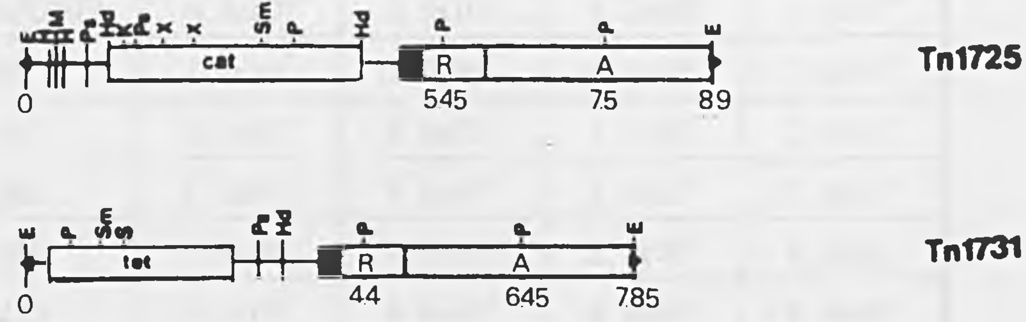


Figure 3.1: Physical and genetic maps of transposons Tn1725 and Tn1731.

█ = Resolution site

R = Resolvase gene A = Transposase gene

Restriction sites: E = EcoRI, H = HpaI, M = MluI,
Ps = PstI, Hd = HindIII, K = KpnI,
X = XbaI, Sm = SmaI, P = PvuII and
S = SalI.

(From Ubben and Schmidt, 1986)

Strain	Conjugation Frequencies			
	Solid		Liquid	
	pRU669	pRU670	pRU669	pRU670
BAH2819	$4.7x10^{-5}$	$2.5x10^{-5}$	$9.3x10^{-6}$	$5.5x10^{-6}$
5055	$1.2x10^{-7}$	$1.7x10^{-7}$	$6.1x10^{-7}$	$7.2x10^{-7}$
2L261	$8.1x10^{-7}$	$9.8x10^{-7}$	$1.1x10^{-7}$	$2.3x10^{-7}$
2L262	$6.1x10^{-7}$	$9.1x10^{-7}$	$2.6x10^{-7}$	$2.8x10^{-7}$
2L266	$7.4x10^{-7}$	$7.7x10^{-7}$	$2.1x10^{-7}$	$3.7x10^{-7}$
2L273	$4.1x10^{-7}$	$6.6x10^{-7}$	$4.1x10^{-7}$	$8.4x10^{-7}$
2L277	$4.2x10^{-7}$	$6.0X10^{-7}$	$1.9x10^{-7}$	$4.0x10^{-7}$
1L919	$7.1x10^{-7}$	$6.2x10^{-7}$	$5.4x10^{-7}$	$4.7x10^{-7}$

Table 3.1: Conjugation frequencies of pRU669 and pRU670 into various strains of K.pneumoniae

Each value is the mean of 3 separate experiments.

A single BAH2819 (pRU669) transconjugant (Ap^R, Km^R, Cm^R) was streaked to confirm purity and plasmid DNA was isolated using the method of Yamamoto et al (1981) to ensure that pRU669 had been successfully transferred.

Subsequent restriction analysis of the transconjugant pRU669 DNA confirmed that it was identical to the pRU669 DNA isolated from the Escherichia coli MM294 host.

3.2.3 Elimination of donor plasmid

Elimination of the donor plasmid was initially performed according to Ubben and Schmidt (1986). An overnight culture grown at 28°C was used to inoculate a 10ml broth to $5x10^6$ cells ml^{-1} which was then grown to a density of 10^8 cells ml^{-1} at 42°C. This was diluted 1:50 and grown again at 42°C before being plated out to single colonies. However, this was not found to be sufficient to eliminate pRU669 from the population and all colonies tested remained Km^R. This observation is consistent with DiJoseph et al (1973) who found that after 9 hours of growth at 42°C, 100% of E.coli 20SO(Rts1) cells remained Km^R. Increasing the temperature from 42°C to 43.5°C did not affect the elimination frequency.

Several authors have suggested that Rts1 only exhibits the temperature dependent instability (Tdi) phenotype in dilute cultures of bacteria (Terawaki et al, 1967; Yokota et al, 1969 and Kaji et al,1982). Subculturing at 42°C was therefore repeated as previously described except that cultures were diluted to approximately $5x10^4$ cells ml^{-1}. Growth and retention of Km^R and Cm^R in terms of viable count were followed over a period of 30 hours.

For the first 17 hours, no increase in viable count nor decrease in the number of plasmid-carrying cells was apparent (Figure 3.2a). This agrees

with other reports suggesting that Rts1 causes temperature sensitive growth in its host (Terawaki et al, 1968; DiJoseph et al, 1973). The precise cause of this phenomenon is not known. Kaji and co-workers have shown that Rts1 exists in linear form at 42°C, but that at 32°C the plasmid is in covalently closed circular (CCC) form (Yamamoto and Kaji, 1977). They propose that the inability of the linear form to circularize during replication somehow affects the growth of the host bacteria at 42°C. This idea has been challenged by Ou (1980), who suggests that thermosensitive growth of Rts1^{+} bacteria and the apparent inhibition of plasmid replication at 42°C is due to mating between cells containing Rts1 caused by a loss of surface exclusion.

After 17 hours, however, K.pneumoniae BAH2819 (pRU669) began to grow in a normal logarithmic fashion, with a doubling time of approximately 50 minutes. Between 17 and 30 hours, the proportion of cells remaining Km^{R} or Cm^{R} as part of the total cell population declined. Other authors do not appear to have followed the growth of Rts1^{+} bacteria for this length of time. At 30°C there was a slight decrease in the proportion of Km^{R} cells within the total population over the 30 hour period (Figure 3.2b) but this effect was far greater at 42°C.

These results (Figure 3.2a) indicate that at this population level, the plasmid pRU669 is slowly lost from the culture at 42°C as judged by the loss of resistance to kanamycin. However, transposition did seem to be occurring as shown by the lower rate of loss of Cm^{R} (Tn1725) as compared to that of Km^{R} (Rts1). The transposition frequency (defined as the number of Cm^{R} cells - number of Km^{R} cells as a proportion of the total viable cell population) was found to be 1.1x10^{-4} insertions per generation (calculated using growth during the logarithmic phase). This compares reasonably well with the 3.5x10^{-3} quoted by Ubben and Schmidt (1986) obtained when using plasmid DNA as the target in an E.coli DH1 background.

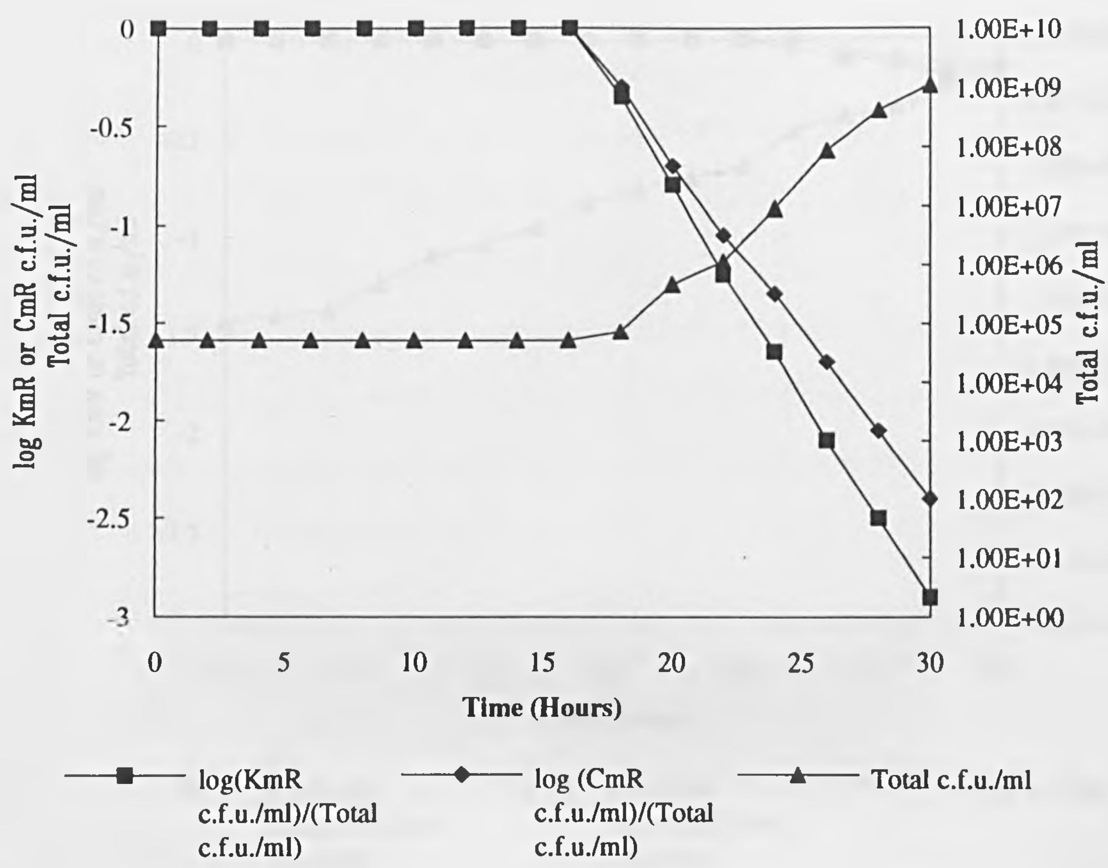


Figure 3.2a:
Growth of K.pneumoniae BAH2819 (pRU669) and segregation kinetics of pRU669 in BAH2819 at 42°C.

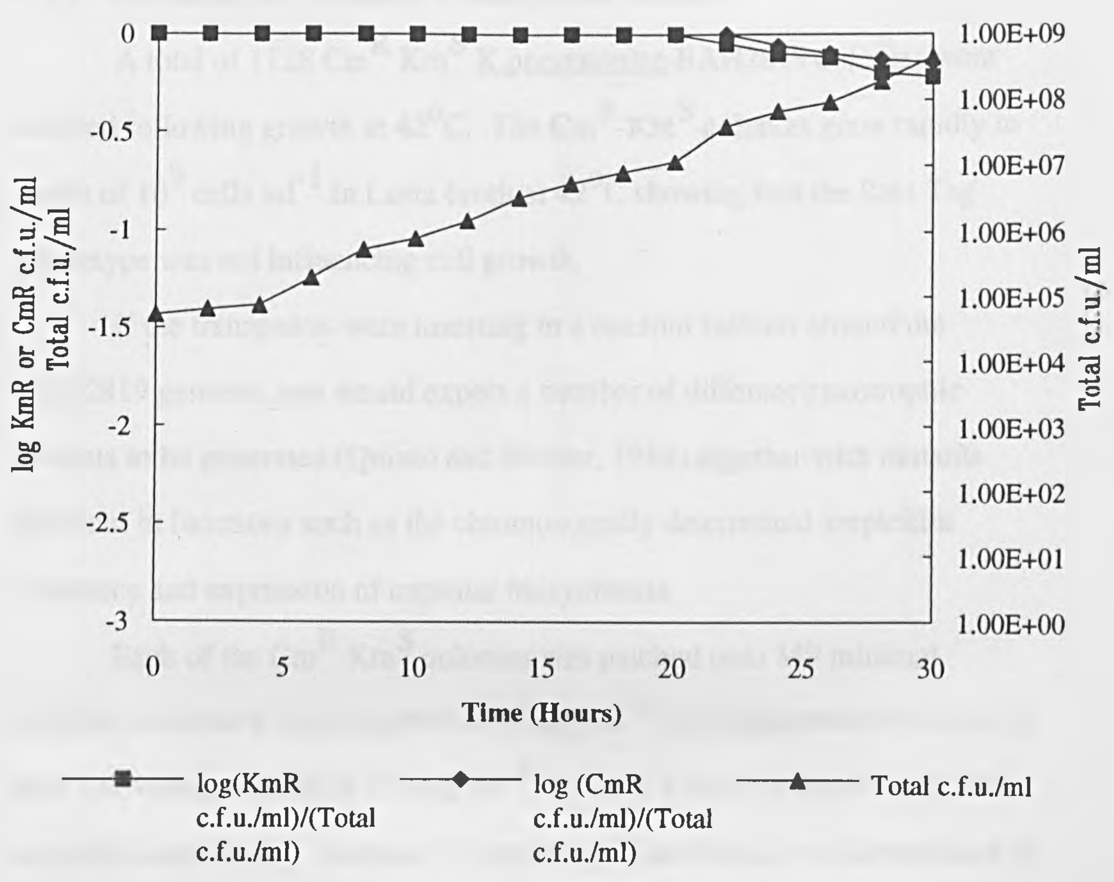


Figure 3.2b
Growth of K.pneumoniae BAH2819 (pRU669) and segregation kinetics of pRU669 in BAH2819 at 30°C.

3.2.4 Screening of prospective transposon mutants

A total of 1128 Cm^R Km^S K.pneumoniae BAH2819 colonies were isolated following growth at 42^0C. The Cm^R Km^S colonies grew rapidly to levels of 10^9 cells ml^{-1} in Luria broth at 42^0C showing that the Rts1 Tsg phenotype was not influencing cell growth.

If the transposon were inserting in a random fashion around the BAH2819 genome, one would expect a number of different auxotrophic mutants to be generated (Quinto and Bender, 1984) together with mutants deficient in functions such as the chromosomally determined ampicillin resistance and expression of capsular biosynthesis.

Each of the Cm^R Km^S colonies was patched onto M9 minimal medium containing chloramphenicol (33µg ml^{-1}) and independently onto LB agar containing ampicillin (100µg ml^{-1}) in order to test for auxotrophy and ampicillin sensitivity. Absence of capsular polysaccharide was determined by plating each Cm^R Km^S isolate onto WF agar containing chloramphenicol (33µg ml^{-1}). This medium strongly encourages capsular polysaccharide production. Any isolates which seemed to be less mucoid than the original BAH2819 strain were to be analysed further with respect to capsule production using CIE and the quellung reaction.

The results (Table 3.2) show that no auxotrophs, ampicillin-sensitive or non-capsular mutants were found during the screenings. This could be due to a number of reasons:-

1) Transposon Tn1725 may have a high degree of specificity for one or a few particular target sequences absent in these genes.

2) Plasmid pRU669 may have undergone deletions resulting in the loss of the kanamycin resistance gene together with the gene/s required for temperature sensitive host growth, while retaining Tn1725.

Mutant phenotype	**Total number isolated**	**Relative frequency**
Km^s Cm^r	1128	100
Ap^s Km^s Cm^r	0	0
Auxotrophs	0	0
Non-capsular	0	0

Table 3.2: Frequencies of isolation of mutants using pRU669 in *Klebsiella pneumoniae* BAH2819

3) Emergence of spontaneous chloramphenicol resistant mutants during growth.

It is known that Tn1725-derived elements do have a preference for insertion into A+T rich sequences and into structures which resemble the transposon's own inverted repeats ("hot spots"; Ubben and Schmidt, 1986). The K.pneumoniae chromosome is actually G+C enriched (Wehmeier et al,1989), but may contain some areas where A+T are the predominant residues. Southern blot analysis of chromosomal DNA taken from 5 Cm^R Km^S BAH2819 derivatives was undertaken using a Tn1725 specific probe in order to ascertain whether or not certain sequences in particular were being targeted by Tn1725. The 2.05kb Pvu II fragment probe was taken from Tn1725 resident in plasmid pRU667 (Ubben and Schmidt, 1986), a pBR322 derivative. Due to its smaller size and higher copy number, this plasmid was easier to isolate and manipulate than pRU669. The hybridisation result revealed that no transposon DNA had inserted into the chromosome of any of the Cm^R Km^S isolates (Figure 3.3a+b). The existence of specific Tn1725 target sites on the BAH2819 resident plasmid was also a possibility, although this was not investigated further.

The Km-resistance gene of Rts1 is flanked by direct repeat sequences and does undergo deletions and inversions in this region (Terawaki et al, 1981). Okawa et al (1987) have shown that the kanamycin resistance gene, together with the gene(s) responsible for the temperature sensitive host growth phenotypes are located on a 3.65kb SalI fragment. Thus, the possibility exists that a deletion may cause the simultaneous loss of both phenotypes.

The direct repeat sequences may also serve as "hot-spots" allowing Tn1725 to transpose itself from its site on pRU669 into this region, leading to disruption of genes encoding Km^R and Tsg phenotypes.

Lanes: 1: pRU667/EcoRI
2: pRU667/SmaI
3: BAH2819/EcoRI
4: BAH2819/SmaI
5: BAH2819 (mutant 1)/EcoRI
6: BAH2819 (mutant 1)/SmaI
7: BAH2819 (mutant 2)/EcoRI
8: BAH2819 (mutant 2)/SmaI
9: BAH2819 (mutant 3)/EcoRI
10: BAH2819 (mutant 3)/SmaI
11: BAH2819 (mutant 4)/EcoRI
12: BAH2819 (mutant 4)/SmaI
13: BAH2819 (mutant 5)/EcoRI
14: BAH2819 (mutant 5)/SmaI

Figure 3.3 a+b: Gel electrophoresis and Southern hybridisation of chromosomal DNA from K.pneumoniae BAH2819 and prospective Tn1725 mutants using 2.05kb PvuII fragment from Tn1725 as probe.

Plasmid pRU669 was unsuccessful as a transposon donor in K.pneumoniae BAH2819. Similar results were obtained using pRU670 (Km^R Tc^R). Transconjugants of other K.pneumoniae strains (Table 3.1) were also examined using both vectors as putative transposon donors. None of the isolates exhibited any signs that random mutagenesis was occurring as judged by patching between 600-1000 Cm^R Km^S individual colonies onto M9 minimal agar following growth at 42^oC.

3.3 Attempts to Use the λ Based Delivery System

3.3.1 Introduction

K.pneumoniae is usually insensitive to E.coli phage λ and therefore is not normally amenable to λ-mediated transposon mutagenesis or other associated molecular genetic techniques.

The LamB protein, a major component of the E.coli outer membrane, serves as the receptor for bacteriophage λ. LamB and all of its functions are thought to be encoded by the lamB gene (Thirion and Hofnung, 1972). This gene has been cloned onto a number of diferent plasmid vectors (de Vries et al, 1984; Clement and Hofnung, 1981; Harkki and Palva, 1985) which can be introduced into other bacterial species in order to extend the host range of λ. This technique has been used successfully to construct λ sensitive derivatives in Salmonella typhimurium (de Vries et al, 1984; Harkki and Palva, 1985), Erwinia species (Ellard et al, 1989) and indeed K.aerogenes (Bloom and Tyler, 1979) and K.pneumoniae (de Vries et al, 1984).

It was hoped to transfer the lamB gene via one of these plasmid vectors, to a number of K.pneumoniae strains and in this way to isolate λ sensitive mutants which could then be subjected to λ::Tn5 transposon mutagenesis.

3.3.2 Transfer of pLB8000 and pTROY9 to K.pneumoniae

Plasmids pLB8000 (Ap^R; Clement and Hofnung, 1981) and pTROY9 (Tc^R; de Vries et al, 1984) were transferred to a number of different K.pneumoniae isolates either by transformation or by conjugation with the aid of the mobilizing plasmid pRK2013, selecting for recipient resistance to ampicillin or tetracycline accordingly (Table 3.3). E.coli MCR106 carries a deletion of the lamB gene and was used as a control.

Strains 5055 and 2L261 were successfully transformed to Ap^R with pLB8000. No attempt was made to transform any of the other strains under test since they were all naturally resistant to ampicillin.

Plasmid pTROY9 was successfully transferred to all strains, except 2L270 and 2L273, by both transformation and conjugation. Colony hybridisation analysis, using an internal SalI fragment of lamB confirmed the presence of pLB8000 and pTROY9 within strain K.pneumoniae 5055 (Figure 3.4). Similar positive results were obtained for all other strains tested. The reason for the failure to transfer the plasmid to strains 2L270 and 2L273 are not clear. Temperatures used in the transformation procedure (growth and heat-shock) of both strains were increased to 43.5^oC in an attempt to disrupt a possible restriction/modification system which may have been present, but this had no effect. It has been noted that the presence of lipopolysaccharide (O-antigen) can have a marked influence on the transformation efficiency of K.pneumoniae (Camprubi et al, 1989) and this may be the case here.

Strain	Transformation		λ^s mutants obtained	Conjugation pTROY9 using (pRK2013)	λ^s mutants obtained
	pLB8000	pTROY9			
5055	+	+	N	+	N
BAH 2819	ND	+	N	+	N
2L261	+	+	N	+	N
3118	ND	+	N	+	N
2L263	ND	+	N	+	N
2L266	ND	+	N	+	N
2L268	ND	+	N	+	N
2L270	ND	-	N	-	N
2L273	ND	-	N	-	N
2L276	ND	+	N	+	N
2L277	ND	+	N	+	N
1L919	ND	+	N	+	N
MCR 106	+	+	Y	ND	ND

Table 3.3: Transfer of LamB$^+$ plasmids to, and λ sensitivity of various K.pneumoniae and E.coli isolates.

ND= Not Determined

+ = Successful plasmid transfer

- = Unsuccessful plasmid transfer

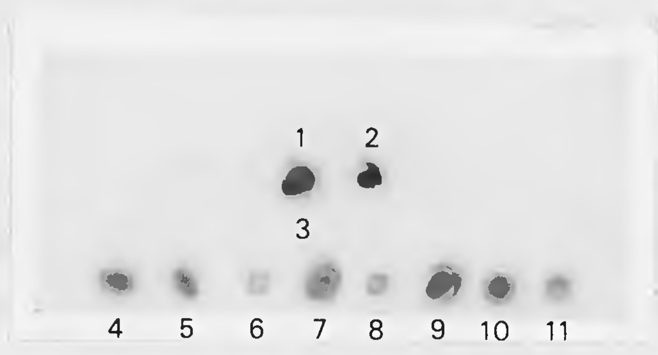


Figure 3.4: Colony hybridisation analysis to confirm presence of pLB8000 and pTROY9 within K.pneumoniae 5055 using 0.7kb SalI fragment from pTROY9 (contains malK distal and lamB sequences).

Key: 1 = E.coli LE392 (pLB8000)
2 = E.coli HB101 (pTROY9)
3 = K.pneumoniae 5055
4-7 = K.pneumoniae 5055 (pLB8000)
8-11 = K.pneumoniae 5055 (pTROY9)

3.3.3 Isolation of λ-sensitive mutants

Two seperate methods were used to test the strains carrying pLB8000 or pTROY9 for sensitivity to λ. Firstly, strains were observed for their ability to propagate λ cI857 and secondly, strains were infected with λ::Tn5 and Km-resistant colony-forming-units were selected on Luria agar plates (+Km). Bacteriophage λ::Tn5 is a suicide vector in Su^- hosts since it carries Oam Pam genes which do not allow phage DNA replication in such a environment.

None of the K.pneumoniae (pLB8000/pTROY9) strains formed plaques when infected with λ cI857, nor did they produce any Km^R colonies when infected with λ::Tn5. However, the control strain E.coli MCR106 (lamB deletion (pTROY9)) successfully propagated λ cI857 and produced round, clear plaques of approximately 1.5mm diameter as well as giving rise to Km^R colonies at a frequency of approximately $1.7x10^{-5}$ per viable cell.

The plasmid pLB8000 expresses the lamB gene at a basal level in vivo but is strongly expressed in vitro with SP6 polymerase because it carries the SP6 promoter (S.A.Benson, pers.comm.to J.M.Pratt). It is therefore not surprising that although laboratory strains of E.coli become λ sensitive due to the basal expression of lamB in vivo, less genetically characterised clinical isolates of K.pneumoniae remain resistant. However, pTROY9 expresses lamB constitutively at high levels and has been shown to successfully convert some strains of K.pneumoniae to λ^S (de Vries et al, 1984).

A number of modifications were made to the infection procedure in order to try to isolate K.pneumoniae (pTROY9) λ^S mutants:

i) K.pneumoniae (pTROY9) cells were infected with λ whilst in the log phase rather than the stationary phase of growth.

ii) Lower concentrations of kanamycin (10-75 $\mu g\ ml^{-1}$) were used to try to select for transposon mutants.

These two alterations were used by Ellard et al (1989) where problems were encountered in isolating Km^R transductants from certain strains of Erwinia containing any of the $lamB^+$ plasmids pTROY9, pHCP2 or pFE1.

iii) Multiplicities of infection (phage:cell ratio) were varied between 50 and 0.1.

It was also thought possible that capsular polysaccharide may have been masking the LamB receptor protein, thus preventing λ absorption. Sodium salicylate has been shown to reduce capsular polysaccharide production in K.pneumoniae (Domenico et al, 1989) and was therefore subsequently incorporated into all media used during the infection experiments at a level of 30 μg ml^{-1}. Washing cells in 1M NaCl for between 1 and 20 min prior to infection has also proved successful in removing extracellular polysaccharide in order to facilitate λ transduction (Ellard et al, 1988 and Steinberger and Beer, 1988).

These methods were used individually, and in conjunction with the modifications already described. However, in this study, none of the alterations, either singly or as a particular combination proved successful in leading to the isolation of λ^S mutants.

Mutants of strain 5055, M10 (K^-O^+) and M1OB (K^-O^-) were transformed with pTROY9 and the presence of the lamB plasmid confirmed as before. Infections were conducted as described previously in order to verify any inhibitory effects of capsular polysaccharide or lipopolysaccharide. Again however, no λ^S derivatives of either strain were discovered, suggesting that neither CPS or LPS were responsible for the failure of λ to infect K.pneumoniae 5055 (pLB8000) or 5055(pTROY9).

In order to test whether or not plasmid structural instability was the cause of the resistance to λ infection, plasmid pTROY9 was re-isolated from its various K.pneumoniae hosts and the DNA used to transform E.coli MCR106. All transformants isolated in this way become λ^S showing that the lamB gene had remained structurally and functionally intact in the K.pneumoniae host background.

The LamB receptor protein has a molecular weight of 47000 (Clement and Hofnung, 1981). Outer membrane protein profiles from E.coli HB101(pTROY9), K.pneumoniae 5055, 5055(pTROY9) and 5055(pLB8000) revealed no differences in this molecular weight area between $LamB^+$ and $LamB^-$ strains of Klebsiella (Figure 3.5). This suggests a number of possibilities: either the LamB protein is not being produced at all; an aberrant form of the receptor is being produced and or that the protein is not being correctly exported or inserted into the outer membrane.

A further problem encountered when using these lam B^+ plasmids has been one of instability. Ellard et al (1989) discovered that one Erwinia (pTROY9) strain lost its ability to be transduced with λ after a single replating. Even the laboratory E.coli strain used to carry pLB8000 loses expression of the LamB phenotype after a short period of storage on agar plates (S.A. Benson pers.comm to J.M. Pratt). The nature of this instability is unknown but may complicate studies of this kind.

In summary, despite the successful introduction of $lamB^+$ plasmids into various strains of K.pneumoniae and careful manipulation of the λ infection procedure, no λ^S derivatives were found.

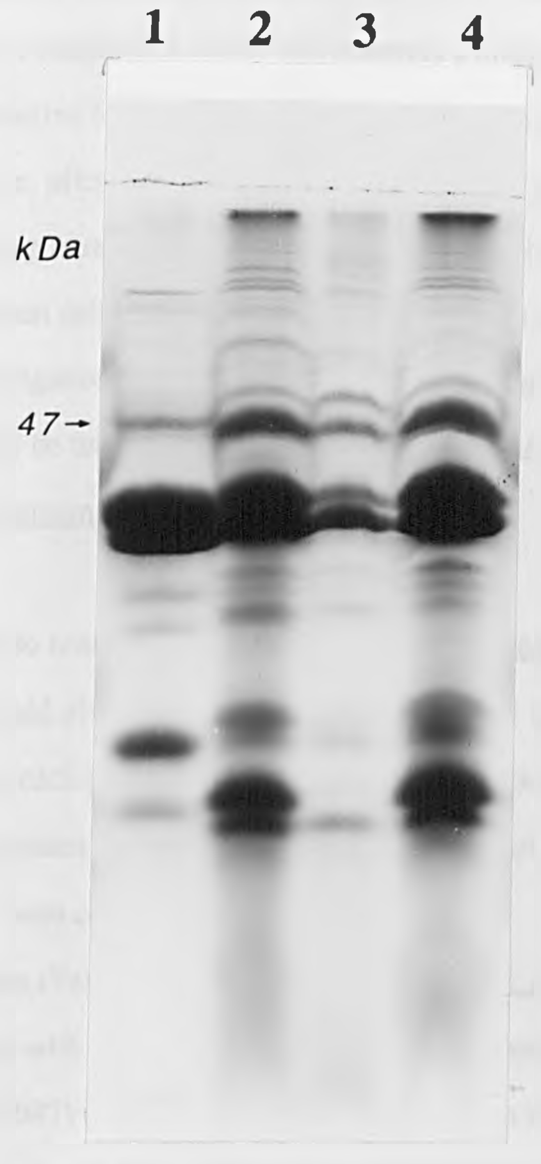


Figure 3.5: Outer membrane profiles of plasmid-containing E.coli and K.pneumoniae strains.

Lanes: 1 = E.coli (pTROY9)
2 = K.pneumoniae 5055
3 = K.pneumoniae 5055 (pLB8000)
4 = K.pneumoniae 5055 (pTROY9)

3.4 Attempts To Use The pHSG415 Delivery System

3.4.1 Introduction

The plasmid pHSG415, a derivative of pSC101, was originally constructed as a containment vector and contains a mutation which renders it temperature-sensitive for replication (Hashimoto-Gotoh et al, 1981). A further derivative, pHSG415::Tn10 contains the cloned transposon Tn10 element (encoding tetracycline resistance) and has previously proved to be a suitable transposon delivery vehicle in K.pneumoniae (Gacesa et al, 1987).

An investigation was carried out in order to determine whether or not this system could be used successfully to mutagenize the available clinical specimens of K.pneumoniae.

3.4.2 Attempts to transfer pHSG415::Tn10 to K.pneumoniae

The plasmid pHSG415::Tn10 was isolated from E.coli C600 and LE392 hosts and each preparation was used to transform a number of K.pneumoniae isolates as well as a plasmid-free strain of E.coli C600. All transformations were conducted at 30°C.

The results (Table 3.4) reveal that none of the K.pneumoniae strains were transformed with pHSG415::Tn10. This is in agreement with the results of Gacesa et al (1987) who could not directly transform a strain of K.pneumoniae known as PG1. This group postulated that an efficient restriction/modification system in strain PG1 did not allow the replication of pHSG415::Tn10. This possibility was not investigated further.

Strain	Transformation	Transfer frequency of pHSG415::Tn10 using R64drd-11	
		From E.coli C600	From E.coli LE392
5055	-	0	0
BAH2819	-	0	0
2L261	-	0	0
3118	-	0	0
24118	-	0	0
2L263	-	0	0
2L264	-	0	0
2L265	-	0	0
2L266	-	0	0
2L268	-	0	0
2L269	-	0	$2.1x10^{-6}$
2L270	-	$2.5x10^{-6}$	$2.4x10^{-6}$
2L271	-	0	0
2L273	-	0	0
2L274	-	0	0
2L275	-	$3.6x10^{-6}$	0
2L276	-	$5.3x10^{-6}$	$4.9x10^{-6}$
2L277	-	$4.7x10^{-6}$	$3.8x10^{-6}$
2L279	-	0	0
E.coli C600	+	ND	ND

Table 3.4: Transfer of pHSG415::Tn10 into various K.pneumoniae isolates using transformation and mobilization techniques.

+ = Successful plasmid transfer

- = Unsuccessful plasmid transfer

ND = Not determined

The mobilizing plasmid R64drd-11 (Meynell and Datta, 1967) has been used to transfer pHSG415::Tn10 to K.pneumoniae (Gacesa et al, 1987) and this strategy was employed using both E.coli strains as donors. Triplicate matings were carried out on a solid agar surface at 30°C and transconjugants were selected on either Luria agar plates containing kanamycin, tetracycline and ampicillin or minimal media agar plates containing kanamycin and tetracycline depending on the K.pneumoniae strain used.

R64drd-11 proved successful in mobilizing pHSG415::Tn10 to a small number of K.pneumoniae strains (Table 3.4) as judged by the conversion of those strains to kanamycin and tetracycline resistance albeit at a low frequency. E.coli C600 was an effective donor of pHSG415::Tn10 to strain 2L275 whilst E.coli LE392 was not. This result was reversed when strain 2L269 was the recipient. The reasons for this are not clear. Both strains were effective donors when K.pneumoniae strains 2L270, 2L276 and 2L277 were the recipients.

Representative transconjugants from strains 2L269, 2L270, 2L275, 2L276 and 2L277 were re-streaked for purity and sub-cultured on 4-5 occasions in the presence of small amounts of kanamycin (20μg ml^{-1}) in an effort to lose R64drd-11 (Sm^R Tc^R) and its interfering tetracycline resistance marker. Only from strain 2L276 were such Sm^S Km^R Tc^R colonies identified. It has been suggested previously that the "mobilization" of pHSG415::Tn10 by R64drd-11 occurs at extremely low frequencies and, moreover, that the "mobilization" is probably passive transfer through the formation of cointegrate plasmids (Hashimoto-Gotoh et al, 1981). This may be the case in transconjugants of 2L269, 2L270, 2L275 and 2L277 where failure to isolate Sm^S Km^R Tc^R cells may have been due to lack of resolution of the cointegrate plasmid. In contrast however, and in agreement

with Gacesa et al (1987) no such problems were encountered with strain 2L276.

Mini-preparations alone of plasmid DNA from Sm^S Km^R Tc^R K.pneumoniae 2L276 cells did not conclusively demonstrate the presence of pHSG415::Tn10 due to the existence of a number of other plasmids in this strain (Figure 3.6a). However, Southern blot analysis, using the complete plasmid pHSG415 (7.1kb) revealed that the plasmid was indeed present in at least two of the transconjugants (Figure 3.6b).

3.4.3 Stability of inheritance of pHSG415::Tn10 in K.pneumoniae at different temperatures

The stability of pHSG415::Tn10 in K.pneumoniae 2L276 at 30°C and 42°C was investigated according to Hashimoto-Gotoh et al, 1981 with the modification that samples were also plated onto Luria agar containing tetracycline to monitor the fate of transposon Tn10. At 42°C, there was a rapid and dramatic reduction in the proportion of plasmid-carrying bacteria within the total cell population (Figure 3.7a). The slightly lower rate of loss of tetracycline resistance also suggests transposition of transposon Tn10. The transposition frequency (defined as in 3.2.3) was approximately $2.3x10^{-4}$ insertions per generation. This figure compares well with the $5x10^{-4}$ reported by Gacesa et al (1987). No loss of pHSG415::Tn10 occurred from 2L276 during 10 hours of growth at 30°C (Figure 3.7b).

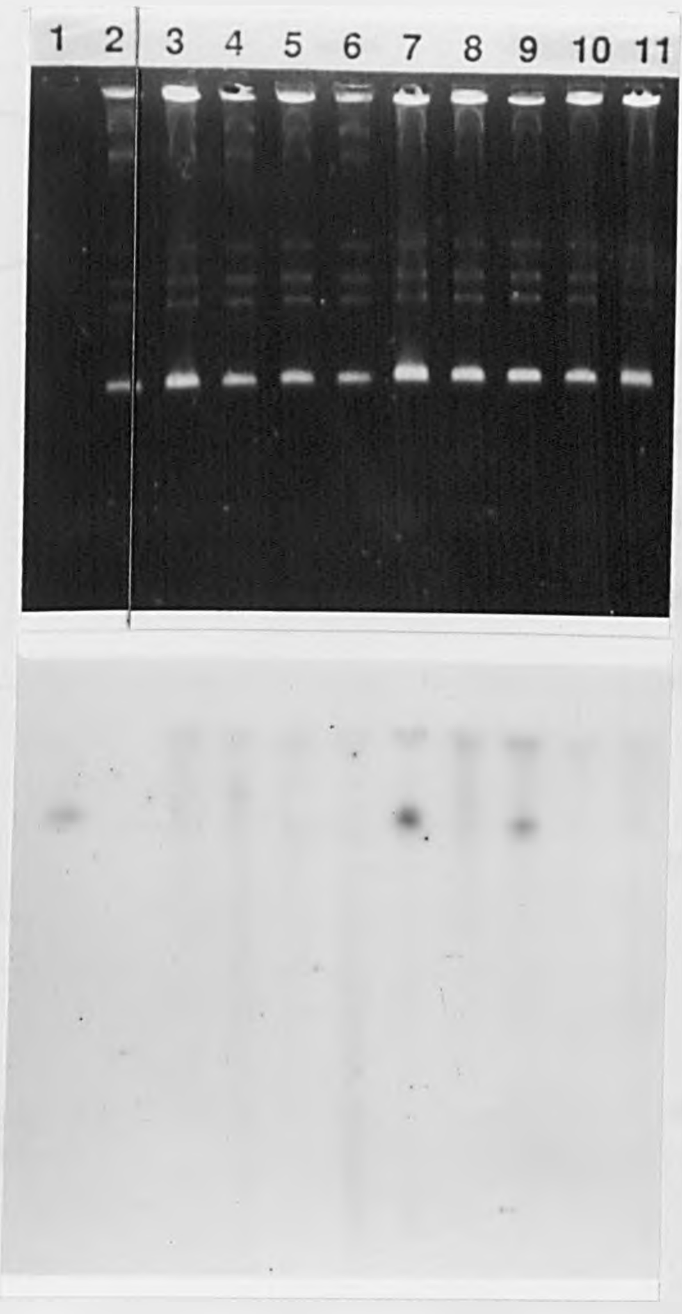


Figure 3.6 a+b: Gel electrophoresis and Southern hybridisation of plasmid DNA from prospective K.pneumoniae 2L276 transconjugants using pHSG415 as a probe.

Lanes: 1 = pHSG415::Tn10
2 = K.pneumoniae 2L276
3-11 = Prospective K.pneumoniae 2L276 transconjugants (Km^R, Cm^R, Tc^R)

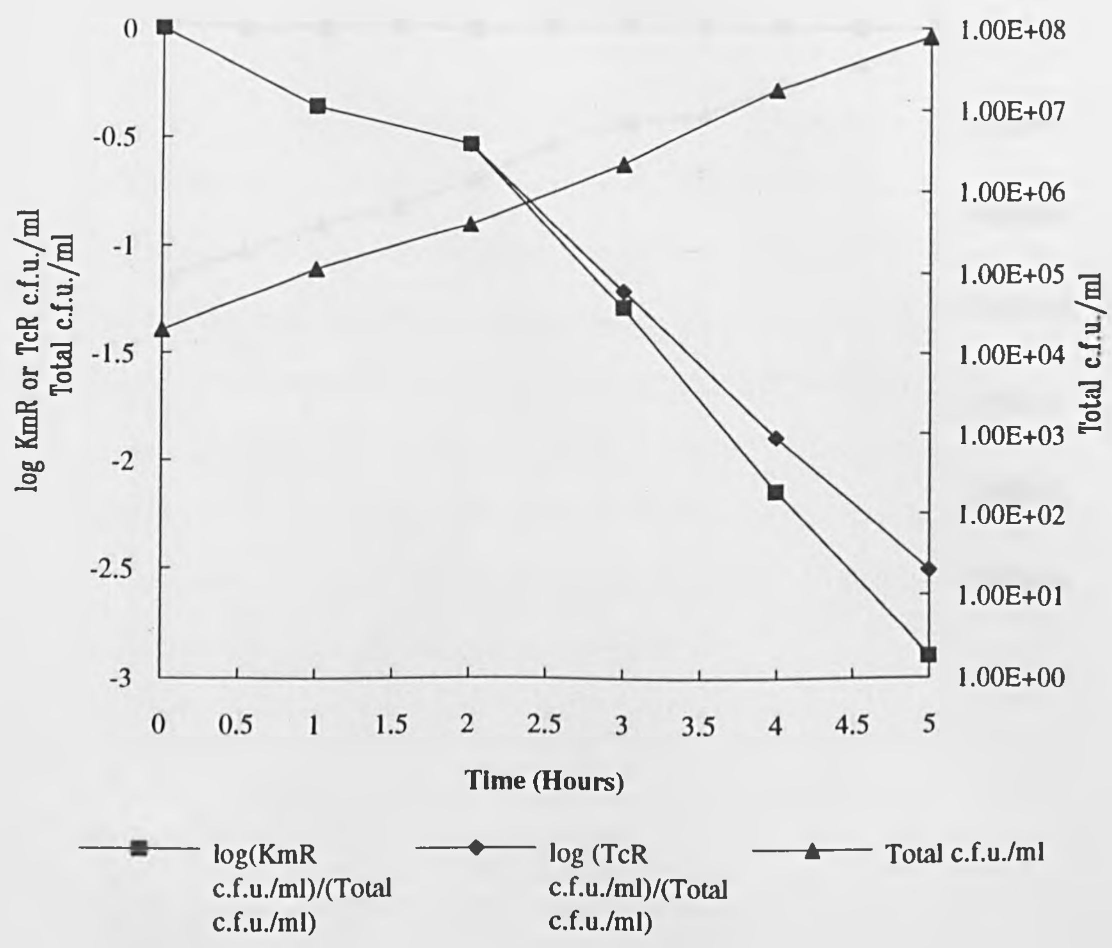


Figure 3.7a
Growth of K.pneumoniae 2L276 (pHSG415::Tn10) and segregation kinetics of pHSG415::Tn10 in K.pneumoniae 2L276 at 42°C.

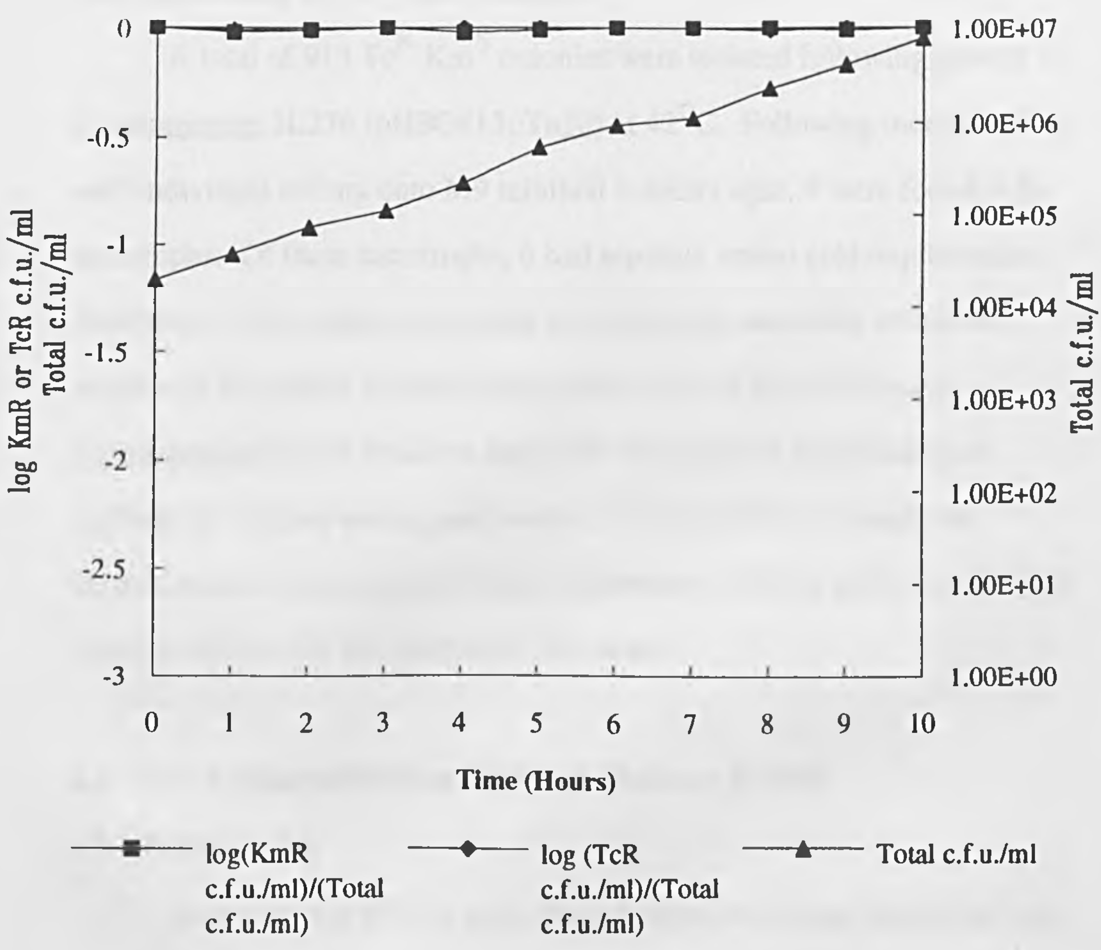


Figure 3.7b
Growth of K.pneumoniae 2L276 (pHSG415::Tn10) and segregation kinetics of pHSG415::Tn10 in K.pneumoniae 2L276 at 30°C.

3.4.4 Screening of Tc^R Km^S colonies

A total of 913 Tc^R Km^S colonies were isolated following growth of K.pneumoniae 2L276 (pHSG415::Tn10) at 42°C. Following inoculation of each individual colony onto M9 minimal medium agar, 9 were found to be auxotrophs. Of these auxotrophs, 6 had separate amino acid requirements. Transposon Tn10 appears therefore to be inserting randomly within the genome of this strain. Unfortunately, this could not be confirmed as K.pneumoniae 2L276 could no longer be serotyped by immunological methods (B. Ayling-smith, pers. comm.). This would have made the identification of non-capsular mutants extremely difficult and it was decided not to continue with the analysis of this strain.

3.5 Use of a Bacteriophage P1-based Delivery System

3.5.1 Introduction

Bacteriophage P1 is a generalised transducing phage which has also been used successfully as a vector for transposon mutagenesis (Quinto and Bender, 1984). The phage infects bacterial cells in the same way as other bacteriophage, but is maintained within the cell in the plasmid prophage state. It is thus often described as a "phasmid". P1 also exhibits an incompatibility effect, i.e. an incoming P1 will not be stably maintained in the presence of an already existing P1 plasmid (Ikeda and Tomizawa, 1968). It is this feature which makes P1 an ideal transposon delivery vehicle.

A P1 bacteriophage carrying a particular antibiotic resistance determinant can be used to infect a bacterial strain, and selection applied for lysogenic cells. Superinfection with a P1::Tn will lead to the rapid elimination of the incoming P1 but not before the Tn element has in a few instances, transposed to the host genome.

This system was examined with regard to its suitability as a transposon delivery system in clinical isolates of K.pneumoniae.

3.5.2 Attempts to isolate P1-sensitive derivatives of K.pneumoniae

Infection of all bacterial strains with P1 was carried out in an attempt to isolate P1-sensitive mutants for use in transposon mutagenesis.

P1 lysogeny was checked by confirming that the chloramphenicol-resistant bacterial cells were capable of releasing phage when grown at 42^0C in the absence of Ca^{2+}. Growth at 42^0C increases the rate of spontaneous induction of P1 lysogens while the lack of Ca^{2+} ions will mean that those P1 phage that have been released cannot re-infect other cells (Gerhardt et al, 1981). E.coli C600 and K.pneumoniae KAY2026 are known to be P1-sensitive and were used as positive controls. All other klebsiellae were regarded as being P1-resistant until demonstrated otherwise.

Some strains gave rise to a large number of spontaneous Cm^R mutants and thus were not used further. Upon infection with P1, only K.pneumoniae strains 5055 and 2L276 gave rise to Cm^R colonies (Table 3.5). Presumptive P1CM lysogens of 2L276 were isolated at a very low frequency and were extremely unstable, consistently reverting to Cm^S after a single subculture. No stable Cm^R derivatives of this strain were ever found and thus the source of Cm^R could not be positively assigned to either P1 lysogeny or spontaneous mutation. However, no Cm^R colonies were discovered from a normal cell population of this strain not infected with P1.

The failure to derive P1-sensitive mutants from other clinical strains was not anticipated, particularly since other K.pneumoniae isolates have shown themselves to be amenable to manipulations of this type (Quinto and Bender,1984; Goldberg et al,1974 and Camprubi et al, 1989). It is conceivable that there is a host restriction/modification barrier to overcome.

Strain	Relevant genotype	P1 lysogen isolation frequency	Titre of phage released (p.f.u.\ml)
5055	$P1^r$	$1.1x10^{-7}$	$3.4x10^{7}$
BAH2819	$P1^r$	0	ND
2L261	$P1^r$	0	ND
24118	$P1^r$	0	ND
2L263	$P1^r$	0	ND
2L265	$P1^r$	0	ND
2L268	$P1^r$	0	ND
2L269	$P1^r$	0	ND
2L270	$P1^r$	0	ND
2L271	$P1^r$	0	ND
2L273	$P1^r$	0	ND
2L274	$P1^r$	0	ND
2L275	$P1^r$	0	ND
2L276	$P1^r$	$3.0x10^{-8}$	ND
2L277	$P1^r$	0	ND
2L279	$P1^r$	0	ND
KAY2026	$P1^s$	$1.2x10^{-4}$	$1.2x10^{8}$
E.coli C600	$P1^s$	$1.5x10^{-3}$	$6.4x10^{9}$

Table 3.5: Isolation of P1CM lysogens from various K.pneumoniae strains.

ND = Not determined

P1 lysogen isolation frequency was defined as: $\frac{\text{Number of P1CM lysogens}}{\text{Total colony forming units}}$

Each figure is the mean of 3 separate experiments.

However, increasing the infection temperature above 30^{0}C will favour the induction of any lysogens that may be formed. Two alternative approaches were adopted to circumvent this difficulty. Firstly, each of the strains involved was grown up overnight at 43.5^{0}C prior to phage addition in an attempt to destroy the host restriction system. Secondly, the infection procedure was initially performed at 43.5^{0}C for varying periods of time ranging from 30 s to 25 min before being transferred to 30^{0}C for whatever time remained of the 30 min infection period. These manipulations were used on each of the strains listed in Table 3.5. This had no visible effect and did not lead to lysogen isolation nor did it increase isolation frequencies for strains 2L276 and 5055.

Evidence for an active restriction/modification system for P1 DNA in K.pneumoniae was obtained from the results for the control strains. The P1 phage used to infect K.pneumoniae KAY2026 had been passaged through the E.coli C600 strain prior to use. Upon induction of KAY2026 a lower phage yield was obtained due to host controlled modification. The P1 lysate obtained from K.pneumoniae KAY2026 was subsequently used to infect the other K.pneumoniae isolates in order to overcome the restriction/modification barrier. This did not seem to affect klebsiella resistance to P1 and no lysogens were obtained. Thus it did not seem likely that the host controlled restriction/modification system was the deciding factor in maintaining the P1-resistance of those K.pneumoniae isolates tested.

The bacterial receptor site for bacteriophage P1 is the lipopolysaccharide core oligosaccharide (Archibald,1980). The lipopolysaccharide sugar chains (O-antigen) have been shown to occlude the P1 receptor leading to diminished lysogen production in K.pneumoniae (Camprubi et al, 1989). However, evidence from the same study also indicated that the presence of capsular polysaccharide on the cell surface did

not affect the accessibility of a P1 derivative to the LPS-core oligosaccharide. Using the mutants M10(K^-O^+) and M10B(K^-O^-) it has been possible to investigate this effect with particular regard to the 5055 strain. The results (Table 3.6) show approximately a 600-fold increase in the number of PICM lysogens isolated from strain MIOB (O^-) as compared to the parent 5055 strain (O^+). However, in contrast to Camprubi et al (1989), the K-antigen did seem to play a minor role in restricting access of PICM bacteriophage to the LPS-core oligosaccharide receptor site as judged by the approximate 3.5-fold increase in lysogen formation in M10 (K^-O^+) over 5055. Camprubi et al (1989) also reported similar lysogeny frequencies between O^- K.pneumoniae and E.coli strains. This study revealed a 20-fold decrease in lysogen formation in the O^- K.pneumoniae strain compared to E.coli C600. However, since these studies utilised E.coli P1 lysates, a 20-50 fold lower efficiency of plating in K.pneumoniae would be expected due to host-controlled modification (J.Lengeler, pers. comm.)

3.5.3 Screening of prospective transposon mutants

A total of 391 Cm^R Km^R K.pneumoniae 5055 colonies were obtained from 11 independent infections with P1 lysates (a frequency of approximately 3.5×10^{-6} per added phage particle).

When patched onto M9 minimal medium, 2 were found to be auxotrophs (0.51%, assuming all 391 Cm^R Km^R colonies are transposon mutants). However, Quinto and Bender (1984) reported in a similar study that only 80% of K.aerogenes Cm^R Km^R colonies were the result of Tn5 transposition from P1 to the chromosome. The proportion of bona-fide transposon mutants generated in this study was not analysed. No non-capsular mutants were identified.

Strain	Isolation Frequency (P1CM lysogens/Total c.f.u.)
5055 (K^+O^+)	1.1×10^{-7}
M10 (K^-O^+)	3.8×10^{-7}
M10B (K^-O^-)	6.7×10^{-5}

Table 3.6: Effect of O and K antigens upon P1CM lysogeny in K.pneumoniae 5055.

Each figure is the mean of 3 separate experiments.

A preliminary investigation revealed that bacteriophage P1 appears to be successful as a vector for Tn5 insertion mutagenesis in K.pneumoniae strain 5055, although this was not confirmed for reasons that will be discussed later (section 3.7).

3.6 Transposon Mutagenesis using the pRT733 Delivery System

3.6.1 Introduction

The plasmid pRT733 (Ap^r, Km^r) is a suicide vector derived originally from pBR322 (Taylor et al, 1989). In this plasmid, the ColE1 origin of replication has been replaced by R6K ori and thus it can only replicate when the π protein is provided in trans such as in an E.coli λ pir lysogen (Kolter et al, 1978).

SM10 λ pir can mobilize pRT733 into K.pneumoniae because it carries a derivative of plasmid RP4 integrated in the bacterial chromosome. This derivative can provide conjugative functions in trans to the mob site on pRT733. Instead of Tn5, plasmid pRT733 carries a derivative, TnphoA (Manoil and Beckwith, 1985) which retains the kan gene as well as the capacity for random insertion within a bacterial genome. Although originally designed for use in the analysis of extracellular protein production, it was hoped that TnphoA could also be used to isolate non-capsular K.pneumoniae mutants.

3.6.2 Isolation of transposon mutants of K.pneumoniae

Rifampicin or streptomycin resistant mutants of K.pneumoniae were isolated in order to provide a criterion for selection against the donor strain. Matings between Rif^r/Str^r klebsiellae and a λ pir lysogen of E.coli SM10(RP4-2-Tc::Mu) containing pRT733 (Ap^r, Km^r) were carried out and transposon mutants were selected directly on LB agar containing kanamycin and rifampicin/streptomycin since pRT733 will not replicate in the K.pneumoniae strains.

Kanamycin resistant colonies were isolated from all matings at frequencies in the 10^{-5}-10^{-6} region (Table 3.7). This is consistent with the findings of Taylor et al, (1989) who used the pRT733 delivery system in Vibrio cholerae.

Three representative Km^R colonies from each mating were biotyped using the API20E system and all were identified as K.pneumoniae.

Between six hundred and one thousand Km^R colonies from each strain were tested for auxotrophy by patching onto M9 minimal salts agar containing kanamycin (Table 3.7). Only strains 5055, 3118, 2L261 and C887 generated auxotrophic mutants. In the majority of K.pneumoniae isolates tested, the pRT733 delivery system was probably unsuccessful in generating random mutations. However, for strains 5055, 3118, 2L261 and C887, the frequencies of auxotrophic mutation produced (0.82%, 1.33%, 0.67% and 0.50%) seem to be reasonably consistent with those previously reported for K.pneumoniae (Gacesa et al, 1987). Two of the auxotrophs generated from strain 5055 were judged to be "leaky", that is a small proportion of those cells that were patched grew on the minimal medium, possibly due to a high reversion frequency. These auxotrophs were discarded and were not included in the calculation of auxotroph generation frequency in this strain. No leaky auxotrophs were generated from the other strains.

Confirmation that random transposon mutagenesis was occurring was obtained from Southern blot analysis. Chromosomal DNA from three Km^R 5055 mutants was examined following digestion with EcoRV, which does not cut within TnphoA, and probed with the 3.4 kilobase Hind III internal fragment of TnphoA. The results show that all 3 mutants selected contain a single TnphoA insertion in different EcoRV fragments (Figure 3.8a+b).

Strain	Isolation frequncy of Km^R colonies	Isolation frequency of auxotrophs from Km^R colonies (%)
5055	$1.5x10^{-5}$	0.82 (7/850)
BAH2819	$2.6x10^{-6}$	0
2L261	$7.2x10^{-6}$	0.67 (4/600)
3118	$9.8x10^{-6}$	1.33 (7/600)
24118	$3.4x10^{-6}$	0
C847	$3.4x10^{-6}$	0
C887	$2.1x10^{-6}$	0.5 (3/600)
C1012	$3.5x10^{-6}$	0
C1300	$1.1x10^{-6}$	0
C2071	$7.2x10^{-6}$	0
C2107	$6.2x10^{-6}$	0
C2445	$1.2x10^{-6}$	0
C3646	$6.1x10^{-6}$	0
C3666	$4.1x10^{-6}$	0

Table 3.7: Transposon mutagenesis and auxotroph generation in various K.pneumoniae isolates using pRT733.

Isolation frequency defined as:

$$\frac{\text{Number of } Km^R \text{ colonies isolated}}{\text{Total colony forming units}}$$

Lanes: 1: Lambda DNA/HindIII
2: pRT733 uncut
3: pRT733/HindIII
4: 5055 uncut chromosomal DNA
5: 5055/EcoRV
6: 5055 mutant 1 uncut chromsomal DNA
7: 5055 mutant 1/EcoRV
8: 5055 mutant 2/EcoRV
9: 5055 mutant 3/EcoRV

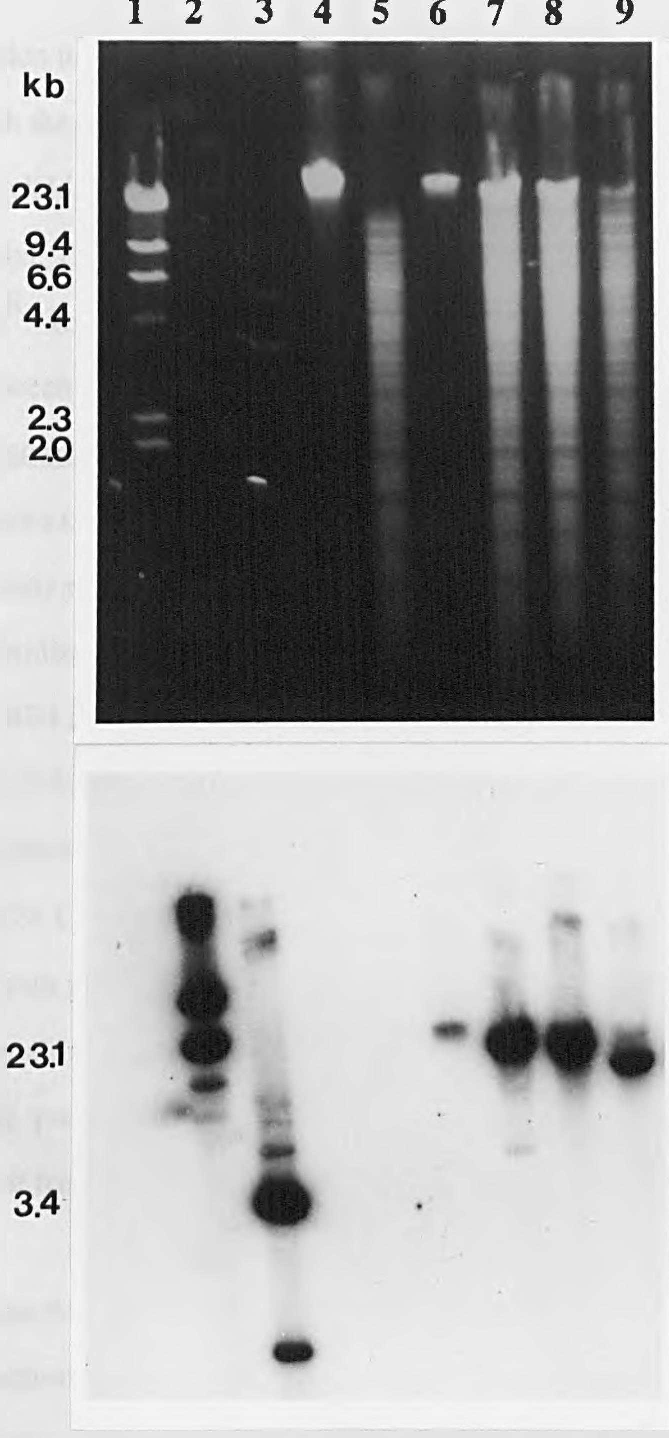


Figure 3.8 a+b: Gel electrophoresis and Southern hybridisation showing random transposon insertion into chromosomal DNA of K.pneumoniae 5055 using a 3.4kb HindIII fragment from TnphoA as a probe.

No hybridisation to the parent 5055 (Km^S) DNA was evident. One potential drawback with the pRT733 transposon delivery system lies with its propensity to yield Ap^R colonies. Taylor et al (1989) reported that as many as 20% of active TnphoA gene fusions in Vibrio cholerae led to the host becoming Ap^R. It was believed that these strains arose from cointegrate formation between the plasmid and the chromosome.

K.pneumoniae strain C887 is naturally Ap^R and thus the generation of such cointegrates could only be investigated using Southern blot analysis. A preliminary study revealed that, of 5 Km^R colonies from strain C887, 1 positively hybridized with a 1.9 kilobase BamHI internal fragment of pRT733 (carrying the RP4 mob region; Miller and Mekalanos, 1988). In strain 5055 (Ap^S), 198 of 1114 Km^R colonies isolated also become Ap^R, a frequency of 17.8%. This phenomenon was not examined in strains 3118 or 2L261. Plasmid pRT733 DNA could not be isolated from any of the Ap^R 5055 colonies nor from the C887 strain which had shown a positive hybridisation reaction. It would seem therefore that the use of the pRT733 vector system in K.pneumoniae promotes cointegrate formation between the plasmid and chromosome at frequencies comparable to those reported previously in other species.

In-frame fusions between phoA and a target gene display alkaline phosphatase activity only if expressed at the cell surface (Manoil and Beckwith, 1985). Strains containing such insertions may be identified by a characteristic blue colony colour on Luria agar plates containing the chromagenic indicator XP. Km^S parent strains 5055, C887, 2L261 and 3118 grown on Luria agar containing XP all exhibited a pale blue colour, probably due to production of host-encoded phosphatase. However, approximately 1 in every 260 Km^R TnphoA insertion mutants of these strains produced a far more vivid blue colour which was believed to represent an active phoA

hybrid protein. Although this was not investigated further and the generation of non-phosphatase producing isolates may be necessary, it is believed that pRT733 together with Tn*pho*A could be used successfully to investigate extracellular protein production and any possible relationship with bacterial virulence in these strains of *K.pneumoniae*.

It would appear that the pRT733 transposon delivery vehicle is a suitable system for the random generation of precise mutations in certain clinical isolates of *K.pneumoniae*. Further studies are required in order that non-capsular mutants may be generated and characterised with respect to the genetic determinants of capsular biosynthesis.

3.7 Discussion

A range of delivery systems have been assessed for their ability to confer random transposon insertion mutagenesis upon the genome of a number of *K.pneumoniae* clinical isolates. Varying degrees of success have been achieved, but no one particular regime seems to be suitable for all strains.

Initial studies have shown that the broad host range plasmid pRT733 (pJM703.1::Tn*pho*A; Taylor *et al*, 1989) appears to deliver Tn*pho*A (Manoil and Beckwith, 1986) in the required random fashion into 4 strains of *K.pneumoniae*. Transposon mutants are selected in a single step thereby providing an extremely fast and reliable method by which to isolate mutations of interest. The generation of Ap^R Km^R colonies (possibly cointegrates) does seem to pose one potential problem with this vector system. However, the use of an Ap^S recipient *K.pneumoniae* strain will allow the straightforward identification of such events.

Major difficulties were encountered with each of the other delivery vehicles. While initially promising, the plasmid pRU669/pRU670 system

(Ubben and Schmidt, 1986) did not seem to transfer the transposons Tn1725/Tn1731 to the K.pneumoniae chromosome. This was despite the K.pneumoniae colonies exhibiting the correct antibiotic resistance profile, i.e. retaining the transposon encoded resistance and losing the plasmid-encoded resistance. A number of theories have been put forward in an attempt to explain this apparent inconsistency.

The problems experienced by other authors (Terawaki et al, 1981; Okawa et al, 1987) when working with Rts1 and plasmids derived from it were also encountered in this study. Accordingly, much time was spent on this vector which could, with hindsight, have been spent more profitably in other directions.

The introduction of $LamB^+$ plasmids into K.pneumoniae strains was successful in several cases. Despite this, no λ^s K.pneumoniae isolates were ever found, even though the structural and functional integrity of the plasmid was maintained in the K.pneumoniae background. The failure of λ to infect such cells appeared to be due to aberrant or non-existent expression of the λ receptor protein LamB in the K.pneumoniae outer membrane. Further work, perhaps involving a LamB-specific antibody probe would be necessary in order to confirm these hypotheses.

Both P1::Tn5 and pHSG415::Tn10 delivery systems appeared to be successful transposon donors within extremely limited host ranges. The use of pHSG415::Tn10 in K.pneumoniae K34 was marred by the discovery that this strain could no longer be serotyped by conventional immunological techniques, thus rendering it useless for further studies into the basis of capsular biosynthesis. Although an efficient transposon mutagenesis vector, bacteriophage P1 was judged less suitable than the pRT733 system and was similarly not used for further analysis. The isolation of transposon mutants was a 2-step rather than a 1-step process, thus making it slower. TnphoA

mutagenesis also appeared to be successful in four strains, rather than just two, extending the range of comparisons during more advanced genetic studies.

In theory, transposon mutagenesis appears to be a relatively straightforward technique. However in practice, numerous difficulties were encountered. Wright et al (1990), prior to their ultimate success, also met with problems and were unable to demonstrate transposition in Vibrio vulnificus with a large number of transposon delivery vectors, including pRT733. However, transposon mutagenesis has been used in K.pneumoniae previously and the large number of obstacles faced during the course of this work were certainly not envisaged. The fact that clinical isolates (probably containing well developed restriction systems) were being studied may be a contributory factor.

CHAPTER 4: Isolation And Characterisation Of Transposon Mutants Deficient In Capsular Polysaccharide Biosynthesis

4.1 Introduction

A vector system has been established that will successfully deliver a transposon into the K.pneumoniae genome in a random fashion.

This chapter describes the isolation and characterisation of non-capsular transposon mutants which are isogenic in all other respects. The use of such mutants will facilitate further analysis of those regions of the K.pneumoniae genome which are involved in the synthesis and expression of the capsular polysaccharide.

4.2 Isolation Of Non-Capsular Mutants Of K.pneumoniae K2 5055

It was decided to concentrate on strain 5055 for three reasons:-

1) Mutants M10 (K^-O^+) and M10B (K^-O^-), derivatives of the parent strain 5055 produced by nitrosoguanidine mutagenesis, were readily available in the laboratory. These would be useful as controls in the analysis of non-capsular transposon mutants.

2) Strain 5055 produces larger amounts of capsular polysaccharide than do the other K.pneumoniae isolates successfully mutagenized with TnphoA, thus making a preliminary visual identification of non-capsular mutants somewhat easier.

3) In contrast to the other K.pneumoniae strains, 5055 is naturally Ap^S which provides a rapid and straightforward mechanism for the removal of pRT733 cointegrate events.

All 850 Km^R colonies of strain 5055 were sub-cultured onto solid Worfel-Ferguson growth medium (containing kanamycin) in order to maximise capsular polysaccharide production. Following 48 hours of growth at 37^oC, plates were examined and cultures which appeared less mucoid than the parent strain were taken for further analysis. A total of three apparently non-mucoid Km^R mutants were isolated from this preliminary screening and

designated as K.pneumoniae 5055-114, 5055-480 and 5055-797. Each of these presumptive non-capsular mutants was then subjected to a battery of tests in order to verify that production of the K2 polysaccharide had been abolished. A further Km^R mutant, 5055-507 was found to have become deficient in urease production (Ure^-). It was decided to use this as a control strain for subsequent experiments.

4.3 Immunological Analysis Of Prospective Mutants

4.3.1 Double immunodiffusion analysis

The production of K2 capsular polysaccharide was tested using a double immunodiffusion procedure. Whole cells of each strain were tested against an anti-K.pneumoniae K2 rabbit antiserum which had been absorbed with the non-capsular derivative strain M10 (K^-O^+), thus rendering the antiserum monospecific towards the K-antigen. The results (Figure 4.1) show that only the control strain 5055-507 gave a positive reaction. This also indicates that the antiserum is indeed monospecific since there is no reaction with either M10 (K^-O^+) or M10B (K^-O^-).

4.3.2 Counter-current immunoelectrophoresis and quellung analysis

Counter-current immunoelectrophoresis (Palfreyman, 1978) and quellung analysis (Kauffmann, 1949) were carried out on the presumptive mutants both locally and independently at the Central Public Health Laboratory, Colindale. The results (Table 4.1) were identical and show that the K^+ strain 5055-507 reacts positively against the corresponding anti-K2 sera whereas each of the three prospective non-capsular mutants elicits a negative reaction.

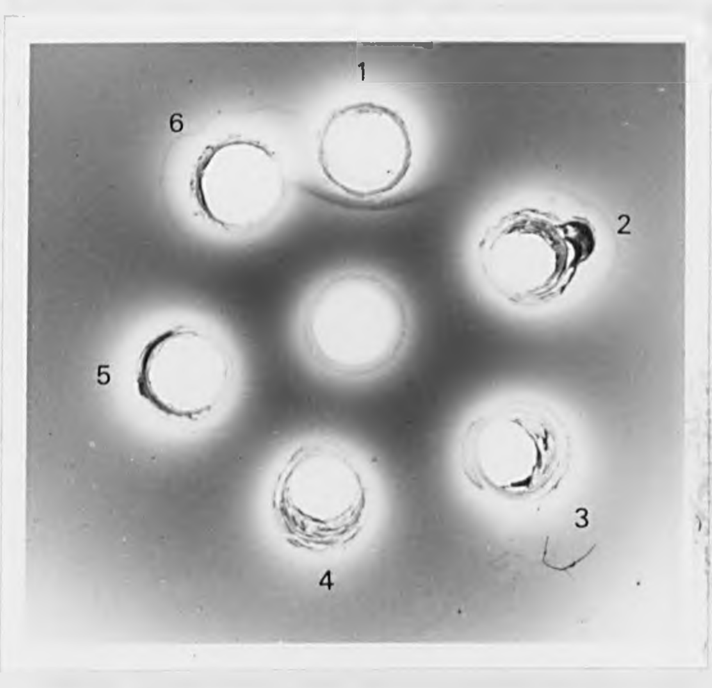


Figure 4.1: Double immunodiffusion analysis of K2 capsular polysaccharide production in prospective K.pneumoniae 5055 non-capsular mutants using anti-K2 monospecific rabbit antiserum.

Wells: 1 = K.pneumoniae 5055-507 whole cells.
2 = K.pneumoniae 5055-114 whole cells.
3 = K.pneumoniae 5055-480 whole cells.
4 = K.pneumoniae 5055-797 whole cells.
5 = K.pneumoniae M10 whole cells.
6 = K.pneumoniae M10B whole cells.

Central well = Monospecific anti-K2 antiserum.

Strain	Reaction			
	CIE		Quellung	
	Liverpool	Colindale	Liverpool	Colindale
5055-507	K2	K2	K2	K2
M10	NT	NT	NT	NT
5055-114	NT	NT	NT	NT
5055-480	NT	NT	NT	NT
5055-797	NT	NT	NT	NT

Table 4.1: Capsular serotyping of K.pneumoniae mutants using counter-current immunoelectrophoresis (CIE) and the quellung reaction.

NT = not typeable

4.3.3 Immune electron microscopy analysis

Immuno-gold labelling with an anti-K2 CPS monoclonal antibody (Trautmann et al, 1988) was used in conjunction with electron microscopy to detect the presence of K2 capsular polysaccharide on the mutant strains. Figure 4.2a clearly shows the aggregation of gold particles around the 5055-507 cell exterior. This was presumed to be a direct result of binding of the anti-mouse antibody-gold conjugate to the K.pneumoniae capsular polysaccharide via the anti-CPS monoclonal antibodies. None of the prospective non-capsular mutants strains showed any appreciable concentration of gold-labelled particles around the cell surface (Figures 4.2b-d). A control experiment in which the anti-CPS monoclonal antibody was omitted showed the same random dispersal of gold particles across the field as in Figures 4.2b-d.

4.4 Electron Microscopy Analysis

Transmission electron microscopy of ruthenium red stained sections of strain 5055-507 revealed the existence of the extracellular capsular polysaccharide, visualized as a somewhat indistinct material surrounding the cell (Figure 4.3a). In contrast, electron micrographs of strains 5055-114, 5055-480 and 5055-797 (Figure 4.3b-d) showed that each of these strains appeared to lack the capsular material.

When taken together, the results from immunological and electron microscopy studies indicate that each of the three prospective mutants is indeed non-capsular. The use of transmission electron microscopy to observe ruthenium red stained cells provides evidence that the mutants are actually failing to express capsular polysaccharide on their cell surfaces rather than producing an immunologically altered form of the K-antigen.

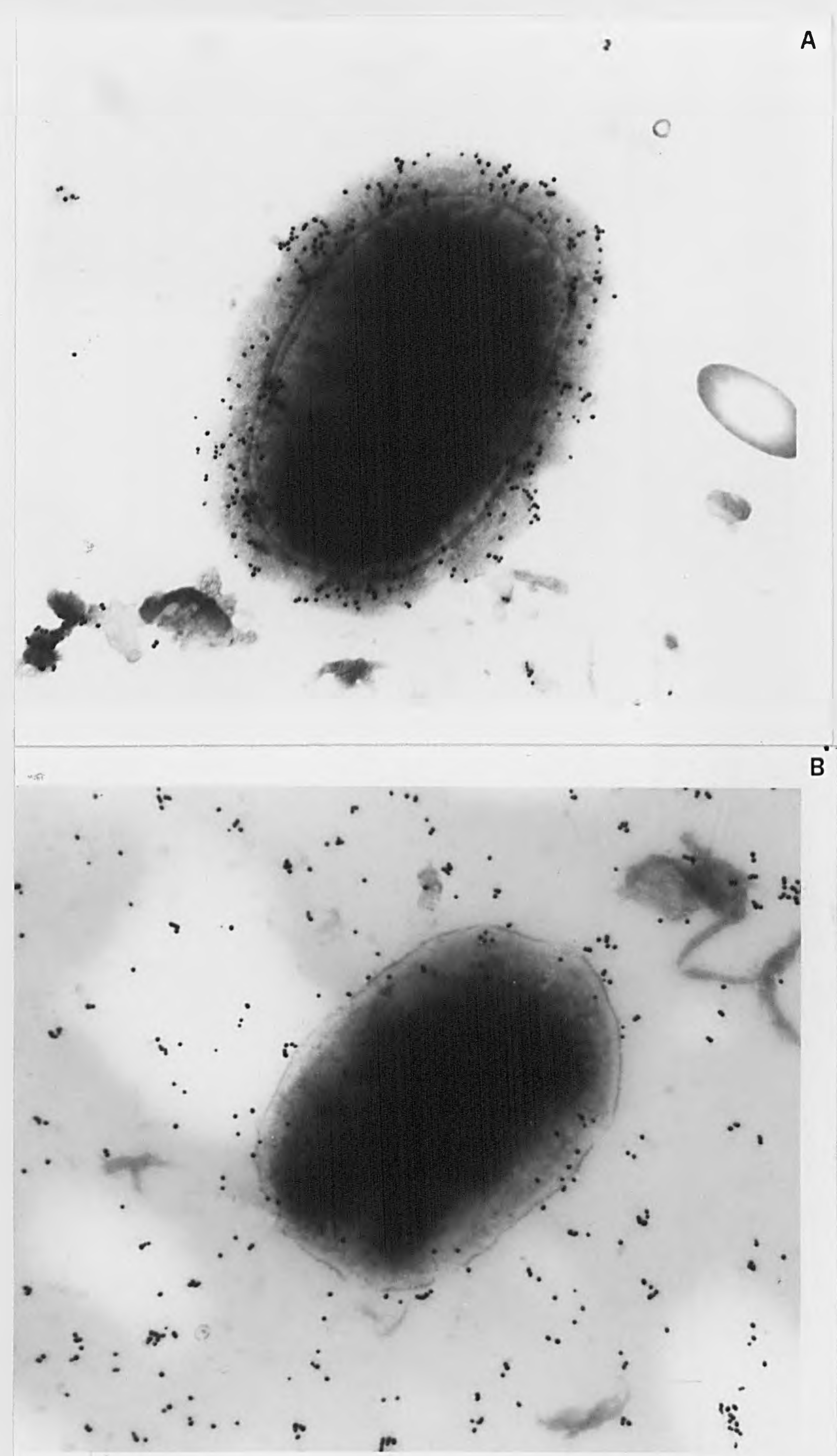
Figure 4.2: Immuno-gold labelling of prospective K.pneumoniae non-capsular mutants using anti-Klebsiella K2 capsular polysaccharide monoclonal antibody.

(a) : 5055-507 (x50000)

(b) : 5055-114 (x50000)

(c) : 5055-480 (x30000)

(d) : 5055-797 (x30000)


A
B

C

D

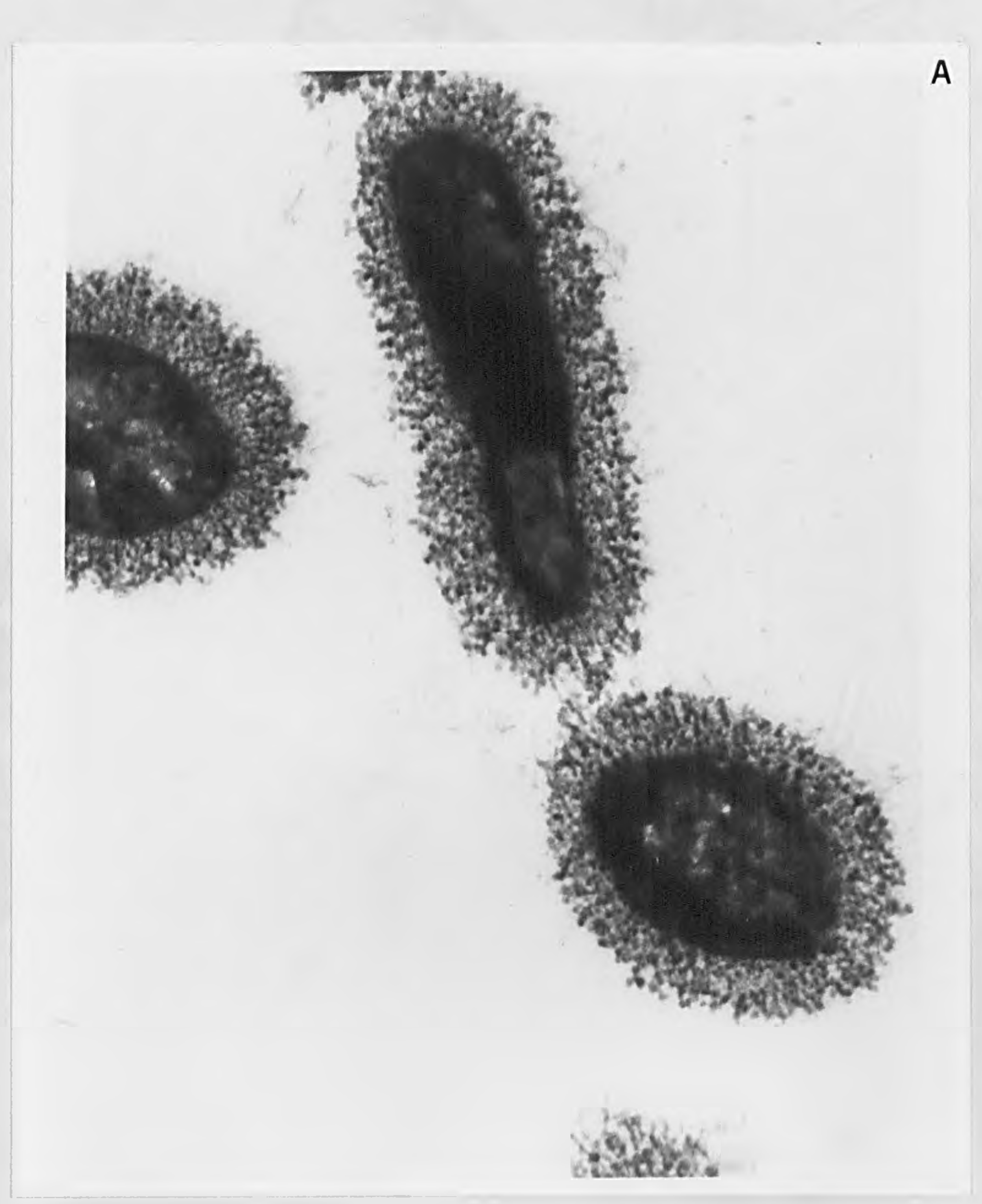
Figure 4.3: Electron micrographs of thin sections of ruthenium red-stained K.pneumoniae capsular and prospective non-capsular mutant strains.

(a) : 5055-507 (x50000)

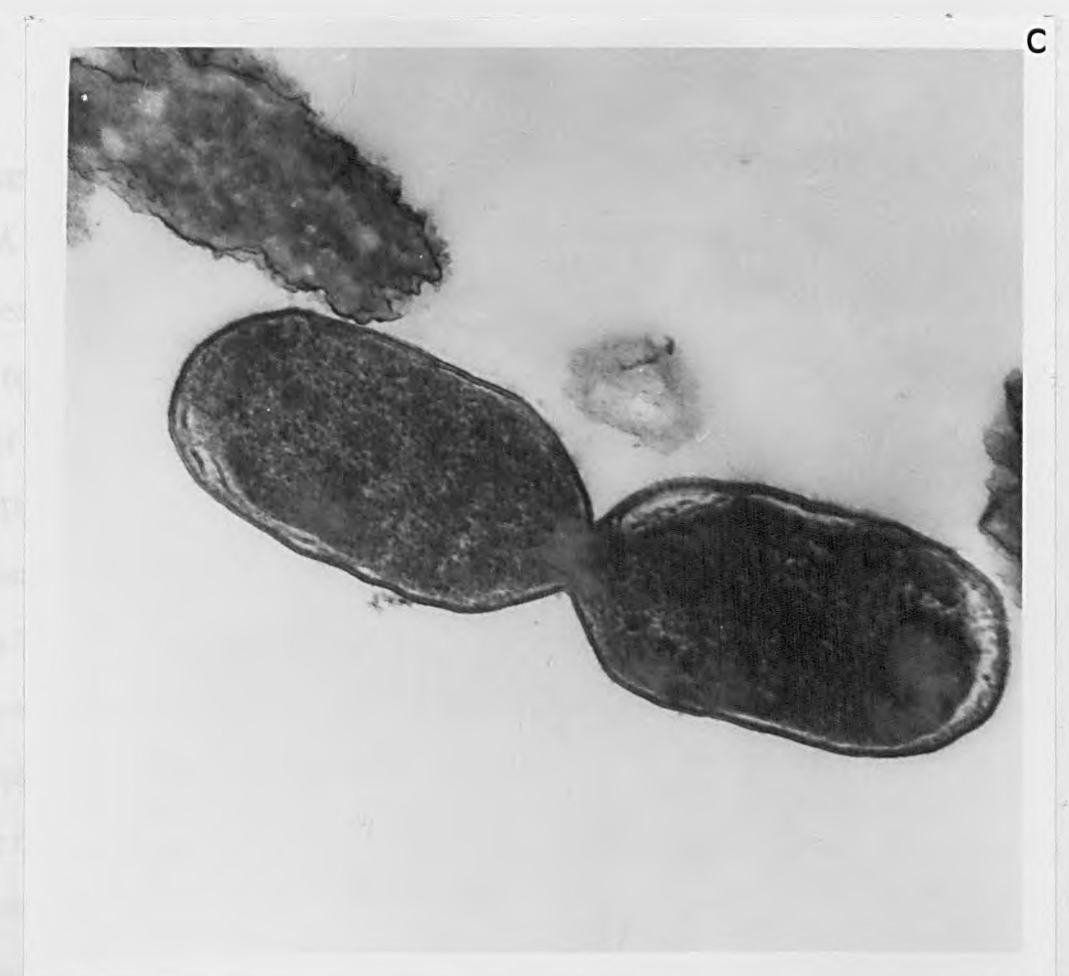
(b) : 5055-114 (x100000)

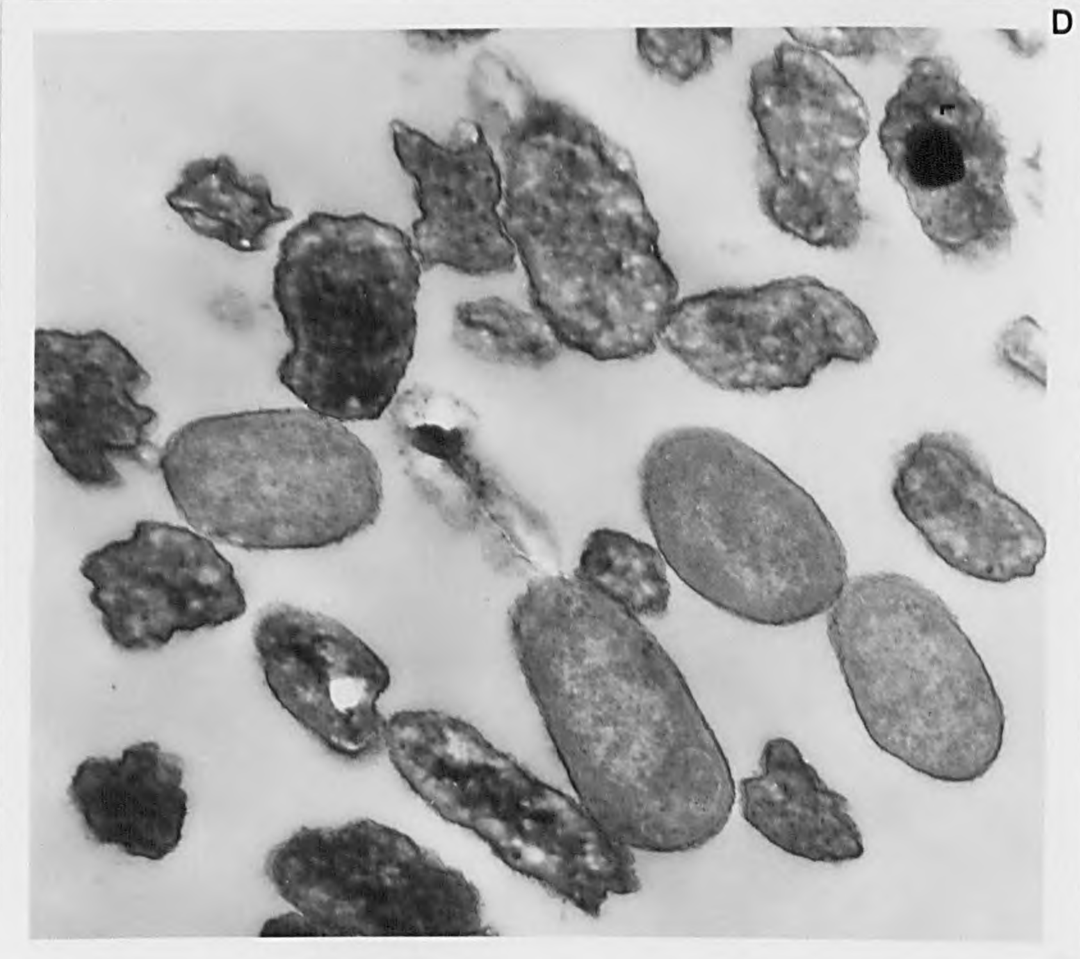
(c) : 5055-480 (x50000)

(d) : 5055-797 (x30000)


A

B


C


D

4.5 Phage Sensitivity Analysis

A bacteriophage typing scheme for Klebsiella isolates has been developed by Gaston et al (1987) and is in regular use as a secondary typing method by the Central Public Health Laboratory, Colindale, to follow the course of a possible epidemic (B.Ayling-Smith, pers. comm).

This system was used independently to type K.pneumoniae 5055 control and non-capsular mutant strains (Table 4.2). The phages shown are part of a 15 phage typing set and are those which gave at least one weak reaction with one of the strains tested. The receptors for each of these bacteriophages are unknown. However, from these results it would seem possible that phage 12 may have a receptor site within the capsular polysaccharide matrix since strong positive reactions occur with K^+ strains while there is no reaction with K^- strains. Equally, phage 4 may have a receptor site that is masked by the CPS such as LPS, since only the K^- strains gave positive reactions. Consequently, phages 4 and 12 may prove useful during other studies as a rapid means to identify the presence or absence of K.pneumoniae K2 polysaccharide on the cell surface. Further work in order to determine the actual receptor sites for both bacteriophage would, however, be required. Strain M10 is a non-capsular mutant generated by chemical mutagenesis. This procedure may have introduced other mutations which could account for the differences seen between this strain and the non-capsular transposon mutants when infected with phages 11 and 13. Infection of these strains with phage 7 was interesting because all three levels of reaction were exhibited within the three non-capsular mutants. The reasons for this are unclear.

Strain	Phages							
	2	3	4	6	7	11	12	13
5055 (K^+O^+)	++	++	-	++	-	++	++	+/-
5055-507 (K^+O^+)	++	++	-	++	-	++	++	+/-
M10 (K^-O^+)	++	++	++	++	+/-	-	-	-
5055-114 (K^-O^+)	++	++	++	++	++	-	-	+/-
5055-480 (K^-O^+)	++	++	++	++	+/-	+/-	-	+/-
5055-797 (K^-O^+)	++	++	++	++	-	+/-	-	+/-

Table 4.2: Phage sensitivity analysis of <u>K.pneumoniae</u> 5055 capsular and non-capsular strains.

++ = Strong reaction (well-defined, clear plaques)

+/- = Weak reaction (irregular, cloudy plaques)

- = No reaction

4.6 Southern Hybridisation Analysis

Although available information seems to indicate that most of the genetic material required for capsular biosynthesis is held on the chromosome of K.pneumoniae (Arakawa et al, 1991: Laakso et al, 1988), other plasmid-encoded determinants may be required for the full expression of the capsule in other species (Barr, 1981; Nassif et al, 1989; McCallum and Whitfield, 1991 and Arakawa et al, 1991).

K.pneumoniae strain 5055 carries a large plasmid which has not been characterised in any way. Southern hybridisation analysis using a 3.4 kilobase internal HindIII fragment of TnphoA as a probe, was used to detect possible transposon insertions into plasmid DNA isolated from each of the non-capsular mutants. The results (Figure 4.4a+b) show that the non-capsular mutant phenotype was not a result of TnphoA insertion into this resident plasmid. This does not however rule out the possibility that the plasmid may contain other information necessary for capsule production.

Southern analysis of chromosomal DNA from the three mutants digested with EcoRV, which does not cut within TnphoA, revealed initially that mutants 114 and 797 contained a single insertion while mutant 480 had three TnphoA insertions. However, subsequent experiments showed that mutant 114 contained a second copy of TnphoA within its chromosomal DNA due to secondary transposition (Figure 4.5a+b). Neither of the other mutants contained further copies of TnphoA.

Mutant strains 5055-114 and 5055-480 appear to share two common sites of insertion in identical EcoRV fragments, at least one of which must be involved in capsule production. However, strain 5055-797 contains an insertion in a seperate EcoRV fragment. Therefore, the genes for capsule production in K.pneumoniae strain 5055 must be organised into at least two EcoRV fragments.

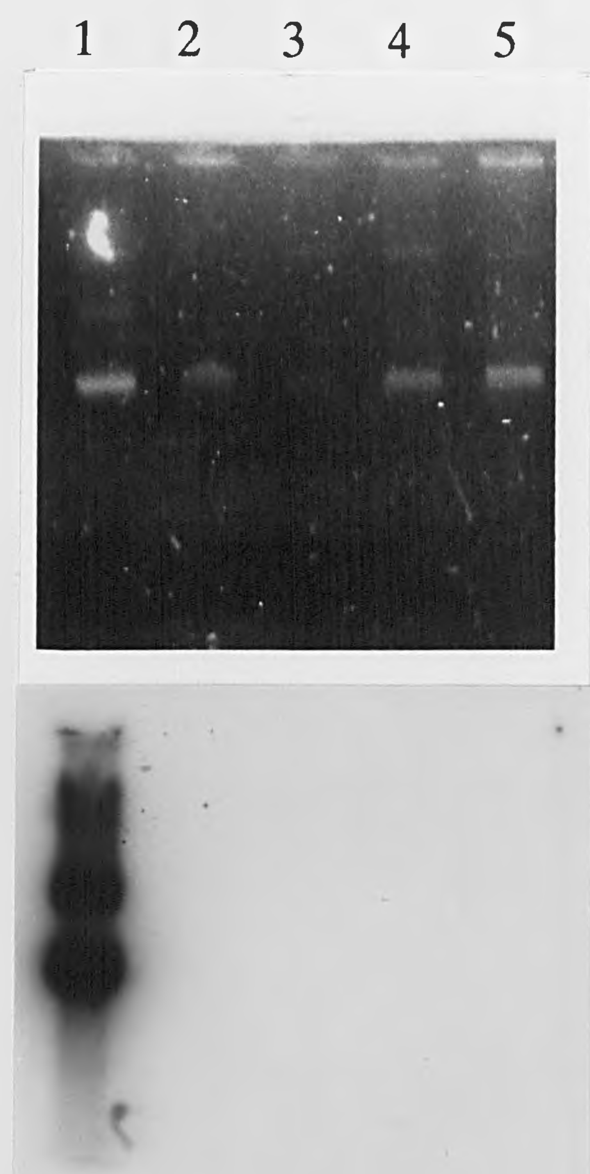
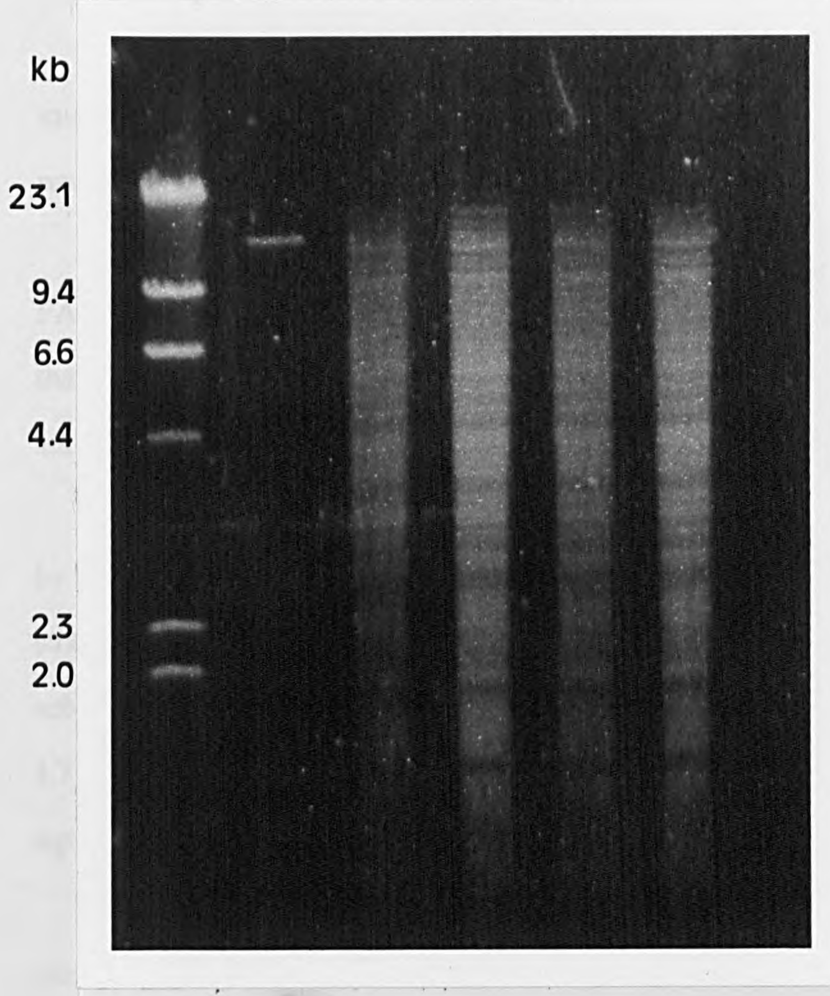


Figure 4.4 a+b: Gel electrophoresis and Southern hybridisation to detect Tn*pho*A insertions into *K.pneumoniae* plasmid DNA using a 3.4kb *Hin*dIII fragment from Tn*pho*A as a probe.

Lanes: 1 = pRT733 (uncut)
2 = *K.pneumoniae* 5055 plasmid DNA (uncut)
3 = *K.pneumoniae* 5055-114 plasmid DNA (uncut)
4 = *K.pneumoniae* 5055-480 plasmid DNA (uncut)
5 = *K.pneumoniae* 5055-797 plasmid DNA (uncut)

Figure 4.5 a+b: Gel electrophoresis and Southern hybridisation of EcoRV-digested chromosomal DNA from K.pneumoniae 5055 and non-capsular derivatives using 3.4kb HindIII fragment from TnphoA as a probe.

Lanes: 1 = Lambda DNA/HindIII
2 = pRT733/EcoRV
3 = 5055/EcoRV
4 = 5055-114/EcoRV
5 = 5055-480/EcoRV
6 = 5055-797/EcoRV


1 2 3 4 5 6
kb
23.1
9.4
6.6
4.4
2.3
2.0
13.0
9.0

4.7 Examination Of The Outer Membrane

The outer membranes from each of the non-capsular transposon mutants were characterized in order to ensure that each was isogenic with the parental strain in all other respects.

Outer membrane proteins were isolated and examined using SDS-PAGE as described in Materials and Methods. The results (Figure 4.6) show that there are no major discernible differences between the parent 5055 strain and any of the non-capsular mutants.

Similarly, lipopolysaccharide was extracted (Osborn, 1966) and analysed by SDS-PAGE as described in Materials and Methods. LPS from mutant strain 5055-797 shows an identical profile to that of the parent with the O-substituted LPS seen above the fast-migrating lipid A core region (Figure 4.7). Interestingly, this strain also appears to contain the HMW-LPS antigen reported by McCallum et al (1989).

The usual ladder pattern seen in LPS could not be resolved despite variation of gel conditions and the amount of LPS loaded. Mutants 5055-114 and 5055-480 also exhibited an identical LPS pattern to the parental strain (data not shown).

4.8 Preliminary Analysis Of Location Of Transposon Insertions

The intracellular contents of the non-capsular mutant strains were released by sonication and by alkaline lysis. There was no accumulation of K2 capsular polysaccharide within the cells as judged by double immunodiffusion analysis using a monospecific anti-K2 CPS antiserum. This would seem to indicate insertion of transposon DNA into K.pneumoniae CPS structural or major regulatory genes rather than genes involved in the translocation of capsular polysaccharide across the cell membrane.

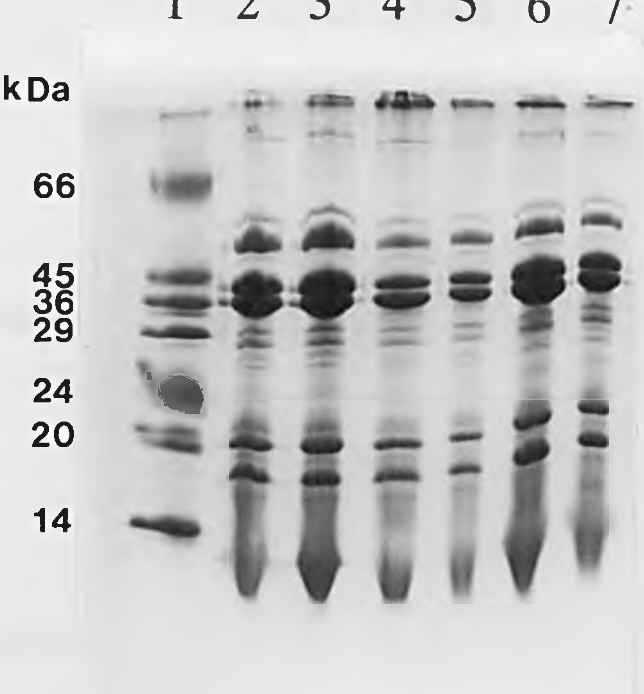


Figure 4.6: SDS-PAGE of outer membrane proteins from K.pneumoniae capsular and non-capsular strains.

Lanes: 1 = Molecular weight protein standards
2 = 5055
3 = M10
4 = M10B
5 = 5055-114
6 = 5055-480
7 = 5055-797

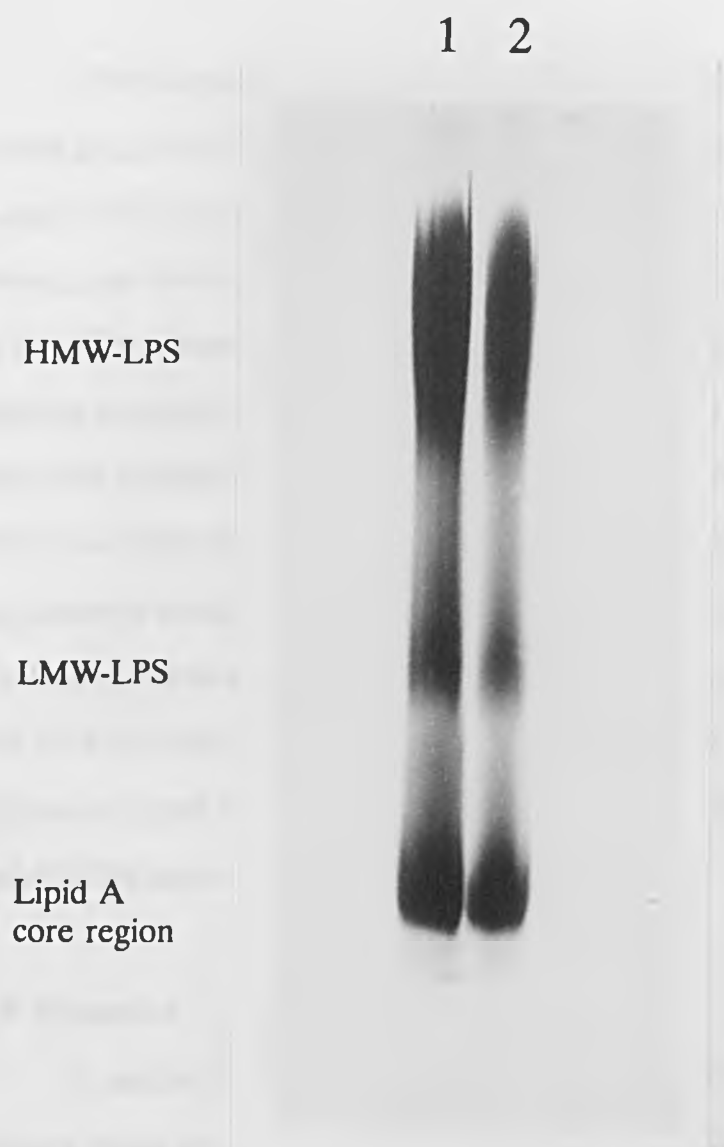


Figure 4.7: SDS-PAGE of lipopolysaccharide from K.pneumoniae capsular and non-capsular mutant strains.

Lanes: 1 = 5055
2 = 5055-797

The plasmid pLV213 contains the rcsA gene from K.pneumoniae K21 (Allen et al, 1987) and has been shown to induce colanic acid production in E.coli at 30°C. The rcsA gene from a K.pneumoniae K20 strain has been shown to be involved in the expression of capsular polysaccharide by virtue of its ability to complement a K20 mutant producing only minute amounts of capsular polysaccharide (McCallum and Whitfield, 1991). The rcsA genes from both serotypes have precisely the same nucleotide sequences. Mutants 5055-114, 5055-480 and 5055-797 were transformed with pLV213 (Cm^R) in an attempt to complement the mutations which led to loss of capsule production. However, no 5055-114 (pLV213), 5055-480 (pLV213) or 5055-797 (pLV213) transformants regained the ability to elicit a K2 polysaccharide capsule as judged by double immunodiffusion analysis using a monospecific anti-K2 CPS antiserum.

4.9 Discussion

A number of groups have reported the isolation of K.pneumoniae mutants which are deficient in capsular polysaccharide biosynthesis (Poxton and Sutherland, 1976; Simoons-Smit et al, 1986; Benedi et al, 1989 and Merino et al, 1989). However in each of these cases, the non-capsular derivatives have been discovered either as spontaneous K^- mutants or after chemical or UV mutagenesis. Thus, the genetic basis for the loss of capsule could not be precisely identified. The insertion of a transposon into the K.pneumoniae capsular biosynthetic genes will also give rise to K^- colonies, except that such mutants have the potential to be mapped with regard to the insertion location, thereby allowing isolation of the genes involved in capsule production. Apart from the recently published work of Arakawa et al (1991), this is the first report of the successful generation of otherwise isogenic, non-capsulated mutants of K.pneumoniae using transposon mutagenesis.

Although multiple Tn*pho*A insertions into chromosomal DNA are not unusual (Donnenberg *et al*, 1990) it was disappointing that only one of the non-capsular mutants 5055-797, had a unique insertion site. Nevertheless, this derivative should prove extremely useful in later studies.

The non-capsular mutants generated during this study were devoid of intracellular K2 CPS accumulation although only a low number of mutants were tested. Arakawa *et al* (1991), also found no evidence of K2 capsular polysaccharide accumulation within the cells of non-capsular K2 transposon mutants. They suggest that the *K.pneumoniae* *cps* gene cluster may be organised diffferently to that of the much studied *E.coli* group II capsular polysaccharide genes and may resemble that of the *E.coli* group I capsules.

CHAPTER 5 : Genetic Analysis of Non-Capsular Mutants

5.1 Introduction

Non-capsular mutants of K.pneumoniae 5055 have been generated using transposon mutagenesis. By virtue of the antibiotic resistance phenotype (Km^R) and the fact that the structure of TnphoA is well known (Rothstein et al, 1980; Manoil and Beckwith, 1985 and Chang et al, 1986), the presence of the transposon gives a clearly identifiable "handle" by which it should prove possible to analyse the DNA flanking the insertion, in this case the Klebsiella genes involved in K2 capsular biosynthesis.

Using specifically chosen restriction enzymes, all or part of the transposon plus flanking regions from one or both sides can be isolated. This will enable a small fragment of Klebsiella DNA to be used to probe a gene library of the parental strain and identify a fully intact Klebsiella cps gene cluster (Arakawa et al, 1991) which could be used to determine the genetic basis of Klebsiella capsule production.

The results in this chapter describe the various strategies which have been employed to clone transposon DNA plus flanking regions from the chromosomal DNA of K.pneumoniae non-capsular transposon mutants. A preliminary analysis of these clones is also presented.

5.2 Attempts to Clone TnphoA plus Klebsiella DNA Using the Cosmid Vector pHC79

The cosmid vector pHC79 (Hohn and Collins, 1980) contains the genes encoding ampicillin and tetracycline resistance and is suitable for the cloning of DNA fragments of up to 40kb. Sau3A-chromosomal fragments from 5055-797 of approximately 25kb and above were purified using sucrose gradients (Maniatis, 1982), ligated into the BamHI site of pHC79 at a molar ratio of approximately 5:1 (vector:fragments) and packaged into phage λ heads as described in Materials and Methods. The Km^R phenotype was used

as a direct selection criteria for clones containing the kan gene of TnphoA. However, within a library of approximately 500 clones (containing inserts), no Km^R colonies were isolated.

5.3 Attempts to Clone TnphoA plus Klebsiella DNA Using Plasmid Vectors

Initial cloning experiments concentrated on using those restriction enzymes which do not cut within TnphoA, for example XbaI, SacI and EcoRV (Taylor et al, 1989). Such enzymes can be utilized to clone the complete transposon plus DNA flanking both ends of the insertion. Gene libraries of XbaI and SacI-digested chromosomal DNA from 5055-797 and 5055-114 were constructed in E.coli LE392 using pUC19 while pBR322 was employed for EcoRV libraries. Despite the isolation of up to 1200 clones per library, no Km^R recombinants were found.

Taylor et al, (1989) used BamHI to isolate fusion joints between phoA and their gene of interest. BamHI and SalI both cut within TnphoA, but at sites distal to the kan gene, thus not disrupting its expression in a suitable host (Figure 5.1). The use of such enzymes will allow flanking DNA to be isolated from one side of the transposon insertion only. A number of enzymes of this type were used in conjunction with pUC19 and/or pBR322 for cloning experiments, but only pUC19/EcoR1 libraries yielded Km^R LE392 recombinants. From the 5055-797 gene library of approximately 1900 clones, 2 Km^R colonies were isolated and recombinant plasmids contained within them were designated pLV750 and pLV751. A library of approximately 2100 clones from strain 5055-114 gave rise to 6 Km^R colonies. Single EcoR1, BamHI and HindIII restriction digests of plasmid DNA revealed that of the 6 5055-114 clones, only 3 were unique and these were designated pLV752, pLV753 and pLV754.

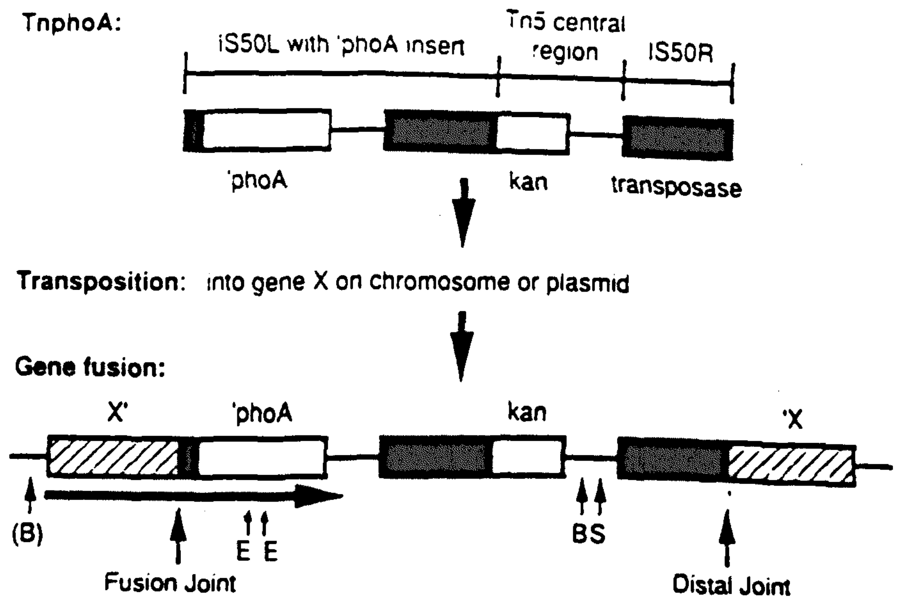


Figure 5.1: Transposon mutagenesis using Tn*pho*A.

Restriction sites: B = *Bam*HI

S = *Sal*I

E = *Eco*RI

Not all restriction sites on Tn*pho*A are shown.

(From Taylor *et al*, 1989).

5.4 Characterisation of TnphoA Recombinant Plasmids

Southern blot analysis of digested plasmid DNA, using the 3.4kb HindIII fragment of TnphoA as a probe, confirmed the presence of TnphoA sequences in each of the recombinant plasmids (Figure 5.2a+b).

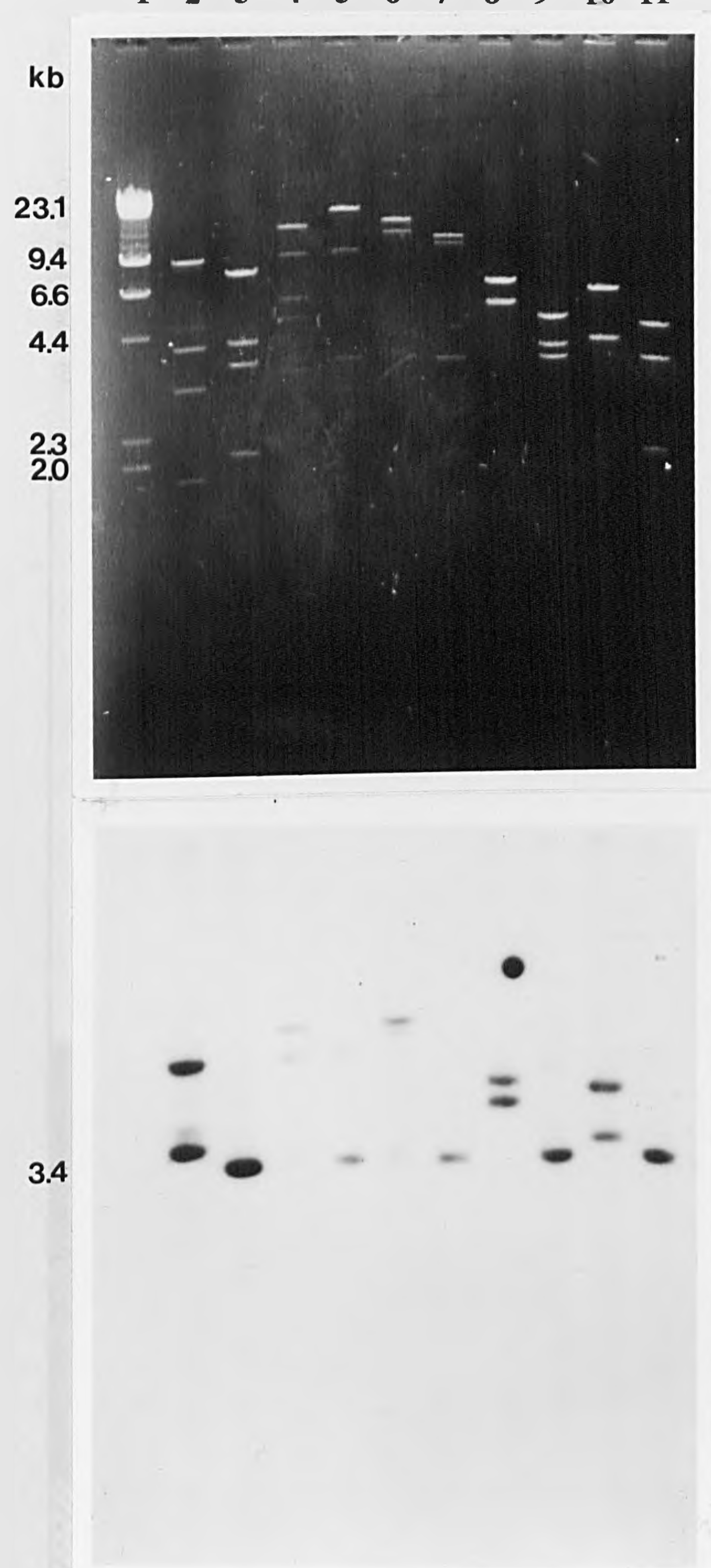
Restriction digest analysis revealed the clones to be carrying inserts of between approximately 7.5kb (pLV754) and 36.3kb (pLV751) and allowed the construction of preliminary restriction maps for pLV750, pLV753 and pLV754 (Figure 5.3). These 3 plasmids appear to represent each of the 3 TnphoA insertions under investigation (1 in 5055-797 and 2 in 5055-114). It was not possible to distinguish at this stage which of the insertions in 5055-114 was responsible for eliminating capsule biosynthesis, or indeed if both were necessary.

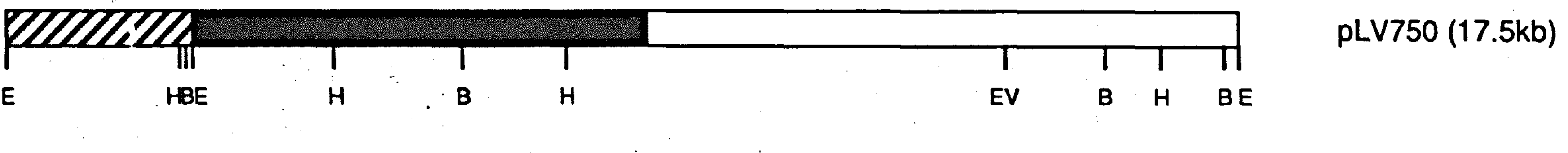
A fragment or sub-clone of pLV750 containing the 8.3kb of Klebsiella chromosomal DNA would appear to be the best choice as an initial probe with which to screen a cosmid gene bank of 5055 genomic DNA. However, pLV753 and pLV754 may be useful in later, more advanced studies.

Recombinant plasmids pLV751 and pLV752 were extremely unstable probably due to the large inserts of Klebsiella DNA contained within them. These plasmids were not mapped.

Figure 5.2 a+b: Gel electrophoresis and Southern hybridisation of recombinant plasmids pLV750, pLV751, pLV752, pLV753 and pLV754 using 3.4kb HindIII fragment from TnphoA as a probe.

Lanes: 1 = Lambda DNA/HindIII
2 = pLV750/BamHI
3 = pLV750/HindIII
4 = pLV751/BamHI
5 = pLV751/HindIII
6 = pLV752/BamHI
7 = pLV752/HindIII
8 = pLV753/BamHI
9 = pLV753/HindIII
10 = pLV754/BamHI
11 = pLV754/HindIII


1
2
3
4
5
6
7
8
9
10
11
kb
23.1
9.4
6.6
4.4
2.3
2.0
3.4



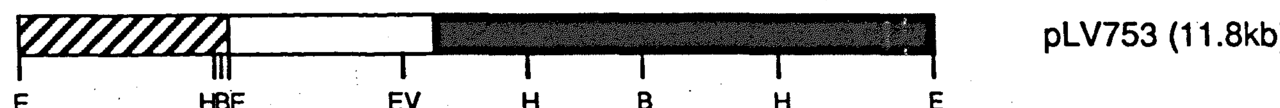


Figure 5.3: Linearised restriction maps of recombinant plasmids pLV750, pLV753 and pLV754

1kb

= pUC19 sequences

= Tn*pho*A sequences

= *Klebsiella* chromosomal DNA sequences

Restriction enzyme sites:
E = *Eco*RI
B = *Bam*HI
H = *Hind*III
EV = *Eco*RV

5.5 Complementation of Non-Capsular Mutations

In an attempt to complement the mutations , each of the 5 recombinant plasmids was introduced, by transformation, into non-capsular mutants strains 5055-114, 5055-480, 5055-797 and M10 with the following exceptions: pLV750 and pLV751 were not used to transform 5055-797 and 5055-114 was not transformed with pLV752, pLV753 and pLV754. In no case were transformants observed to have regained the ability to express Klebsiella K2 capsular polysaccharide.

5.6 Discussion

The single TnphoA insertion which abolished capsular polysaccharide production has been cloned from K.pneumoniae 5055-797 together with a portion of Klebsiella DNA flanking the insertion to one side. Although unproven at this stage, it is hoped that this region represents an integral component of the capsular biosynthesis machinery which can be used to probe a cosmid gene library in order to isolate the intact gene cluster necessary for the production of Klebsiella K2 capsular polysaccharide.

Arakawa et al (1991) used a 15kb probe from a transposon mutant in similar experiments, but could not isolate the complete cps gene cluster from a gene library of several thousand clones. It was later discovered that recombinant plasmids carrying the cps gene cluster caused the clone to grow so slowly that it could not be distinguished from satellite colonies. This group did, however, isolate a recombinant plasmid carrying a 23kb DNA fragment containing a single Tn5 insertion from a non-capsular mutant of K.pneumoniae strain Chedid, a serotype K2 reference strain. This plasmid, designated pCPS7B06, had the ability to produce a K2 capsule on the surface of K.pneumoniae non-capsulated mutants of other serotypes. From this evidence, they concluded that pCPS7B06, together with an unidentified gene,

gene X, provided the complete genetic information necessary for the biosynthesis of Klebsiella K2 capsular polysaccharide. Gene X was thought to have been disrupted in the chromosome of the mutant strain from which pCPS7B06 had been isolated. Complementation by an intact gene X in other serotype mutants allowed pCPS7B06 to convert the non-capsular variants to $K2^+$.

E.coli HB101 required the presence of rmpA (Nassif et al, 1989), in addition to pCPS7B06, to allow the production of K2 capsular polysaccharide, although the presence of the K2 capsule did not increase the virulence of the E.coli strain. It was also speculated that the organisation of Klebsiella capsule genes may be similar to that of E.coli group I capsular polysaccharide.

Unfortunately, attempts during this study to complement non-capsular mutants with the recombinant plasmids were unsuccessful. This was perhaps unsurprising given that pLV750, pLV753 and pLV754 contain only relatively small inserts of between 1.0kb and 8.4kb and that the inserts in pLV753 and pLV754 may contain Klebsiella DNA which has no involvement in capsular biosynthesis.

Arakawa et al (1991) also suggested that the Klebsiella cps gene cluster appeared to be relatively well conserved among various K2 isolates. Given that this is the case, it would be interesting to compare intact cps genes from 5055 with those from Chedid. This would give a valuable insight into the genetic basis of K2 capsular polysaccharide production in Klebsiella and further studies could involve comparisons with other capsule-producing species such as E.coli, N.meningitis and H.influenzae.

CHAPTER 6: Influence of the K.pneumoniae K-Antigen on Serum Killing and Phagocytosis

6.1 Introduction

Previous studies using either spontaneous K^- mutants or those derived from chemical or UV mutagenesis have generally advanced the theory that the O-antigen provides the necessary protection from complement-mediated serum killing in K.pneumoniae and that the K-antigen plays little or no role (Tomas et al, 1986; Williams et al, 1983 and McCallum et al, 1989). Capsular polysaccharide does however appear to be the sole factor in mediating resistance of K.pneumoniae to phagocytosis in the presence of complement (Williams et al, 1983, 1986 and Simoons-Smit et al,1986).

Transposon mutagenesis has allowed the isolation of non-capsular mutants with an insertion at a single site within the gene of interest. Such mutants will be isogenic with the parental strain in all other respects and will permit the contribution of the capsular polysaccharide in resistance to serum killing and phagocytosis to be determined for K.pneumoniae.

6.2 Influence of the K.pneumoniae K-antigen on Serum Killing

Serum resistance experiments were carried out as described in Materials and Methods and the results are shown in Figure 6.1. The parental strain K.pneumoniae 5055 grew rapidly in fresh non-immune pooled human serum (PHNS) while the non-capsular mutants M10 and 5055-797 also grew, but at a slower rate than the parental strain. This is in contrast to the results of Williams et al (1983) who found that normal human serum was bacteriostatic for M10. The vast majority of the M10B population was rapidly killed by the effects of PHNS within 30 minutes (under 1% survival). However, after approximately 2 hours the population began to increase in number again. Reversion of K.pneumoniae strains from O^- to O^+ in non-immune serum has been reported previously (Williams and Tomas et al, 1990) and it is postulated that the observed rise in M10B numbers was due to

the growth of spontaneous serum-resistant, possibly O^+, mutants of M10B. Further analysis, involving the isolation and characterisation of the LPS from serum-resistant mutants would be necessary in order to confirm this theory. No evidence of this phenomenon has been documented in previous studies with this strain. K^+ revertants of M10, M10B or 5055-797 were not found following incubation in PHNS. All four strains grew rapidly in heat-inactivated serum.

6.3 Influence of the K.pneumoniae K-antigen on Phagocytosis

Each of the four strains 5055, M10, 5055-797 and M10B was tested for susceptibility to phagocytosis by PMNL's either with or without pre-opsonisation of the bacterial cells using a luminol-enhanced chemiluminesence system (Allen,1977).

Only strain M10B exhibited a recognisable chemiluminesence (CL) response when incubated with PMNL's without pre-opsonisation (Figure 6.2a).

Figure 6.2b shows that pre-incubation with non-immune human serum led to the production of significant CL responses for both unencapsulated strains (M10 and 5055-797) as well as M10B, confirming the findings of others in suggesting non-capsulate K.pneumoniae strains can be sufficiently opsonised by complement to allow phagocytosis by PMNL's (Williams et al, 1983,1986). Strain 5055 did not produce any detectable PMNL CL response above that of the control (PMNL's + no bacteria, Figure 6.2b) and required pre-opsonisation with antiserum containing anti-CPS antibodies in order to produce a CL response (Figure 6.2c).

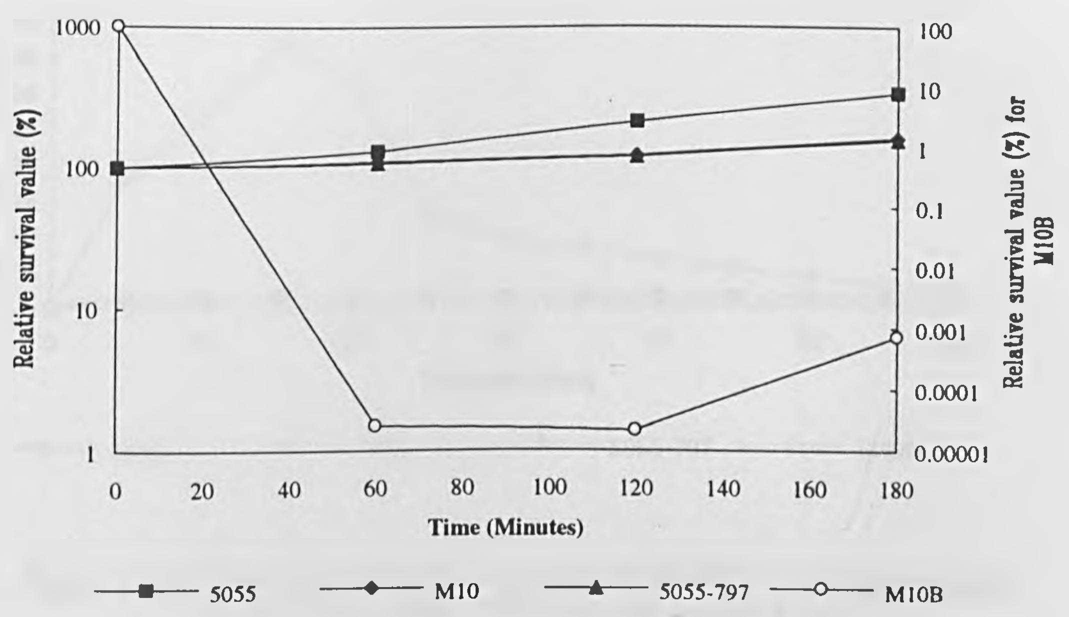


Figure 6.1: Effect of non-immune human serum upon K.pneumoniae strains 5055, M10 5055-797 and M10B.

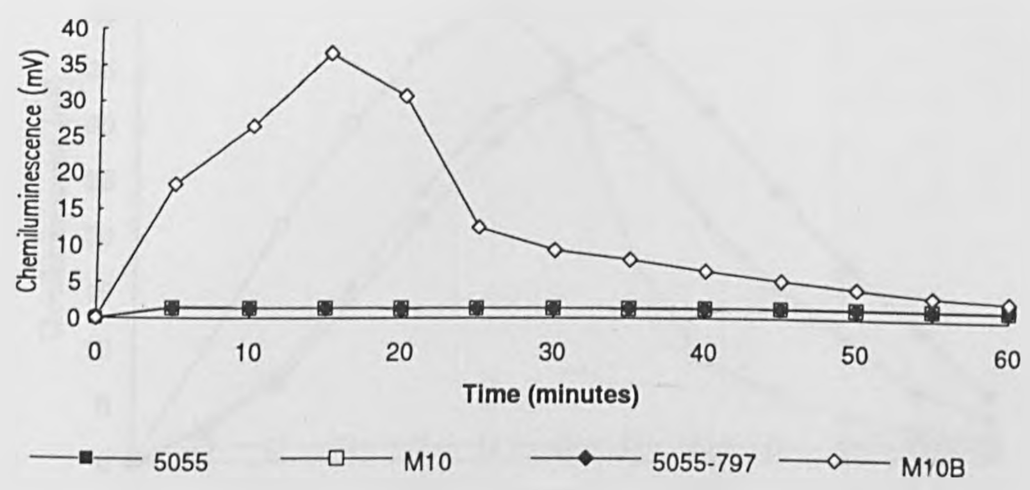


Figure 6.2a: Chemiluminescent response of PMNL's to K.pneumoniae strains 5055, M10, 5055-797 and M10B in the absence of serum pre-treatment.

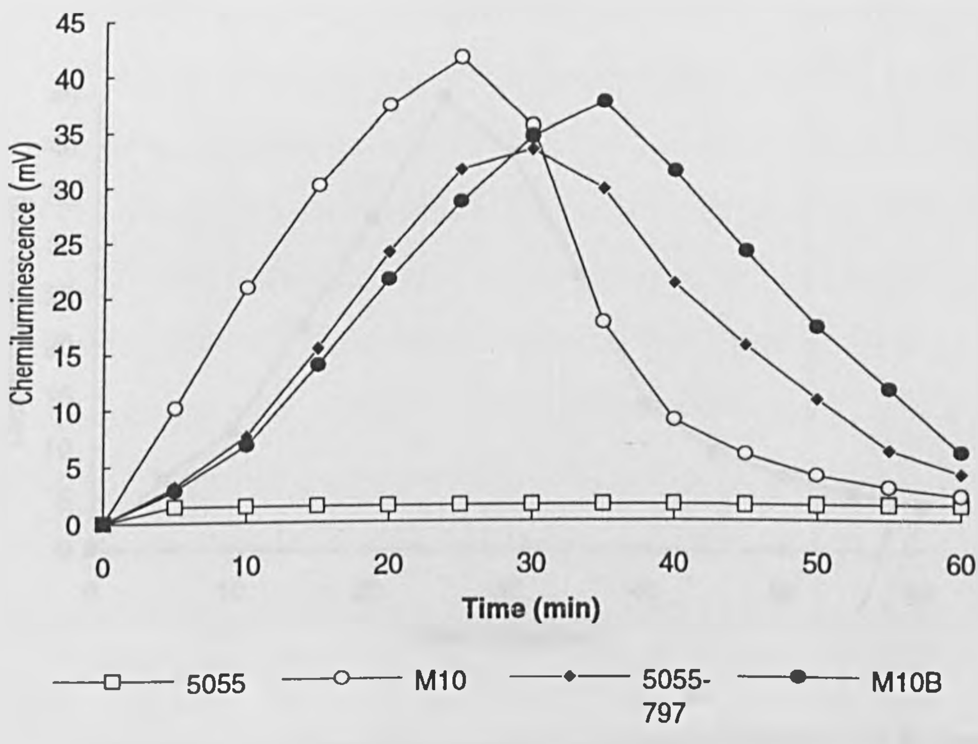


Figure 6.2b: Chemiluminescent response of PMNL's to K.pneumoniae strains 5055, M10, 5055-797 and M10B following opsonisation with non-immune human serum.

Serum concentration = 30%

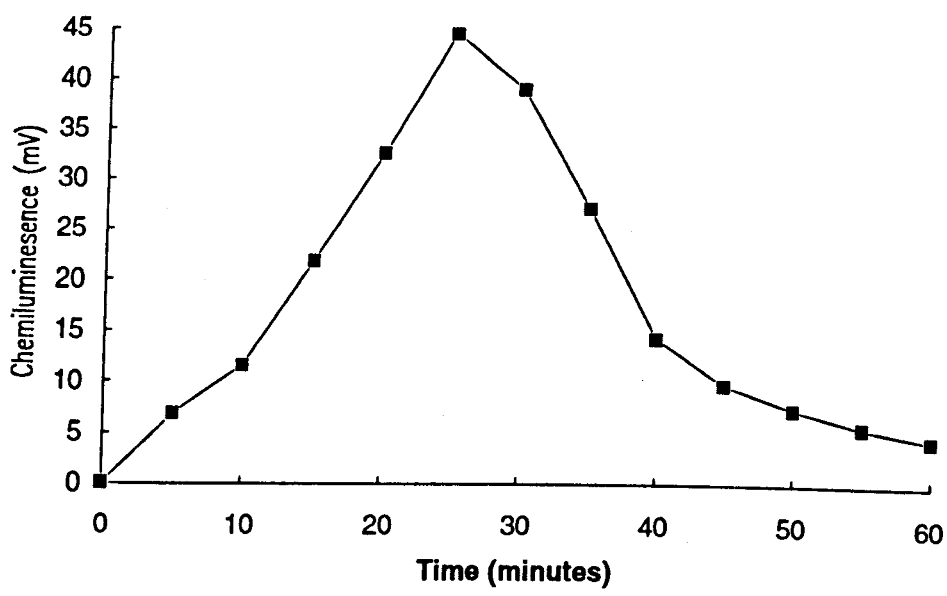


Figure 6.2c: Chemiluminescent response of PMNL's to K.pneumoniae 5055 following opsonisation with monospecific anti-Klebsiella K2 CPS antiserum.

Serum concentration = 30%

6.4 Discussion

The results of this study have confirmed the findings of a number of other authors (Tomas et al, 1986; Williams et al, 1983 and McCallum et al, 1989) in observing that the capsular polysaccharide of K.pneumoniae of strain 5055 does not determine resistance to the effects of normal human serum. It may however play some minor role in view of the fact that 5055 grew at a faster rate in PNHS than either M10 or 5055-797. These findings did differ somewhat from those of Williams et al (1983) in that they found PNHS to be bacteriostatic for M10 whereas this study showed that M10 and 5055-797 both grew reasonably well in this environment. This may be due to differences in the method of preparation of the bacteria for the respective experiments. Following the results of their work, Williams et al (1983) recommended that future studies of this type should involve using bacteria grown under conditions resembling those found in vivo. All strains for these experiments were grown on M9 minimal media containing 5% heat-inactivated serum, thus allowing the bacteria to adapt and perhaps to grow more quickly when introduced into PNHS.

The majority of a population of strain M10B cells were rapidly killed by the effects of PNHS adding further weight to the argument that it is actually the O-antigen which is responsible for serum resistance. However, having been reduced to a very low level, they then began to divide again suggesting possible O^- to O^+ reversion. The fact that no M10, M10B or 5055-797 K^+ revertants were found on preliminary screenings on Worfel-Ferguson media suggests again that the O-antigen is of primary importance in determining serum resistance. More specifically, McCallum et al (1989) have demonstrated that a high-molecular-weight (HMW) fraction of the Klebsiella O1 LPS is critical in determining serum resistance possibly by virtue of the ability of these long chain LPS molecules to bind complement at a distance

away from the cell surface. K.pneumoniae is known to contain this HMW-LPS (P.Williams pers. comm. to J.R. Saunders) although the contribution to serum resistance of this virulence determinant was not fully assessed during the course of this work.

In agreement with Williams et al (1986) the presence of the O-antigen was also found to be important in protecting the cells from phagocytosis in the absence of complement. However when complement was present, unencapsulated K.pneumoniae cells were opsonised and became susceptible to phagocytosis. Expression of capsular polysaccharide by strain 5055 served to fully protect the cells from phagocytosis in the presence or absence of complement. Only when this strain was pre-opsonised with a K2-CPS specific monoclonal antibody did phagocytosis occur. K.pneumoniae CPS is therefore able either to block activation or deposition of complement onto the cell surface thus preventing opsonisation (Williams and Tomas, 1990). Although the precise mechanism by which this is achieved is not yet clear, C3b is not thought to be deposited on the surface of encapsulated strains (Williams, 1987) nor is K66-CPS able to activate complement in vitro (Ciurana and Tomas, 1987).

Chapter 7: Closing Discussion

Attempts have been made to generate non-capsular transposon mutants in K.pneumoniae using a number of different delivery strategies. No single system has proved to be entirely suitable for all strains of K.pneumoniae, although the majority enjoyed some degree of success amongst a narrow host range.

Stable non-capsular mutants of K.pneumoniae 5055 were finally generated using the suicide vector pRT733 (pJM703.1::TnphoA; Taylor et al, 1989). Auxotrophs and urease-negative mutants were also generated with relative ease and it is believed that this system could be widely applied to studies of other virulence determinants in K.pneumoniae. In particular, investigations into extracytoplasmic proteins could be enhanced bearing in mind the facility of TnphoA activity when part of a gene fusion that leads to the expression of membrane, periplasmic, outer membrane or extracellular hybrid proteins. This would only be suitable provided that non-phosphatase producing strains were available.

It is a distinct possibility that some of the gene products involved in the expression of capsular polysaccharide on the Klebsiella cell surface may be periplasmic or outer membrane proteins in which case TnphoA could prove to be a particularly useful tool in future analyses of the precise nature and function of such proteins.

A large number of Klebsiella clinical isolates remained refactory to the transposon mutagenesis systems used during this work and other regimes, such as the R388 rep(ts)::Tn5 system (Sasakawa and Yoshikawa, 1987) used successfully in K.pneumoniae by Arakawa et al (1991), may need to be utilized. Alternatively, it may be necessary to develop new delivery systems and/or transposons in order to facilitate similar genetic studies of these strains.

The TnphoA fusion joints were cloned from the non-capsular mutants chromosomes into pUC19. Unfortunately, insufficient time was available to analyse these recombinant plasmids other than to produce preliminary restriction maps. This was extremely disappointing since a detailed analysis coupled with further experiments had originally been envisaged.

As mentioned previously further work could include the use of these plasmids or sub-clones thereof to probe a cosmid gene library of K.pneumoniae 5055 in the hope of identifying an intact capsule biosynthesis region. The isolation of such a region would then allow direct comparisons to be made with pCPS7B06 (Arakawa et al, 1991).

Plasmid pCPS7B06 carries a 23kb region of Klebsiella DNA that is sufficient to allow the production of K2 capsular polysaccharide in non-capsular mutants of various serotypes of K.pneumoniae. Detailed studies of such clones may provide information as to the organisation of those genes involved in capsular biosynthesis and expression. For instance, it is unknown whether they are organised in a similar fashion to the E.coli group II capsular polysaccharides (Boulnois et al, 1987) or in a quite different manner as has been speculated by Arakawa et al (1991).

In addition to pCPS7B06, the rmpA gene (Nassif et al, 1989) was necessary in order to elicit Klebsiella K2 capsule production on the E.coli cell surface (Arakawa et al, 1991). Up until this report, it was thought that two distinct regulatory systems operated to control discrete aspects of extracellular polysaccharide production in Klebsiella. The rcsA gene (Allen et al, 1987) is known to be involved in the expression of type-specific K-antigen (McCallum and Whitefield, 1991) while rmpA was believed only to play a role in controlling the expression of a seperate mucoid polysaccharide (Nassif et al, 1989). The rmpA gene has so far only been identified in strains of Klebsiella which carry a large virulence plasmid. However, some strains

of Klebsiella do not carry any large plasmids (Whitfield and McCallum, 1991). Clarification of the precise nature and function of both of these putative regulatory systems is therefore a prerequisite to the understanding of any relationship between them and indeed to the full comprehension of capsular expression in Klebsiella.

RcsA proteins have also been isolated from E.coli (Gottesman et al, 1985), Erwinia amylovora (Coleman et al, 1990) and Erwinia stewartii (Torres-Cabassa et al, 1987). There appear to be high degrees of homology between the E.coli, Klebsiella and E.amylovora RcsA proteins (Coleman et al, 1990; Torres-Cabassa, unpublished results). All have been shown to perform similar functions to the Klebsiella RcsA protein, acting either directly or indirectly as a transcriptional activator in the biosynthesis of extracellular polysaccharide.

Sequencing of the cloned Klebsiella DNA from the relevant recombinant plasmids would be relatively straightforward and may also yield information as to possible functions for these areas of the chromosome. However, this is unlikely to be of any real value without the isolation of the full genetic machinery necessary for capsule production.

Results presented in this study here have confirmed the work of other authors in identifying the role of K.pneumoniae K2 capsular polysaccharide in protection against phagocytosis by PMNL'S (Williams et al, 1983; Simoons-Smit et al, 1986 and Ciurana et al, 1987).

This work has however, contrasted with previous reports in its use of transposon-induced K^- mutants. Such mutants are more likely to be isogenic in all other respects than K^- mutants derived from chemical mutagenesis. The growth conditions used during the study probably also simulate conditions in vivo to a greater extent than those used previously.

The minor or null role for K.pneumoniae K2 capsular polysaccharide in resistance to the bactericidal effects of serum was also confirmed (Williams et al, 1983; Simoons-Smit et al, 1986 and Tomas et al, 1986).

BIBLIOGRAPHY

Albesa, I. (1989). Klebsiella pneumoniae haemolysin adsorption to red blood cells. Journal of Applied Bacteriology **67**, 263-266.

Allen, P. M. (1986). Genetics and role in pathogenicity of Klebsiella capsules. Ph.D thesis, University of Liverpool, Liverpool, UK.

Allen, P. M., Fisher, D., Saunders, J. R. and Hart, C. A. (1987). The role of capsular polysaccharide K21b of Klebsiella and of the structurally related colanic-acid polysaccharide of Escherichia coli in resistance to phagocytosis and serum killing. Journal of Medical Microbiology **24,** 363-370.

Allen, P. M., Hart, C. A. and Saunders, J. R. (1987). Isolation from Klebsiella and characterization of two rcs genes that activate colanic acid capsular biosynthesis in Escherichia coli. Journal of General Microbiology **133,** 331-340.

Allen, R. C. (1977). Evaluation of serum opsonic capacity by quantitating the initial chemiluminescent response from phagocytizing polymorphonuclear leukocytes. Infection and Immunity **15**, 828-833.

Appleyard, R. K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from Escherichia coli K12. Genetics **39,** 440.

Amako, K., Meno, Y. and Takade, A. (1988). Fine structures of the capsules of Klebsiella pneumoniae and Escherichia coli K1. Journal of Bacteriology **170,** 4960-4962.

Arakawa, Y., Ohta, M., Wacharotayankun, R., Mori, M., Kido, N., Ito, H., Komatsu, T., Sugiyama, T. and Kato, N. (1991). Biosynthesis of Klebsiella K2 capsular polysaccharide in Escherichia coli HB101 requires the functions of rmpA and the chromosomal cps gene cluster of the virulent strain Klebsiella pneumoniae Chedid (01:K2). Infection and Immunity **59**, 2043-2050.

Archibald, A. R. (1980). Virus receptors, Part 1, Bacterial Viruses. Chapman and Hall, London.

Athana, A., Ofek, I. and Keisari, Y. (1989). Lectinophagocytosis-mediated by interaction between a macrophage surface lectin and capsular polysaccharides of Klebsiella pneumoniae. 89th Annual Meeting of the American Society for Microbiology, New Orleans USA (Abstract B104).

Auerswald, E. A., Ludwig, G. and Schaller, H. (1981). Structural analysis of Tn5. Cold Spring Harbor Symp. Quant. Biol. **45**, 107-113.

Baer, H., Bringaze, J. K. and McNamee, M. (1954). The effect of the doses of bacterial polysaccharide antigen in antibody production in mice. Journal of Bacteriology **67**, 123-127.

Barr, J. G. (1981). Spontaneous loss and transfer of plasmid-mediated K21 antigen synthesis in Klebsiella pneumoniae. Journal of Clinical Pathology **34**, 434-438.

Batshon, B. A., Baer, H. and Shaffer, M. F. (1963). Immunological paralysis in mice by *Klebsiella pneumoniae* type 2 polysaccharide. Journal of Immunology **90**, 121-6.

Bauernfeind, A. (1984). Epidemiological typing of *Klebsiella* by bacteriocins. Methods in Microbiology **16**, 213-228.

Belas, R., Mileham, A., Simon, M. and Silverman, M. (1984). Transposon mutagenesis of marine *Vibrio* spp. Journal of Bacteriology **158**, 890-896.

Bender, R. A., Snyder, P. M., Bueno, R., Quinto, M. and Magasanik, B. (1983). Nitrogen regulation system of *Klebsiella aerogenes*: the *nac* gene. Journal of Bacteriology **156**, 444-446.

Benedi, V. J., Ciurana, B. and Tomas, J. M. (1989). Isolation and characterization of *Klebsiella pneumoniae* unencapsulated mutants. Journal of Clinical Microbiology **27**, 82-87.

Berg, D. E. (1977). DNA insertion elements, plasmids and episomes. Eds Bukhan, A.I., Shapiro, J.A. and Adyha, S.L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 205-212.

Bloom, F. R. and Tyler, B. (1979). Introduction of bacteriophage Lambda into *Klebsiella aerogenes*. Journal of Bacteriology **139**, 680-682.

Bodey, G. P., Elting, L. S., Rodriguez, S. and Herandez, M. (1989). *Klebsiella* Bacteremia. Cancer **64**, 2368-2376.

Bolivar, R., Rodriguez, R. L., Greene, P. J., Berlach, M. C., Heynecker, H. L. and Boyer, H. W. (1977). Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene **2**, 95-113.

Bopp, L. H., Baltch, A. L., Hammer, M. C., Franke, M. A., Smith, R. P. and Lutz, F. (1991). Isolation and characterization of a transposon-induced cytotoxin-deficient mutant of Pseudomonas aeruginosa. Infection and Immunity **59**, 708-711.

Boulnois, G. J. and Jann, K. (1989). Bacterial polysaccharide capsule synthesis, export and evolution of structural diversity. Molecular Microbiology **3**, 1819-1823.

Boulnois, G. J. and Roberts, I. S. (1990). Genetics of capsular polysaccharide production in bacteria. Current Topics in Microbiology and Immunology **150**, 1-18.

Boulnois, G. J., Roberts, I. S., Hodge, R., Hardy, K. R., Jann, K. B. and Timmis, K. N. (1987). Further analysis of the cloned K1 capsule biosynthesis genes of Escherichia coli: definition of three functional regions for capsule production. Molecular and General Genetics **200**, 242-246.

Boulnois, G. J., Varley, J. M., Sharpe, G. S. and Franklin, C. H. (1985). Transposon donor plasmids, based on ColIb-P9, for use in Pseudomonas putida and a variety of other Gram negative bacteria. Molecular and General Genetics **200**, 65-67.

Boyer, H. W. and Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in Escherichia coli. Journal of Molecular Biology **41**, 459-472.

Brill, J. A., Quinlan-Walshe, C. and Gottesman, S. (1988). Fine-structure mapping and identification of two regulators of capsule synthesis in Escherichia coli K-12. Journal of Bacteriology **170**, 2599-2611.

Broom, M. J. and Beebe, R. D. (1988). Emphysematous septic arthritis due to Klebsiella pneumoniae. Clinical Orthopaedics and Related Research **226**, 219-221.

Brown, M. G. M., Weston, A., Saunders, J. R. and Humphreys, G. O. (1979). Transformation of Escherichia coli C600 by plasmid DNA at different phases of growth. FEMS Microbiology Letters **5**, 219-222.

Bryan, C. S., Reynolds, K. L. and Brenner, E. R. (1983). Analysis of 1186 episodes of gram-negative bacteremia in non-university hospitals: The effects of antimicrobial therapy. Rev. Infect. Dis. **5**, 629-643.

Brun-Buisson, C., Legrand, P., Philippon, A., Montravers, F., Ansquer, M. and Duval, J. (1987). Transferable enzymatic resistance to third-generation cephalosporins during nosocomial outbreak of multiresistant Klebsiella pneumoniae. The Lancet **2**, 302-306.

Bullen, J. J. and Griffiths, E. (1987). Iron and Infection -Molecular, Physiological and Clinical Aspects. John Wiley and Sons, Chichester, UK.

Camprubi, S., Regue, M. and Tomas, J. (1989). The influence of lipopolysaccharide on the transformation efficiency of Klebsiella pneumoniae. Canadian Journal of Microbiology **35**, 735-737.

Camprubi, S., Sabater, E., Regue, M. and Tomas, J. M. (1989). The infuence of the O-antigen and the capsular polysaccharide on the establishment of P1Cmts lysogeny in Klebsiella pneumoniae. FEMS Microbiology Letters **60**, 67-70.

Casewell, M. and Talsania, H. G. (1979). Predominance of certain Klebsiella capsular types in hospitals in the United Kingdom. Journal of Infection **1**, 77-79.

Chang, C. N., Kuang, W-J and Chen, E. Y. (1986). Nucleotide sequence of the alkaline phosphatase gene of Escherichia coli. Gene **44**, 121-125.

Choy, Y. M. and Dutton, G. G. S. (1973). The structure of the capsular polysaccharide from Klebsiella K-type 21. Canadian Journal of Chemistry **51**, 198-207.

Ciurana, B. and Tomas, J. M. (1987). Role of lipopolysaccharide and complement in susceptibility of Klebsiella pneumoniae to non-immune serum. Infection and Immunity **55**, 2741-2746.

Ciurana, B., Williams, P. and Tomas, J. M. (1987). Physico-chemical surface properties and phagocytosis of Klebsiella pneumoniae K66:01.

Molecular Biology and Infectious Diseases, Centenary Symposium of the Institut Pasteur, Paris, France.

Clement, J. M. and Hofnung, M. (1981). Gene sequence of the λ receptor, an outer membrane protein of E.coli K12. Cell **27,** 507-514.

Close, J. C. and Rodriguez, R. L. (1982). Construction and characterization of the chloramphenicol resistance gene cartridge: a new approach to the transcriptional mapping of extrachromosomal elements. Gene **20,** 305-316.

Coleman, M., Pearce, R., Hitchin, E., Busfield, F., Mansfield, J. W. and Roberts, I. S. (1990). Molecular cloning, expression and nucleotide sequence of the rcsA gene of Erwinia amylovora, encoding a positive regulator of capsule expression: evidence for a family of related capsule activator proteins. Journal of General Microbiology **136,** 1799-1806.

Connolly, J. C., McLean, C. and Tabaqchali, S. (1984). The effect of capsular polysaccharide and lipopolysaccharide of Bacteroides fragilis on polymorph function and serum killing. Journal of Medical Microbiology **17,** 259-271.

Cryz, S. J. (1990). Klebsiella polysaccharide vaccines. In Bacterial Vaccines, Alan R. Liss Inc, 87-103.

Cryz, S. J., Furer, E. and Germanier, R. (1983). Simple model for the study of Pseudomonas aeruginosa infections in leukopenic mice. Infection and Immunity **39,** 1067-1072.

Cryz, S. J., Furer, E. and Germanier, R. (1984). Experimental Klebsiella pneumoniae burn wound sepsis: Role of capsular polysaccharide. Infection and Immunity **43**, 440-441.

Cryz, S. J., Furer, E. and Germanier, R. (1986). Immunisation against fatal experimental Klebsiella pneumoniae pneumonia. Infection and Immunity **54**, 403-407.

Cryz, S. J., Mortimer, P. M., Mansfield, V. and Germanier, R. (1986). Seroepidemiology of Klebsiella bacteremic isolates and implications for vaccine development. Journal of Clinical Microbiology **23**, 687-690.

Darveau, R. P. and Hancock, R. E. W. (1983). Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough Pseudomonas aeruginosa and Salmonella typhimurium strains. Journal of Bacteriology **155**, 831-838.

De Vries, G. E., Raymond, C. K. and Ludwig, R. A. (1984). Extension of bacteriophage lambda host range: Selection, cloning and characterization of a constitutive lambda receptor gene. Proceedings of the National Academy of Sciences USA **81**, 6080-6084.

DiJoseph, C. G., Bayer, M. E. and Kaji, A. (1973). Host cell growth in the presence of the thermosensitve drug resistance factor, Rts1. Journal of Bacteriology **115**, 399-410.

DiJoseph, C. G. and Kaji, A. (1974). Molecular nature of the deoxyribonucleic acid of a thermosensitve R factor, Rts1. Journal of Bacteriology **120,** 1364-1369.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980). Broad host range DNA cloning systems for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proceedings of the National Acadamy of Science, USA **77,** 7347-7351.

Domenico, P., Diedrich, D. L. and Straus, D. C. (1985). Extracellular polysaccharide production by Klebsiella pneumoniae and its relationship to virulence. Canadian Journal of Microbiology **31,** 472-478.

Domenico, P., Hopkins, T. and Cunha, B. A. (1990). The effect of sodium salicylate on antibiotic susceptibility and synergy in Klebsiella pneumoniae. Journal of Antimicrobial Chemotherapy **25,** 343-351.

Domenico, P., Johanson, W. G. Jr. and Straus, D. C. (1982). Lobar pneumonia in rats produced by clinical isolates of Klebsiella pneumoniae. Infection and Immunity **37,** 327-335.

Domenico, P., Schwartz, S. and Cunha, B. A. (1989). Reduction of capsular polysaccharide production in Klebsiella pneumoniae by sodium salicylate. Infection and Immunity **57,** 3778-3782.

Donnenberg, M. S., Calderwood, S. B., Donohue-Rolfe, A., Keusch, G. T. and Kaper, J. B. (1990). Construction and analysis of TnphoA mutants of

enteropathogenic Escherichia coli unable to invade HEp-2 cells. Infection and Immunity **58,** 1565-1571.

Drake, C. R., Roberts, I. S., Jann, B., Jann, K. and Boulnois, G. J. (1990). Molecular cloning and expression of the genes encoding the Escherichia coli K4 capsular polysaccharide, a fructose-substituted chondroitin. FEMS Microbiology Letters **66,** 227-230.

Duguid, J. P. (1951). The demonstration of bacterial capsules and slime. Journal of Pathology and Bacteriology **63,** 673-683.

Duguid, J. P. (1959). Fimbriae and adhesive properties in Klebsiella strains. Journal of General Microbiology **21,** 271-286.

Dutton, G. G. S. and Folkman, T. E. (1980). Structural investigation of the capsular polysaccharide of Klebsiella serotype K44. Carbohydrate Research **78,** 305-315.

Dutton, G.G.S. and Yang, M.T. (1972). 4, 6-0-(1-carboxyethylidene)-D-Mannose as a structural unit in capsular polysaccharide of Klebsiella K-type5. Canadian Journal of Chemistry **50,** 2382-2384.

Echarti, C., Hirschel, B., Boulnois, G. J., Varley, J. M., Waldvogel, F. and Timmis, K. N. (1983). Cloning and analysis of the K1 capsule biosynthesis genes of Escherichia coli: Lack of homology with Neisseria meningitidis group B DNA sequences. Infection and Immunity **41,** 54-60.

Edwards, S. W., Say, J. E. and Hart, C. A. (1987). Oxygen-dependent killing of Staphylococcus aureus by human neutrophils. Journal of General Microbiology **133,** 3591-3597.

Ehrenworth, L. and Baer, H. (1956). The pathogenicity of Klebsiella pneumoniae for mice: the relationship to the quantity and rate of production of type-specific capsular polysaccaride. Journal of Bacteriology **72,** 713-717.

Ellard, F. M., Cabello, A. and Salmond, G. P. C. (1989). Bacteriophage λ–mediated transposon mutagenesis of phytopathogenic and epiphytic Erwinia species is strain dependent. Molecular and General Genetics **218,** 491-498

Fader, R. C. and Davis, C. P. (1980). Effect of piliation on Klebsiella pneumoniae infection in rat bladders. Infection and Immunity **30,** 554-561.

Fader, R. C. and Davis, C. P. (1982). Klebsiella pneumoniae induced experimental pyelitis: the effect of piliation on infectivity. Journal of Urology **128**, 197-201.

Filip, C., Fletcher, G., Wulff, J. L. and Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. Journal of Bacteriology **115,** 717-722.

Finke, A., Roberts, I., Boulnois, G., Pazzani, C. and Jann, K. (1989). Activity of CMP-2-keto-3-deoxyoctulosonic acid synthetase in Escherichia coli strains expressing the capsular K5 polysaccharide: implication for K5 polysaccharide biosynthesis. Journal of Bacteriology **171,** 3074-3079.

Frosch, M., Weisgerber, C. and Meyer, T. F. (1989). Molecular characterization and expression in Escherichia coli of the gene complex encoding the polysaccharide capsule of Neisseria meningitidis group B. Proceedings of the National Academy of Science USA **86,** 1669-1673.

Gacesa, P., Leaves, G. S. and Weightman, A. J. (1987). Genetic analysis of Klebsiella pneumoniae alginate lyase by transposon Tn10 mutagenesis. Hydrobiologia **151/152,** 571-575.

Gahan, L. C., Sandford, P. A. and Conrad, H. E. (1967). The structure of the serotype 2 capsular polysaccaride of Aerobacter aerogenes. Biochemistry **6,** 2755-2766.

Garcia de la Torre, M., Romero-Vivas, J., Martinez-Beltran, J., Guerrero, A., Meseguer, M. and Bouza, E. (1985). Klebsiella bacteremia: An analysis of 100 episodes. Reviews of Infectious Diseases **7,** 143-150.

Gaston, M. A., Ayling-Smith, B. A. and Pitt, T. L. (1987). New bacteriophage typing scheme for subdivision of the frequent capsular serotypes of Klebsiella spp. Journal of Clinical Microbiology **25,** 1228-1232.

Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. (1981). Manual of methods for general bacteriology. American Society for Microbiology, USA.

Gerlach, G. F. and Clegg, S. (1988a). Cloning and characterization of the gene cluster encoding type 3 (MR/K) fimbriae of Klebsiella pneumoniae. FEMS Microbiology Letters **49,** 377-383.

Gerlach, G. F. and Clegg, S. (1988b). Characterization of two genes encoding antigenetically distinct type-1 fimbriae of Klebsiella pneumoniae. Gene **64,** 231-240.

Glynn, A. A. and Howard, C. J. (1970). The sensitivity to complement of strains of Escherichia coli related to their K antigens. Immunology **18,** 331-346.

Goldberg, M. B., DiRita, V. J. and Calderwood, S. B. (1990). Identification of an iron-regulated virulence determinant in Vibrio cholerae, using TnphoA mutagenesis. Infection and Immunity **58,** 55-60.

Goldberg, R. B., Bender, R. A. and Streicher, S. L. (1974). Direct selection for P1-sensitive mutants of enteric bacteria. Journal of Bacteriology **118,** 810-814.

Gottesman, S., Trisler, P. and Torres-Cabassa, A. (1985). Regulation of capsular polysaccharide synthesis in Escherichia coli K-12: characterisation of three regulatory genes. Journal of Bacteriology **162,** 1111-1119.

Grossman, N., and Leive, L. (1984). Complement activation via the alternative pathway by purified Salmonella lipopolysaccharide is affected by its structure but not its O-antigen chain length. Journal of Immunology **132,** 376-385.

Guerry, P., Leblanc, D.J. and Falkow, S. (1973). General method for the isolation of plasmid deoxyribonucleic acid. Journal of Bacteriology **116,** 1064-1066.

Haddy, R. I., Klimberg, S. and Epting, R. J. (1987). A two center review of bacteremia in the community hospital. J. Fam. Pract. **24,** 253-271.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. Journal of Molecular Biology **166,** 557-580.

Hancock, I. C. and Poxton, I. R. (1988). Bacterial cell surface techniques. John Wiley and Sons LTD.

Harkki, A. and Palva, E. T. (1985). A LamB expression plasmid for extending the host range of phage λ to other enterobacteria. FEMS Microbiology Letters **27,** 183-187.

Hart, C. A., Gibson, M. F. and Buckles, A. (1981). Variation in skin and environmental survival of hospital gentamycin-resistant enterobacteria. Journal of Hygiene **87,** 277-285.

Hashimoto-Gotoh, T., Franklin, F. C. H., Nordheim, A. and Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors. I.Low copy number, temperature sensitive, mobilization-defective pSC101-derived containment vectors. Gene **16,** 227-235.

Higashi, Y., Strominger, J. L. and Sweeley, C. C. (1967). Structure of a lipid intermediate in cell wall peptidoglycan synthesis: A derivative of a C_{55} isoprenoid alcohol. Proceedings of the National Academy of Science USA **57,** 1798-1803.

Hitchcock, P. J. and Brown, T. M. (1983). Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. Journal of Bacteriology **154,** 269-277.

Hohn, B. and Collins, J. (1980). A small cosmid for efficient cloning of large DNA fragments. Gene **11,** 291-298.

Hotte, B., Rath-Arnold, I., Puhler, A. and Simon, R. (1990). Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by Xanthomonas campestris pv. campestris. Journal of Bacteriology **172,** 2804-2807.

Ikeda, H. and Tomizawa, J. (1968). Prophage P1: an extrachromosomal replication unit. Cold Spring Harbor Symp. Quant.Biol. **33,** 791-795.

Jann, K. and Jann, B. (1983). The K antigens of Escherichia coli. Prog. Allergy. **33,** 53-79.

Jarvis, W. R., Munn, V. P., Highsmith, A. K., Culver, D. H. and Hughes, J. M. (1985). The epidemiology of nosocomial infections caused by Klebsiella pneumoniae. Infect. Control. **6,** 68-75.

Joiner, K. A., Brown, E. J. and Frank, M. M. (1984). Complement and bacteria: chemistry and biology in host defence. Annual Reviews of Immunology **2**, 461-491.

Joiner, K. A., Grossman, N., Schmetz, M. and Leive, L. (1986). C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by _Salmonella montevideo_. Journal of Immunology **136**, 710-715.

Jorgensen, R. A., Rothstein, S. J. and Reznikoff, W. S. (1979). A restriction enzyme cleavage map of Tn_5_ and location of a region encoding neomycin resistance. Molecular and General Genetics **177**, 65-72.

Kadurugamuwa, J. L., Anwar, H., Brown, M. R. W., Hengstler, B., Kunz, S. and Zak, O. (1988). Influence of cephalosporins and iron on surface protein antigens of _Klebsiella pneumoniae in vivo_. Antimicrobial Agents and Chemotherapy **32**, 364-368.

Kaji, A., Yoshimoto, H., Yamamoto, T. and Finvers, S. (1982). Studies on various phenotypes of drug resistance factor, Rts1. Drug resistance in Bacteria. Eds Mitsuhashi, S. Japan, Sci, Soc, Tokyo/Thieme Stratton, NY.

Kato, N., Kato, O. and Nakashima, I. (1976). Effect of capsular polysaccharide of _Klebsiella pneumoniae_ on host resistance to bacterial infections. Induction of increased susceptibility to infections in mice. Japanese Journal of Microbiology **20**, 163-172.

Kato, N., Nakashima, I. and Ohta, M. (1975). Interferon production in mice by the capsular polysaccharide of Klebsiella pneumoniae. Infection and Immunity **12**, 1-6.

Kauffmann, F. (1949). On the serology of the Klebsiella group. Acta Pathol. Microbiol. Scand. **26**, 381-406.

Kenne, L. and Lindberg, B. (1983). Bacterial polysaccharides **2**, 287-320. In The Polysaccharides. Edited by Aspinall, G. O. New York Academic Press.

Kikuchi, N., Hiramune, T., Taniyama, H. and Yanagawa, R. (1987). Difference of virulence in causing metritis in horses between heavily encapsulated and non-capsulated strains of Klebsiella pneumoniae capsular type 1. Japanese Journal of Veterinary Research **35**, 263-273.

Klipstein, F. A., Engert, R. F. and Houghten, R. A. (1983). Immunological properties of purified Klebsiella pneumoniae heat-stable enterotoxin. Infection and Immunity **42**, 838-841.

Kolter, R., Inuzuka, M. and Helinski, D. R. (1978). Transcomplementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell **15**, 1199-1208.

Korhonen, T. K., Tarkka, E., Ranta, H. and Haahtela, K. (1983). Type 3 fimbriae of Klebsiella sp.: molecular characterisation and role in bacterial adhesion to plant roots. Journal of Bacteriology **155**, 860-865.

Kroll, J. S., Hopkins, I. and Moxon, E. R. (1988). Capsule loss in H.influenzae type b occurs by recombination-mediated disruption of a gene essential for polysaccharide transport. Cell **53**, 347-356.

Kroll, J. S., Loynds, B., Brophy, L. N. and Moxon, E. R. (1990). The bex locus in encapsulated Haemophilus influenzae: a chromosomal region involved in capsule polysaccharide export. Molecular Microbiology **4**, 1853-1862.

Kroncke, K-D., Boulnois, G., Roberts, I., Bitter-Suermann, D., Golecki, J. R., Jann, B. and Jann, K. (1990). Expression of the Escherichia coli K5 capsular antigen: immunoelectron microscopic and biochemical studies with recombinant E.coli. Journal of Bacteriology **172**, 1085-1091.

Kushner, S.R. (1978). An improved method for transformation of Escherichia coli with ColE1-derived plasmids. In Genetic Engineering,eds Boyer, H.B. and Nicosia, S. (Elsevier/North Holland, Amsterdam).

Kuypers, J. M., Heggen, L. M. and Rubens, C. E. (1989). Molecular analysis of a region of the group B streptococcus chromosome involved in type III capsule expression. Infection and Immunity **57**, 3058-3065.

Laakso, D. H., Homonylo, M. K., Wilmot, S. J. and Whitfield, C. (1988). Transfer and expression of the genetic determinants for O and K antigen synthesis in Escherichia coli 09:K(A)30 and Klebsiella sp. 01:K20, in Escherichia coli K12. Canadian Journal of Microbiology **34**, 987-992.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head protein of bacteriophage T4. Nature **277,** 680-685.

Lam, B. S., Strobel, G. A., Harrison, L. A. and Lam, S. T. (1987). Transposon mutagenesis and tagging of fluorescent Pseudomonas: antimycotic production is necessary for control of Dutch elm disease. Proceedings of the National Academy of Science USA **84,** 6447-6451.

Leying, H., Suerbaum, S., Kroll, H-P., Stahl, D. and Opkferkuch, W. (1990). The capsular polysaccharide is a major determinant of serum resistance in K-1-positive blood culture isolates of Escherichia coli. Infection and Immunity **58,** 222-227.

Liang-Takasaki, C., Grossman, N. and Leive, L. (1983). Salmonella activate complement differentially via the alternate pathway depending on the structure of their lipopolysaccharide O-antigen. Journal of Immunology **130,** 1867-1871.

Ma, M. Y., Mulligan, M. E., Friedman, M. H. and Anderson, M. S. (1983). Prognosis of Klebsiella bacteremia: Possible association with antimicrobial usage and bacterial resistance. In Easmon, C. S. F. and Gaya, H. (Eds.): Second International Symposium on Infections in the Immunocompromised Host. New York: Academic Press.

Maayan, M. C., Ofek, I., Medalla, O. and Aronson, M. (1985). Population shift in mannose-specific fimbriated phase of Klebsiella pneumoniae during experimental urinary tract infection in mice. Infection and Immunity **49,** 785-789.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, USA.

Manoil, C. and Beckwith, J. (1985). Tn*pho*A: A transposon probe for protein export signals. Proceedings of the National Academy of Sciences USA **82,** 8129-8133.

Martin, W. J., Yu, P. K. W. and Washington, J. A. (1971). Epidemiological significance of *Klebsiella pneumoniae*. A 3 month study. Mayo Clinic Proc. **46,** 785-791.

McCabe, W. R., Carling, P. C., Bruins, S. and Greely, A. (1975). The relation of K antigen to virulence in *Escherichia coli*. Journal of Infectious Diseases **131,** 6-10.

McCallum, K. L., Schoenhals, G., Laakso, D., Clarke, B. and Whitfield, C. (1989). A high-molecular-weight fraction of smooth lipopolysaccharide in *Klebsiella* serotype 01:K20 contains a unique O-antigen epitope and determines resistance to nonspecific serum killing. Infection and Immunity **57,** 3816-3822.

McCallum, K. L. and Whitfield, C. (1991). The *rcs*A gene of *Klebsiella pneumoniae* 01:K20 is involved in expression of the serotype-specific K (capsular) antigen. Infection and Immunity **59,** 494-502.

McGowan, J. E. Jr. (1985). Changing etiology of nosocomial bacteremia and fungemia and other hospital-acquired infections. Rev. Infect. Dis. **7** (Suppl.), S357.

McLaughlin, R. E. and Hughes, T. A. (1989). Transposon mutagenesis and complementation of the fructokinase gene in Rhizobium leguminosarum biovar trifolii. Journal of General Microbiology **135,** 2329-2334.

Meno, Y. and Amako, K. (1990). Morphological evidence for penetration of anti-O antibody through the capsule of Klebsiella pneumoniae. Infection and Immunity **58,** 1421-1428.

Merino, S., Ciurana, B., Sabater, E. and Tomas, J. M. (1989). A new and rapid method for the isolation of K^- isogenic mutants from Klebsiella pneumoniae. Journal of Microbiological Methods **9,** 287-295.

Merrick, M., Filser, M., Kennedy, C. and Dixon, R. (1978). Polarity of mutations induced by insertion of transposons Tn5, Tn7, Tn10 into the nif gene cluster of Klebsiella pneumoniae. Molecular and General Genetics **165,** 103-111.

Merrick, M. J., Gibbins, J. R. and Postgate, J. R. (1987). A rapid and efficient method for plasmid transformation of Klebsiella pneumoniae and Escherichia coli. Journal of General Microbiology **133,** 2053-2057.

Meshulam, T., Obedeanu, N., Merzbach, B. and Sobel, J. D. (1984). Phagocytosis of mucoid and nonmucoid strains of Pseudomonas aeruginosa. Clinical Immunology and Immunopathology **32,** 151-165.

Meynell, E. and Datta, N. (1967). Mutant drug resistant factors of high transmissibility. Nature **214,** 885-887.

Miller, V. L. and Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins virulence determinants in Vibrio cholerae requires toxR. Journal of Bacteriology **170,** 2575-2583.

Mizuta, K., Ohta, M., Mori, M., Hasegawa, T., Nakashima, I. and Kato, N. (1983). Virulence for mice of Klebsiella strains belonging to the 01 group: relationship to their capsular (K) types. Infection and Immunity **40,** 56-61.

Montgomerie, J. Z. and Ota, J. K. (1980). Klebsiella bacteremia. Archives of Internal Medicine **140,** 525-527.

Morrison, D. C. and Kline, L. F. (1977). Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides. Journal of Immunology **118,** 362-368.

Nakashima, I. and Kato, N. (1974). Non-specific stimulation of immunoglobulin synthesis by capsular polysaccharide of Klebsiella pneumoniae. Immunology **27,** 179-193.

Nassif, X., Fournier, J-M., Arondel, J. and Sansonetti, P. J. (1989). Mucoid phenotype of Klebsiella pneumoniae is a plasmid-encoded virulence factor. Infection and Immunity **57,** 546-552.

Nassif, X., Honore, N., Vasselon, T., Cole, S. T. and Sansonetti, P. J. (1989). Positive control of colanic acid synthesis in Escherichia coli by rmpA and rmpB, two virulence-plasmid genes of Klebsiella pneumoniae. Molecular Microbiology **3,** 1349-1359.

Ohta, M., Mori, M., Nakashima, I. and Kato, N. (1979). Adjuvant action of capsular polysaccharide of Klebsiella pneumoniae on antibody response. Further purification of the active substance. Microbiol. Immunol. **23,** 805-813.

Okawa, N., Tanaka, M., Finver, S. and Kaji, A. (1987). Identification of the Rts1 DNA fragment responsible for temperature-sensitive growth of host cells harboring a drug resistance factor, Rts1. Biochem. Biophys. Res. Commun **142,** 1084-1088.

Okawa, N., Yoshimoto, H. and Kaji, A. (1985). Identification of an Rts1 DNA fragment conferring temperature-dependent instability to vector plasmids. Plasmid **13,** 88-98.

Orskov, I. (1956). Immunological paralysis induced in rabbits by a heavily capsulated Klebsiella strain. Acta Pathol. Micrbiol. Scand. **38,** 375-379.

Orskov, I. and Fife-Asbury, M. A. (1977). New Klebsiella capsular antigen, K82, and the deletion of five of those previously assigned. Int. J. System. Bacteriol. **27,** 386-387.

Orskov, F. and Orskov, I. (1978). Serotyping of Enterobacteriaceae with special emphasis on K antigen determination. In Methods in Microbiology **11**, 37-38. Edited by Norris, J. R. and Bergen, T.

Osborn, M. J. (1966). Preparation of lipopolysaccaride from mutant strains of Salmonella. Methods of Enzymology **8**, 161-164.

Ou, J. T. (1980). Mating due to loss of surface exclusion as a cause for thermosensitive growth of bacteria containing the Rts1 plasmid. Molecular and General Genetics **180**, 501-510.

Palfreyman, J. M. (1978). Klebsiella serotyping by counter-current immunoelectrophoresis. Journal of Hygiene. Cambridge **81**, 219-225.

Pollack, M. (1976). Significance of circulating capsular antigen in Klebsiella infections. Infection and Immunity **13**, 1543-1548.

Poxton, I. R. and Sutherland, I. W. (1976). Isolation of rough mutants of Klebsiella aerogenes and their synthesis of polysaccharides. Journal of General Microbiology **96**, 195-202.

Pruzzo, C., Deblia, E. A. and Satta, G. (1980). Identification of the major adherence ligand of Klebsiella pneumoniae in the receptor for coliphage T7 and alteration of Klebsiella adherence properties by lysogenic conversion. Infection and Immunity **30**, 562-569.

Pruzzo, C., Deblia, E. A. and Satta, G. (1982). Mannose-inhibitable adhesions and T3-T7 receptors of Klebsiella pneumoniae inhibit phagocytosis and intracellular killing by human polymorphonuclear leucocytes. Infection and Immunity, **36**, 949-956.

Pruzzo, C. and Satta, G. (1988). Capsular antigenic variations by lysogenic conversion in Klebsiella pneumoniae: relationship with virulence. Current Microbiology **16**, 259-263.

Pruzzo, C., Valisena, S. and Satta, G. (1983). Laboratory and wild-type Klebsiella pneumoniae strains carrying mannose-inhibitable adhesins and receptors for coliphages T3 and T7 are more pathogenic for mice than are strains without such receptors. Infection and Immunity **39**, 520-527.

Przondo-Hessek, A. and Pulverer, G. (1983). Hemagglutinins of Klebsiella pneumoniae and Klebsiella oxytoca. Zbl. Bakteriol. Hyg. I. Abt. Orig. A **255**, 472-478.

Quinto, M. and Bender, R. A. (1984). Use of bacteriophage P1 as a vector for Tn5 insertion mutagenesis. Applied and Enviromental Microbiology **47**, 436-438.

Reid, J. H. and Patrick, S. (1984). Phagocytic and serum killing of capsulate and non-capsulate Bacteroides fragilis. Journal of Medical Microbiology **17**, 247-257.

Riser, E. and Noone, P. (1981). Klebsiella capsular type versus site of isolation. Journal of Clinical Pathology **34**, 552-555.

Riser, E., Noone, P. and Thompson, R. E. M. (1978). The use of a fluoresence typing method in an epidemiological study of Klebsiella infection in a London hospital. Journal of Hygiene **80**, 43-56.

Robbins, J. B., Schneerson, R., Egan, W. B., Vann, W. and Liu, D. T. (1980). Virulence properties of bacterial capsular polysaccharides - unanswered questions. In The Molecular Basis of Microbial Pathogenicity. Edited by Smith, H., Skehel, J. J. and Turner, M. J.: Verlag Chemie, Weinheim.

Roberts, I., Mountford, R., High, N., Bitter-Suermann, D., Jann, K., Timmis, K. and Boulnois, G. (1986). Molecular cloning and analysis of genes for production of K5, K7, K12 and K92 capsular polysaccharides in Escherichia coli. Journal of Bacteriology **168**, 1228-1233.

Roberts, I., Mountford, R., Hodge, R., Jann, K. B. and Boulnois, G. J. (1988). Common organization of gene clusters for production of different capsular polysaccharides (K Antigens) in Escherichia coli. Journal of Bacteriology **170**, 1305-1310.

Rosner, J. L. (1972). Formation, induction and curing of bacteriophage P1 lysogenes. Virology **49**, 679-689.

Rothstein, S. J., Jorgensen, R. A., Yin, J. C.-P., Yong-Di, Z., Johnson, R. C. and Reznikoff, W. S. (1980). Genetic organisation of Tn5. Cold Spring Harbor, Symposia on Quantitative Biology **45**, 99-105.

Rubens, C. E., Wessels, M. R., Heggen, L. M. and Kasper, D. L. (1987). Transposon mutagenesis of type III group B Streptococcus: Correlation of capsule expression with virulence. Proceedings of the National Academy of Science USA **84,** 7208-7212.

Sasakawa, C. and Yoshikawa, M. (1987). A series of Tn5 variants with various drug-resistance markers and suicide vector for transposon mutagenesis. Gene **56,** 283-288.

Serushago, B. A., Mitsuyama, M., Handa, T., Koga, T. and Nomoto, K. (1989). Role of antibodies against outer-membrane proteins in murine resistance to infection with encapsulated Klebsiella pneumoniae. Journal of General Microbiology **135,** 2259-2268.

Schmid, E. N., Menge, B. and Lickfeld, K. G. (1981). Capsule fine structure in thin slices of Klebsiella pneumoniae biovar d (Richard). Journal of Ultrastructure Research **75,** 41-49.

Schwacha, A., Cohen, J.A., Gehring, K.B. and Bender, R.A. (1990). Tn1000-mediated insertion mutagenesis of the histidine utilization (hut) gene cluster from Klebsiella aerogenes: Genetic analysis of hut and unusual target specificity of Tn1000. Journal of Bacteriology 172, 5991-5998.

Silver, R. P., Finn, C. W., Vann, W. F., Aaronson, W., Schneerson, Kretschmer, P. J. and Garon, C. F. (1981). Molecular cloning of the K1 capsular polysaccharide genes of E.coli. Nature (London) **289,** 696-698.

Silverblatt, F. J. and Weinstein, R. J. (1979). Enterobacteriaceae. In Principles and Practice of Infectious Diseases. Edited by Marriott, G. L., Douglas, R. G. and Benott, S. E., John Wiley, New York.

Simon, R., Priefer, U. and Puhler, A. (1983). A broad host range mobilization system for in-vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1**, 784-791.

Simoons-Smit, A. M. (1987). Typing, virulence and epidemiology of Klebsiella species. Ph.D. thesis, Free University, Amsterdam, The Netherlands.

Simoons-Smit, A. M., Verweij-Van Vught, A. M. J. J., De Vries, P. M. J. M. and Maclaren, D. M. (1987). Comparison of biochemical and serological typing results and antimicrobial susceptibility patterns in the epidemiological investigation of Klebsiella spp. Epidemiol. Inf. **99**, 625-634.

Simoons-Smit, A. M., Verweij-Van Vught, M. J. J., Kanis, I. Y. R. and Maclaren, D. M. (1984). Virulence of Klebsiella strains in experimentally induced skin lesions in the mouse. Journal of Medical Microbiology **17**, 67-77.

Simoons-Smit, A. M., Verweij-Van Vught, M. J. J., Kanis, I. Y. R. and MacLaren, D. M. (1985). Chemiluminescence of human leukocytes stimulated by clinical isolates of Klebsiella. Journal of Medical Microbiology **19**, 333-338.

Simoons-Smit, A. M., Verweij-Van Vught, M. J. J. and MacLaren, D. M. (1986). The role of K antigens as virulence factors in Klebsiella. Journal of Medical Microbiology **21,** 133-137.

Slopek, S., Przondo-Hessek, A., Milch, H. and Deak, S. (1967). A working scheme for bacteriophage typing of Klebsiella bacilli. Arch. Immunol. Ther. Exp. **16,** 589-599.

Southern, E. (1975). Detection of specific sequences among DNA fragments seperated by gel eletrophoresis. Journal of Molecular Biology **98,** 503.

Smith, A. N., Boulnois, G. J. and Roberts, I. S. (1990). Molecular analysis of the Escherichia coli K5 kps locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Molecular Microbiology **4,** 1863-1869.

Steinberger, E.M. and Beer, S.V. (1988). Creation and complementation of pathogenicity mutants of Erwinia amylovora. Mol. Plant. Microbe. Interact. **1,** 135-144.

Steinhauer, B. W., Eickhoff, T. C., Kislak, J. W. and Finland, M. (1966). The Klebsiella-Enterobacter-Serratia division. Clinical and epidemiologic characteristics. Ann. Int. Med. **65,** 1180-1194.

Stendahl, O. (1983). The physicochemical basis of surface interaction between bacteria and phagocytic cells. In Medical Microbiology **3**. Role of the envelope in the survival of bacteria in infection, p137-152. Edited by

Easmon C. S. F., Brown, M. R. W., Jeljazewicz, J. and Lambert, P. A., Academic Press.

Stendahl, O., Normann, B. and Edebo, L. (1979). Infuence of O and K antigens on the surface of Escherichia coli in relation to phagocytosis. Acta Pathologica et Microbiologica Scandinavica Sect. B. **87,** 85-91.

Straus, D. C. (1987). Production of an extracellular toxic complex by various strains of Klebsiella pneumoniae. Infection and Immunity **55,** 44-48.

Straus, D. C., Atkisson, D. L. and Garner, C. W. (1985). Importance of a lipopolysaccharide-containing extracellular toxic complex in infections produced by Klebsiella pneumoniae. Infection and Immunity **50,** 787-795.

Streicher, S.L., Bender, R.A. and Magasanik, B. (1975). Genetic control of glutamine synthetase in Klebsiella aerogenes. Journal of Bacteriology **121,** 320-331.

Sutherland, I. W. (1972). Bacterial exopolysaccharides. Advances in Microbial Physiology **8,** 143-213.

Sutherland, I. W. (1977). Bacterial exopolysaccharides - their nature and production. In Surface carbohydrates of the prokaryotic cell, p27-96. Edited by Sutherland, I. W., London & New York: Academic Press.

Sutherland, I.W. and Norval, M. (1970). The synthesis of exopolysaccharide by Klebsiella aerogenes membrane preparations and the

involvement of lipid intermediates. Biochem. Journal (London) **120,** 567-576.

Tamaoki, J., Sakai, N., Isono, K., Kanemura, T., Takeyama, K. and Takizawa, T. (1991). Lipopolysaccharide from Klebsiella pneumoniae inhibits Na^+ absorption in canine tracheal epithelium. Infection and Immunity **59,** 716-717.

Taylor, R. K., Manoil, C. and Mekalanos, J. J. (1989). Broad host-range vectors for delivery of TnphoA: use in genetic analysis of secreted virulence determinants of Vibrio cholerae. Journal of Bacteriology **171,** 1870-1878.

Terawaki, Y., Kakizawa, H., Takayash, H. and Yoshikawa, M. (1968). Temperature sensitivity of cell growth in Escherichia coli associated with the temperature sensitive R(KM) factor. Nature **219,** 284-285.

Terawaki, Y., Kobayashi, Y., Matsumoto, H. and Kamio, Y. (1981). Molecular cloning and mapping of a deletion derivative of the plasmid Rts1. Plasmid **6,** 222-234.

Terawaki, Y. and Round, R. (1972). Replication of the R factor Rts1 in Proteus mirabilis Journal of Bacteriology **109,** 492-498.

Terawaki, Y., Takayasu, H. and Akiba, T. (1967). Thermosensitive replication of a kanamycin resistance factor. Journal of Bacteriology **94,** 687-690.

Terman, J. W., Alford, R. H., and Bryant, R. E. (1972). Hospital-acquired Klebsiella bacteremia. Amer. J. Med. Sci. **264**, 191-196.

Thirion, J.P. and Hofnung, M. (1972). On some genetic aspects of phage λ resistance in E.coli K12. Genetics **71**, 207-216.

Tomas, J. M., Benedi, V. J., Ciurana, B. and Jofre, J. (1986). Role of capsule and O antigen in resistance of Klebsiella pneumoniae to serum bactericidal activity. Infection and Immunity **54**, 85-89.

Torres-Cabassa, A., Gottesman, S., Frederick, R. D., Dolph, P. J. and Coplin, D. L. (1987). Control of extracellular polysaccharide synthesis in Erwinia stewartii and Escherichia coli K-12: a common regulatory function. Journal of Bacteriology **169**, 4525-4531.

Trautmann, M., Cryz, S. J., Sadoff, J. C. and Cross, A. S. (1988). A murine monoclonal antibody polysaccharide is opsonic in vitro and protects against experimental Klebsiella pneumoniae infection. Microbial Pathogenesis **5**, 177-187.

Troy, F.A. (1979). The chemistry and biosynthesis of selected bacterial capsular polymers. Annual Reviews of Microbiology **33**, 519-560.

Troy, F. A., Frerman, F. E. and Heath, E. C. (1971). The biosynthesis of capsular polysaccharide in Aerobacter aerogenes. Journal of Biological Chemistry **246**, 118-133.

Tsai, C-M. and Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Analytical Biochemistry **119,** 115-119.

Ubben, D. and Schmitt, R. (1986). Tn1721 derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41,** 145-152.

Van Oss, C. J. and Gillman, C. F. (1972). Phagocytosis as a surface phenomenon. Journal of the Reticuloendothelial Society **12,** 283-292.

Verweij-van Vught, A. M. J. J, Namavar, F., Peerbooms, P. G. H., Sparrius, M and McLaren, D. M. (1984). The role of different K antigens of Escherichia coli in phagocytosis by polymorphonuclear leukocytes. Journal of Medical Microbiology **17,** 141-150.

Wehmeier, U., Sprenger, G. A. and Lengeler, J. W. (1989). The use of λplac-Mu hybrid phages in Klebsiella pneumoniae and the isolation of stable Hfr strains. Molecular and General Genetics **215,** 529-536.

Westphal, O. and Jann, K. (1965). Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure. Methods of Carbohydrate Chemistry **5,** 83-91.

Williams, P., Brown, M. R. W. and Lambert, P. A. (1984). Effect of iron deprivation on the production of siderophores and outer membrane proteins in Klebsiella aerogenes. Journal of General Microbiology **130,** 2357-2365.

Williams, P., Chart, H., Griffiths, E. and Stevenson, P. (1987). Expression of high affinity iron uptake systems by clinical isolates of Klebsiella. FEMS Microbiology Letts. **44**, 407-412.

Williams, P., Lambert, P. A. and Brown, M. R. W. (1988). Penetration of immunoglobulins through the Klebsiella capsule and their effect on cell-surface hydrophobicity. Journal of Medical Microbiology **26**, 29-35.

Williams, P., Lambert, P. A., Brown, M. R. W. and Jones, R. J. (1983). The role of the O and K antigens in determining the resistance of Klebsiella aerogenes to serum killing and phagocytosis. Journal of General Microbiology **129**, 2181-2191.

Williams, P., Lambert, P. A., Haigh, C. G. and Brown, M. R. W. (1986). The influence of the O and K antigens of Klebsiella aerogenes on surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. Journal of Medical Microbiology **21**, 125-132.

Williams, P. and Tomas, J. M. (1990). The pathogenicity of Klebsiella pneumoniae. Reviews in Medical Microbiology **1**, 196-204.

Wright, A., Dankert, M., Fennessey, P. and Robbins, P.W. (1967). Characterization of a polyisoprenoid compound functional in O-antigen biosynthesis. Proceedings of the National Academy of Science USA **57**, 1798-1803.

Wright, A., Simpson, L.M., Oliver, J.D. and Morris, J.G. (1990). Phenotypic evaluation of acapsular transposon mutants of Vibrio vulnificus. Infection and Immunity **58**, 1769-1773.

Yamamoto, T., Finver, S., Yokota, T., Bricker, J. and Kaji, A. (1981). The region controlling the thermosensitive effect of plasmid Rts1 on host growth is seperate from the Rts1 replication region. Journal of Bacteriology **146**, 85-92.

Yamamoto, T. and Kaji, A. (1977). Replication of thermosensitve Rts1 plasmid deoxyribonucleic acid at the nonpermissive temperature. Journal of Bacteriology **132**, 90-99.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**, 103-119.

Yokochi, T., Nakashima, I. and Kato, N. (1977). Effect of capsular polysaccharide of Klebsiella pneumoniae on the differentiation and functional capacity of macrophages cultured in vitro. Microbiol. Immunol. **21**, 601-610.

Yokochi, T., Nakashima, I. and Kato, N. (1979). Further studies on generation of macrophages in in vitro cultures of mouse spleen cells and its inhibition by the capsular polysaccharide of Klebsiella pneumoniae. Microbiol. Immunol. **23**, 487-499.

Yokota, T., Kanamaru, Y., Mori, R. and Akiba, T. (1969). Recombination between a thermosensitve kanamycin resistance factor and a

non-thermosensitve multiple drug resistance factor. Journal of Bacteriology **98,** 863-873.