MECHANISM OF UPTAKE OF THE CATECHOLIC (7)-α-FORMAMIDO CEPHALOSPORIN BRL 41897A BY *KLEBSIELLA PNEUMONIAE*

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Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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SUMMARY

The catecholic cephalosporin BRL 41897A is resistant to β-lactamases and is taken up by bacteria via the iron transport system. The uptake of this antibiotic in *E. coli* uses the Fiu and Cir outer membrane proteins, whereas in *P. aeruginosa* it enters via the pyochelin transport system. In this thesis mutants of *K. pneumoniae* resistant to BRL 41897A were isolated using Tn*phoA* mutagenesis and used to study the mechanism of uptake of BRL 41897A by *K. pneumoniae*. The activity of BRL 41897A towards the parent strain (M10) was increased in iron depleted media, whereas no significant differences in the resistant (KSL) mutants were observed. Three mutants (KSL19, KSL38 and KSL59) produced decreased amounts of certain iron-regulated outer membrane proteins. The uptake of ^{55}Fe-BRL 41897A by M10 in iron-deficient medium was higher than in iron-rich medium. This result indicated the involvement of an iron transport system in the uptake of BRL 41897A by *K. pneumoniae*. Uptake by the KSL mutants in iron-defient culture was higher than that by M10. This result, supported by analysis of outer membrane and periplasmic proteins of the KSL mutants, indicates that loss of one outer membrane protein can be compensated by over expression of other outer membrane and/or periplasmic proteins. However, the increased uptake of BRL 41897A by the KSL mutants did not reflect increased activity towards these strains, indicating that there are defects in the transport of BRL 41897A resulting in failure to reach the penicillin binding protein target sites in the cytoplasmic membrane. Southern blotting of chromosomal digests and sequencing in one mutant (KSL19) showed that only one copy of Tn*phoA* was inserted into its chromosome. A putative Tn*phoA* inserted gene in KSL19, designated *kslA*, carrying a signal sequence was identified. Transformation of a fragment containing the *kslA* gene into KSL19 cells restored the sensitivity to BRL 41897A to that of the parent strain. Data base peptide sequence searches revealed that the *kslA* gene in the KSL19 has some amino acid homology with the *E. coli* ExbD protein, which is involved in stabilisation of the TonB protein.

Key words: *Klebsiella pneumoniae*, BRL 41897A, resistant, uptake, iron transport.

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Abbreviations

ABC	ATP-binding cassette
AMPS	ammonium persulphate
ARI	acute respiratory infections
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate (X-phosphate)
bp	base pairs
BSA	bovine serum albumin
CAA	casamino acids
cm	centimetre(s)
CM	cytoplasmic membrane
Da	daltons
DNA	deoxyribonucleic acid
DR	direct repeat
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
ESBL	extended-spectrum β-lactamase
ET	electrophoretic type
g	gramme(s)
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IPTG	isopropyl β-D-thiogalactopyranoside
IR	inverted repeat

IROMP	iron-regulated outer membrane proteins
IS	insertion sequences
l	litre(s)
LB	Luria broth
LPS	lipopolysaccharide
M	mole(s) per litre
MDR	multi drug resistance
MIC	minimum inhibitory concentration
μ	micro
mg	milligram(s)
ml	millilitre(s)
MOPS	morpholinopropane sulphonic acid
NBT	nitro blue tetrazolium
NCTC	National Collection of Type Cultures
nm	nanometre(s)
NNIS	National Nosocomial Infections Surveillance
OD	optical density
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PG	peptidoglycan
pI	isoelectric points

PMF	proton motive force
rpm	revolutions per minute
Sarkosyl	N-lauroylsarcosine, sodium salt
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBP	transferrin-binding protein
TBS	tris buffered saline
TBS/Tween	tris buffered saline with added Tween 20 (0.3%)
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris	tris (hydroxymethyl) amino ethane
TSB	tryptone soy broth
UTI	urinary tract infection
V	volt(s)
v/v	volume by volume
w/v	weight by volume
WHO	world health organization
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YE	yeast extract

CHAPTER 1

GENERAL INTRODUCTION

1.1. Bacterial resistance to antimicrobial drugs

1.1.1. Evolution of drug resistance

Soon after the introduction of penicillin in the 1940s, penicillin-resistant staphylococci plagued hospitals even though community-acquired infections were initially unaffected. About a decade after the introduction of the drugs to which resistance was directed, in the late 1950s and early 1960s, reports of multidrug resistance on transferable genetic elements called "plasmids" appeared in Japan, Latin America, Europe and the USA. Resistance may emerge from a mutation in an intrinsic chromosomal or viral gene or, as in microorganisms, by acquisition of exogenous genetic material bearing single or multiple resistance determinants.

Initially the resistant bacteria were largely confined to the gastrointestinal tract, for example, *Escherichia coli, Salmonella* and *Shigella*. At the time, while the finding was important, many doubted that the problem would spread to other kinds of bacteria. But in the 1960s and 1970s Gram-negative rods, such as *Klebsiella, Pseudomonas, Proteus*, and *Enterobacter* species, emerged as important drug resistant pathogens (Levy, 1994).

By the mid 1970s, organisms of the respiratory and genito-urinary tract, such as *Haemophillus influenzae* and *Neisseria gonorrhoeae*, became hosts for drug-resistance determinants. Single and multidrug resistances were traced to the acquisition of antibiotic-resistance genes from drug-resistant bacteria of the gastrointestinal tract (Levy, 1994). By the early 1980s, many hospitals had also begun

to identify methicillin-resistant *Staphylococcus aureus* infection, which have become a persistent problem, causing bacteremias, pneumonias and wound infections and spreading from large teaching hospitals to smaller community hospitals. By the early 1990s, resistance to methicillin was detected in 20-40% of all *S. aureus* isolates reported to the National Nosocomial Infections Surveillance (NNIS) System in the USA. In many cases these isolates were only susceptible to vancomycin (Cohen, 1994).

Another nosocomial pathogen, the enterococcus, was also becoming increasingly resistant to drug treatment. Enterococci cause a variety of nosocomial infections, from urinary tract and wound infections to bacteremia. The organism is intrinsically resistant to various antibiotics, and patients often require a combination of drugs, including penicillins and aminoglycosides, for effective treatment. During the 1980s, *Enterococcus* species became increasingly drug resistant, and many infections could only be treated with vancomycin. Unfortunately, resistance to vancomycin soon followed and, by early 1993, almost 14% of enterococci isolated from intensive-care units in hospitals in the USA participating in the NNIS System were resistant to vancomycin. Many of these drug-resistant infections are essentially untreatable (Cohen,1994).

A final recent nosocomial problem has been the emergence of multidrug-resistant *Mycobacterium tuberculosis*. Although these organisms also occurred in community-acquired infections, several recent outbreaks have occurred in hospitals, particularly affecting patients infected with HIV. Some of these organisms are resistant to as many as seven of the standard antituberculosis agents. Multi-drug resistant *M. tuberculosis* not only poses a dilemma for the treatment of the individual

patient with active tuberculosis, but it also poses problems for preventive therapy for patients and health-care workers who are exposed to these drug-resistant organisms. (Cohen, 1994)

1.1.2. Community infections

Until the 1970s, antimicrobial resistance was viewed primarily as a problem of the hospital. However, the emergence worldwide of drug resistant *Neisseria gonorrhoea* and *Haemophilus influenzae* in the 1970s demonstrated that antimicrobial resistance is also a problem for the community. In the USA, increasing frequencies of drug resistance were soon recognized in community-acquired infections. From 1979 to 1989, the frequency of drug resistance among *Salmonella* isolates increased from 16% to 32% (Lee *et al*, 1992). By 1986, 32% of *H. influenzae* isolates were resistant to ampicillin. By the 1990s, 32% of *N. gonorrhoeae* isolates were resistant to penicillin or tetracycline and resistance to fluoroquinolones was being recognized. In 1986, 32% of *Shigella* isolates were resistant to trimethoprim-sulfamethoxazole, an agent commonly used in the treatment of shigellosis (Tauxe *et al.*,1990).

A more significant global problem involves *Shigella dysentriae* type 1A (the Shiga bacillus), which causes epidemic dysentery that may be associated with a mortality of 15%. Since the early 1970s, drug-resistant strains of *S. dysenteriae* have caused epidemics in various parts of the world, particularly in central Africa. By the 1990s, strains of *S. dysenteriae* isolated in Burundi were resistant to all oral antimicrobial agents available in that country, rendering this infection unresponsive to antimicrobial treatment (Ries *et al.*, 1994).

Increasing antimicrobial resistance in *Streptococcus pneumoniae*, however,

may pose the most important public health dilemmas. This organism is a common cause of bacteremia, pneumonia and meningitis, and may account for 40% all of episodes of acute otitis media in children. It has been estimated that these middle-ear infections are the most frequent reasons for consulting pediatricians in the USA, causing an estimated 24 million visits a year. Since the 1950s, pneumococci had remained remarkably susceptible to penicillin. In the 1970s, resistance to penicillin was recognized and, by the late 1970s, isolated epidemics of multidrug-resistant pneumococcal disease were reported from South Africa. During the 1980s, penicillin-resistant and multiple-drug resistant *S. pneumoniae* were reported in various areas in the USA. Many of the drug-resistant pneumococcal infections were among children and, in some cases, serious infections required treatment with vancomycin. A recent report from Tenessee identified ten children with meningitis or other serious, invasive pneumococcal infections, bacterial isolates from whom were resistant to both penicillin and extended-spectrum cephalosporins (Cohen, 1994).

1.1.3. Factors contributing to resistance

Each organism is affected by a different combination of factors. Multiple studies have shown the importance of antibiotic use in the emergence of drug resistance. Antibiotics are used extensively in both humans and animals, and often this use is inappropriate. Drugs are often used to treat the wrong kind of infection, at the wrong dosage or for the wrong period of time. All of these inappropriate uses lead to unnecessary selective pressure for the emergence of drug resistance (Sanders and Sanders,Jr., 1985). Although antimicrobial use is an important factor in the emergence of resistance, almost universally, various other factors facilitate the

subsequent transmission of a drug-resistant organism, and the success of this transmission determines the frequency of antibiotic resistance and whether this organism poses a public health problem. Additional factors influence the emergence of resistance for other pathogens. An increase in people susceptible to infection can lead to increases in resistance. People are living longer and, for many diseases, susceptibility to infection increases with age. The epidemic of infections with HIV has been partly responsible for the resurgence of drug-resistant tuberculosis. In the developing world, inadequate sanitary and hygienic conditions can facilitate the transmission of drug resistant organisms. This can be exacerbated by the breakdown of social structures or of public health measures that may accompany revolutions or wars. Such destabilizing factors are likely to be important in the emergence of the drug-resistant *S. dysenteriae* that plagued Africa since the late 1970s. Similarly, inadequate infection-control practices or personnel in hospital can lead to the increased spread of drug resistant infections. With the current scope of international travel and commerce, a drug-resistant organism that emerges in one area can be transmitted quickly to another (Munoz *et al.*, 1992). Breakdown of public health measures also occurs in the developed world. In the USA, the assumption that tuberculosis had been controlled led to reductions in public health programs to control tuberculosis. This, in combination with immigration, the epidemic of HIV infection and incomplete or in appropriate drug treatment, has led to the resurgence of tuberculosis in the USA (Bottger, 1994; Cohen, 1994).

1.1.4. Antimicrobial resistance

Bacteria are capable of resisting or avoiding the action of antibiotics in a

number of ways, the most important mechanisms are:

(1) Production of enzymes which inactivate the antibiotics.

This mechanism has provided the biggest obstacle to the effective chemotherapy of infectious diseases. The most striking example is the production of β-lactamase: enzymes which inactivate penicillins and cephalosporins by hydrolysing the β-lactam ring (Sykes and Matthew, 1976; Jacoby, 1994; Naumoski *et al*, 1992). Other important examples are enzymes which inactivate antibiotics by the addition of groups which destroy their antimicrobial activity. Adenylating, phosphorylating and acetylating enzymes are the most important factors responsible for resistance to the aminoglycosides (Davies and Smith, 1978).

(2) Modification of the target site so that it is insensitive to the antibiotic.

This mechanism has been recognised for many years but has not posed a major therapeutic problem. An example is the resistance of pneumococci to sulphonamides, which is due to a decreased affinity of the target enzyme, tetrahydropteroic acid synthetase, for the sulphonamide (Wolf and Hotchkiss, 1963). In a few species, resistance has emerged by the development of altered penicillin-binding proteins (PBPs) with decreased affinity for β-lactam antibiotics. This mechanism of resistance is found mainly in bacteria that are naturally transformable, including *S. pneumoniae, N. gonorrhoeae, N. meningitidis* and *Haemophilus influenzae*. PBP-mediated resistance has also been found in enterococci and in *S. aureus*, which are not naturally transformable but, in these cases, a novel low affinity PBP has been acquired, rather than there being a decrease in the affinities of the normal PBPs (Archer and Neimeyer, 1994). The emergence of PBP-mediated resistance to β-lactam antibiotics in naturally transformable species appears to have occurred by interspecies

recombination. Resistance to penicillin in non-β-lactamase producing *N. gonorrhoeae* is due to the production of low-affinity forms of PBP1 and PBP2, combined with reductions in the permeability of the cell envelope to penicillin (Dowson *et al.*, 1994). Resistance to β-lactams due to altered PBP has been reported in clinical isolates of *N. gonorrhoeae* (Dougherty *et al.*, 1980), *S. pneumoniae* (Hakenbeck *et al.*,1980), and *S. aureus* (Hayes *et al.*, 1981).

(3) Prevention of access of the antibiotic to the target site.

The development and introduction of antibiotics which are resistant to inactivating enzymes has altered the selective pressure for the emergence of resistant strains. Consequently the pattern of antibiotic resistance is beginning to change. Resistance caused by a reduced level of antibiotic uptake has been demonstrated in many laboratory strains of bacteria (Foulds and Chai, 1978; Harder *et al.*,1981; Sawai *et al.*, 1982). The PBP targets for β-lactam antibiotics are located in the cytoplasmic membrane, probably on the outer surface (Spratt, 1980 and 1994; Ghuysen, 1994). Therefore, β-lactams only need to penetrate the cell wall in order to reach their site of action (Di Rienzo *et al.*, 1978; Lambert, 1983 and 1988a). Any changes in wall composition which affect the rate of penetration of antibiotics are likely to alter the sensitivity, both to β-lactams and to antibiotics which act at intracellular sites, since all of them must first pass across the cell wall before reaching the transport systems in the cytoplasmic membrane. In addition to wall permeability changes resulting from phenotypic variation, many envelope mutants have been shown to possess dramatically altered permeability characteristics which influence their antibiotic susceptibility (Grundstrom *et al.*,1980; Osborn and Wu, 1980; Hancock and Bellido, 1992).

(4) Antibiotic resistance mediated by active efflux.

Active efflux may first be recognized when de energized cells accumulate more of a substance than do energized cells. Initially, the studies of efflux dealt with simple cations (Na^+, K^+, Ca^{++}) which were extruded by an energy-dependent process linked to the proton motive force (PMF) or ATP. This transport occurred via cation-specific export proteins. Later, resistance to tetracycline, was linked to its energy-dependent efflux in Gram-negative bacteria (McMurry *et al.*, 1980 and 1983). Low level quinolone efflux systems may contribute to quinolone resistance in susceptible strains. They appear to play an important role in enhancing resistance in cells that already manifest a decreased uptake of quinolones, e.g., in *Mar* mutants of *E. coli* (Cohen *et al.*, 1988 and 1989). Active efflux has also been characterised in some quinolone-resistant mutants of *S. aureus* (Kaatz *et al.*, 1991). Studies on mutants of *Bacillus subtilis* selected for resistance to rhodamine 6G have revealed chromosomal resistance by efflux not only to this agent but also to other structurally unrelated agents including ethidium bromide, chloramphenicol and puromycin (Neyfakh *et al.*, 1991). Today, active efflux is a common drug-resistance mechanism in bacteria (Levy, 1992). Several of these efflux systems that confer multi drug resistance (MDR) use the proton-motive force as a source of energy, but an ATP-requiring efflux system, such as the arsenite-antimonite-resistance pump is also found in *E. coli*. Resistance to erythromycin in *Staphylococcus* is mediated by an ATP-binding cassette (ABC) transporter, and several antibiotic-producing *Streptomyces* strains protect themselves by ABC-transporter-mediated active efflux of the antibiotic (Ouellette *et al.*, 1994).

1.1.5. Control of antimicrobial resistance

During the 1980s, a number of international efforts focused on the problem of drug resistance. A meeting in Santo Domingo, Dominican Republic in 1981 emphasized the global nature of antibiotic resistance. At the time, developing countries were more compromised by drug resistance than were the industrial nations; resistance in the industrial countries could be managed by newer antibiotics that developing countries could not afford to buy. From this meeting emerged the Statement on Antibiotic Misuse, signed by the participants (Levy, 1994). Later in 1981, the WHO in Geneva convened a workshop of international experts to assess the problem and develop guidelines towards dealing with it.

During the period 1983-1986, the Forgarty International Center of the National Institutes of Health sponsored six task forces involving more than 100 experts from over 30 countries, which examined different aspects of drug resistance as a global issue. Their work has served as a model for other country-wide efforts. The study included examination of the different elements influencing drug usage and resistance: global antibiotic use, worldwide prevalence of drug resistance, regulation of antibiotic prescribing and use, social and behavioural factors, education and economic issues. Unfortunately, these efforts did not produce the effects sought. The problem continued to simmer because too few took it seriously enough to take aggressive action. Resistance now accompanies common bacterial, viral and fungal infectious disease-causing agents, so steps must be taken to reverse the situation (Levy, 1994).

Understanding the epidemiological factors that result in the emergence and transmission of antimicrobial resistance defines two general areas for control: efforts to decrease the selective pressures for the emergence and persistence of antibiotic

resistance and to decrease the transmission of drug-resistant organisms. Several specific actions are necessary to control antimicrobial resistance:

(1) Better surveillance systems are needed to define more accurately the frequency and geographical distribution of antimicrobial resistance. Such data may be useful not only in identifying public health priorities, but also in making recommendations to physicians for using antimicrobial agents. Epidemiological studies are also necessary to define more precisely the risk factors that influence resistance and to evaluate potential control strategies.

(2) One way to decrease transmission is by effective use of vaccines. It would prevent the transmission of the pathogen itself and thus circumvent the problem of antimicrobial resistance.

(3) Greater emphasis on hygiene, sanitation and practices to control infection, involving improving living standards in many areas of the developing world, would also decrease transmission.

(4) More prudent use of existing antimicrobial agents is extremely important, which involves evaluating the current uses in both humans and animals. The use of narrow-spectrum agents, appropriate dosage and duration of antimicrobial use, stricter requirements for use of a particular drug and better diagnostic methods may lead to more effective and prudent use of antimicrobial agents.

(5) Finally, the pharmaceutical industry should be encouraged to develop new agents to treat infections with organisms that are resistant to current antimicrobials (Cohen, 1994).

In order to control antibiotic resistant organisms, one approach is to use current antibiotics in ways that encourage the re-establishment of susceptible flora.

A second direct approach to the problem would be to discover novel antibiotic structures that microorganisms have not encountered before. Alternatively, drugs could be designed that circumvent or block resistance mechanisms (Levy, 1994).

In this thesis, the main emphasise has been placed upon studies of the uptake of a catecholic cephalosporin (BRL 41897A) by *Klebsiella pneumoniae* and the mechanism of resistance towards this antibiotic. BRL 41897A was designed to be taken up via iron-transport systems of Gram-negative bacteria, and to resist the attack of β-lactamases produced by bacteria. In order to create *K. pneumoniae* mutants resistant to BRL 41897A, Tn*phoA* mutagenesis was employed. *K. pneumoniae* was chosen in this study because it has an important role both as a nosocomial pathogen as a carrier of multi drug resistance genes.

1.2. Klebsiella pneumoniae

Klebsiella pneumoniae (originally named *Klebsiella aerogenes*) is not a common cause of infection in the community, however it has emerged as an important nosocomial pathogen after the introduction of antibiotics (Montgomerie and Oka, 1980; McGowan, 1985), showing a particularly high incidence of antibiotic resistance (Ullmann, 1978). These nosocomial infections are seldom the sole cause of death but in many cases are contributory factors.

Infection with *K. pneumoniae* is associated with a number of predisposing factors. A Public Health Laboratory Service report in 1982 showed that 49% of patients with *K. pneumoniae* infections were elderly and that 30% had concurrent urinary tract infections (UTI) (Young, 1982). These bacteria are among the five most frequently reported pathogens from nosocomial UTI. Their spread and establishment

in urologic wards have resulted in numerous endemic and epidemic outbreaks. *K. pneumoniae* is also an important pathogen in neonates, where it causes sepsis, meningitis and necrotising enterocolitis (Hill *et al.*, 1974). It has also often been found in the respiratory tract, especially in patients with pre-existing respiratory disease (Johanson *et al.*, 1972).

It has been reported that in addition to *E. coli*, *K. pneumoniae* carries plasmid-mediated β-lactamases that confer high-level resistance to cefoxitin and other 7-α-methoxy-β-lactams as well as to oxymino-β-lactams. To date, such extended-spectrum enzymes have been found in isolates from many parts of the world, including South Korea, Greece (Jacoby and Medeiros, 1991), France (Nouvellon *et al.*, 1994), Europe in general (Pornull *et al.*, 1993), Israel (Reish *et al.*, 1993), Australia (Eisen *et al.*, 1995), India (Chhibber and Bajaj, 1995), and the USA (Martinez-Martinez *et al.*, 1996).

The occurrence of extended-spectrum β-lactamases (ESBL) in Gram-negative bacilli, especially *Klebsiella* spp. and *E. coli*, has recieved much attention. ESBLs confer resistance to the newer cephalosporins and monobactams, creating serious problems. The majority of these enzymes are located on transferable plasmids and are related to the TEM- or SHV-β-lactamases (Sirot *et al.*, 1987; Chanal *et al.*, 1989; Weber *et al.*, 1990)). The enzymes show broader substrate-profiles caused by mutations, which lead to minor alterations in the amino acid configuration of the active site of the original enzyme. Since 1989 plasmid-mediated ESBLs in *K. pneumoniae* and *E. coli* conferring resistance to cefamycins have also been reported (Meyer *et al.*, 1993). These enzymes resemble chromosomally-mediated enzymes, with isoelectric points (pI) higher than 8.0 and are less inhibited by clavulanic acid

than the common plasmid-mediated enzymes (Bauernfeind *et al.*, 1989; Papanicolaou *et al.*, 1990). A European collaborative study during 1987-1988 investigated resistance to β-lactam antibiotics among Gram-negative septicaemia isolates (Pornull *et al.*, 1993). *E. coli* and *Klebsiella* spp. comprised 29% and 9% of the isolates, respectively. Four per cent of the *E. coli* isolates and 7% of the *Klebsiella* isolates were reported to show reduced susceptibility or resistance (MIC >2 mg/L) to ceftazidime, cefotaxime and/or aztreonam. Thirty-three of these isolates were tested and fifteen of them showed the reported reduced susceptibility or resistance when retested. All nine strains produced at least one β-lactamase of chromosomal origin with a pI range of 6.6-8.2. The conclusion was that *E. coli* and *Klebsiella* spp. from the European Study Group on Antibiotic Resistance septicaemia isolates were found to produce ESBLs of both chromosomal and plasmid origin (Pornull *et al.*, 1993).

In 1993 an outbreak of multiresistant *Klebsiella* in a neonatal intensive care unit was reported from the Children's Hospital and Beilinson Medical Center, Petah Tiqwa, Israel. All infections developed at least 5 days after admission to the unit (range, 5-40 days). Four infants had septicaemia and one had urinary tract infection. Three of the infected infants died. All *Klebsiella* isolates were resistant to ampicillin, cefotaxime, cefuroxime, co-amoxiclav, mezlocillin, chloramphenicol, gentamicin, and ceftazidime (except in two); all were susceptible to imipenem, amikacin and quinolones (Reish *et al.*, 1993).

Workers from the Monash Medical Centre in Australia reported a major outbreak of clinical infections by multiresistant *K. pneumoniae*, mainly involving patients in the Newborn Services Unit with limited spread to adult patients. Distinctive polymorphisms generated by the PCR technique of random amplified

polymorphic DNA analysis were utilized for strain differentiation in a fashion similar to ribotyping. Species differentiation of an increasing number of bacteria was studied using either single or multiple overlapping, randomly designed primers. The investigation showed initially the predominance of a single epidemic strain that was transmitted between patients in the Newborn Services Unit but was later also found in other pediatric and adult patients (Eisen *et al.*, 1995).

In India, acute respiratory infections (ARI) account for 14.3% of deaths during infancy and 15.4% of deaths between 1 and 5 years of age. Each year at least 630,000 persons die in India because of ARI, and pneumonia alone accounts for 70% of such deaths. Morbidity and mortality due to pneumonia continue to rank high in other countries. Among Gram-negative bacteria, *K. pneumoniae* alone accounts for 25-43% of nosocomial pneumonias, thus making it the most common agent in this disease process (Chhibber and Bajaj, 1995). In studies on *K. pneumoniae* strains obtained from the sputum of a patient with pneumonia at the Massachusetts General Hospital (Boston, Mass.), Martinez-Martinez *et al.* (1996) found that resistance to cefoxitin (MICs >64 μg/ml) is associated with the loss of the 35-kDa porin. George *et al.* (1995) found that *K. pneumoniae* mutants isolated on the basis of resistance to chloramphenicol or nalidixic acid, exhibited cross-resistance to a wide range of antibiotics including tetracycline, penicillins and cephalosporins, nalidixic acid, norfloxacin and puromycin. A reduced level of one of the major OMPs (equivalent to OmpF in *E. coli*) was also observed, however loss of OmpF alone could not account for the MDR (multi drug resistance) phenotype. A DNA fragment from the *K. pneumoniae* MDR1 mutant conferred on *E. coli* multiple-antibiotic resistance and other phenotypes (a substantial reduction in the level of OmpF, and changes in the

energy-dependent uptake of tetracycline and chloramphenicol) essentially identical to those observed in the *K. pneumoniae* MDR1 mutant. However, the region cloned in this study did not contain the putative *Klebsiella* equivalent of the *marRAB* region of *E. coli*, as the 3.0 kb *Bam*HI cloned fragment was not detected by hybridization of the *marRAB* probe in *K. pneumoniae*.

1.3. The catecholic 7α-formamido cephalosporin BRL41897A

The 7α-formamido cephalosporins BRL 41897A and BRL 42948A (Figure 1.1) were developed by workers at SmithKline Beecham in a chemical programme designed to identify β-lactamase stable antibiotics (Basker *et al.*, 1986).

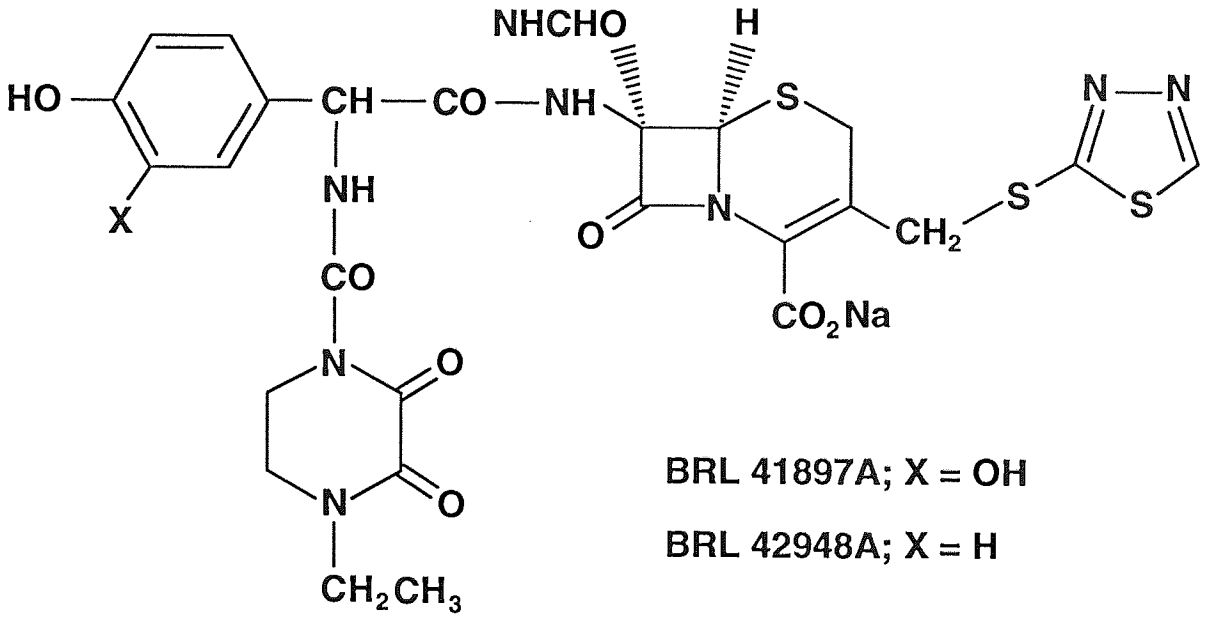


Figure 1.1. The structure of the 7(α)-formamido cephalosporins, BRL 41897A and BRL 42948A.

The antibacterial activity of a α-formamido penicillin and some preliminary structure-activity relationships have been published, followed by comparison of activity of some 7α-substituted cephalosporins with the corresponding 7α-formamido analogue (Basker *et al.*, 1986). The advantage of the 7α-substituted cephalosporins over the unsubstituted derivative is their greater stability towards bacterial β-lactamases. In an attempt to improve upon this activity, several other ureido and acyl amino cephalosporins were introduced. Results showed that the 7α-formamido cefoperazone analogue was the most potent of the cephalosporins tested, being at least 2-4 fold more active than other ureido or acyl amino derivatives.

It was found that introduction of a 3-[(N-methyltetrazolyl) thiomethyl] group at C-3 or 4-hydroxy substituent into the phenyl ring results in a 2-fold increase in activity. However, further substitution, giving the 3,4-dihydroxy derivative, results in a pronounced increase in activity against Gram-negative organisms, albeit at the expense of some activity against Gram-positive bacteria. This compound possessed very potent activity particularly against strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Basker *et al.*, 1984).

The potent activity of catechol-substituted β-lactam antibiotics results from their ability to penetrate the outer membrane via the TonB-dependent high affinity iron transport systems which are expressed when bacteria are grown under conditions of iron limitation (Watanabe *et al.*,1987; Sanada *et al.*,1988; Critchley *et al.*,1991; Hazumi *et al.*,1992). BRL 41897A containing the formamido group at the 7α position and two hydroxyl groups at positions 3 and 4 of the phenyl ring, was used in this study to investigate iron uptake mechanisms in *K. pneumoniae*

1.4. Iron in living organisms

Iron is the second most abundant metal (after aluminium) and the fourth most abundant element in the earth's crust. It is required for growth by most organisms, although not at the level of a macronutrient (Weinberg, 1978); only the lactic acid bacteria do not seem to require iron for their metabolism (Archibald, 1983). The iron content of microorganisms varies considerably. For example, plankton, bacteria and fungi contain approximately 3.5, 0.25 and 0.13 mg per gram dry weight, respectively. There are also considerable species differences in the requirement of iron to support growth. The common enteric bacteria need 0.02-0.03 mg per litre of iron (0.5μM) in the medium for maximum growth, while pseudomonads require four-fold higher levels (Neilands, 1974). Hartmann and Braun (1981) found that *Escherichia coli* K-12 could grow aerobically at an iron concentration as low as 0.05 μM and the growth rate increased with increasing iron concentration between 0.05 and 2 μM. Radioactive tracer techniques revealed a cellular iron content of 4 nano moles per mg dry weight.

The importance of iron for living organisms is underlined by its role in a large number of proteins which require its presence for their activity. Firstly, there are proteins involved in the reversible binding of oxygen in animals (haemoglobins and myoglobins, Dickerson and Geis, 1983), plants (leghaemoglobin, Ellfolk, 1972) and invertebrates (haemerythrin, Crichton and Charloteaux Wauters, 1987). Iron is also required to activate enzymes involved in electron transfer, cytochromes, hydrogenase, ferridoxin and succinate dehydrogenase (Neilands, 1981; Dallman, 1986). In many organisms iron is involved in nitrogen fixation and in chlorophyll synthesis (Neilands, 1981). In addition, iron is required for the activity of a number of enzymes in

microbial and mammalian cells which participate in oxygen metabolism, such as superoxide dismutase (which has an important role in the intracellular phase of the pathogenesis of *Shigella flexneri*, Frauzan, 1990), catalase and peroxidase (Halliwell and Gutteridge, 1985), various amino acid hydroxylases and dioxygenases (Feigelson and Brady, 1974; Nozaki and Ishimura, 1974; Wigglesworth and Baum, 1980; Braun, 1985) and ribonucleotide reductase, which is necessary for transformation of ribonucleotide diphosphates to their corresponding deoxy derivatives required for DNA synthesis (Reichard and Ehrenberg, 1983). Although iron has a very important role in biological systems, it can also be extremely toxic. Iron acts as a catalyst to produce the highly reactive hydroxide radical from superoxide anion and hydrogen peroxide (Flitter *et al* , 1983; Griffiths, 1987a):

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe} OH^{\cdot} + OH^- + O_2$$

Hydroxide radicals are involved in the destruction of biological membranes via peroxidation of lipids, and in the scission of DNA (Weinberg, 1989). In order to survive, aerobic bacteria have enzymes to prevent the production of hydroxide radicals; peroxidase and catalase for removing hydrogen peroxide, and superoxide dismutase for removing superoxide anions (Griffiths, 1987b).

1.5. Acquisition of iron by microorganisms

Iron has a very low solubility in aqueous solutions. In these solutions, iron can exist in two oxidation states, Fe^{2+} and Fe^{3+}, the predominant form being Fe^{3+}. Accepting 10^{-23}M as the solubility constant of $Fe(OH)_3$, the maximum concentration

of free iron at physiological pH is 10^{-17}M (Neilands, 1984) and in normal human serum, the amount of free iron in equilibrium with transferrin-bound iron has been estimated to be approximately 10^{-18}M (Payne, 1988), an extremely low concentration compared with that needed for maximum growth. Consequently microorganisms have evolved various strategies to obtain sufficient quantities of iron to support their metabolism and growth (Hantke, 1987). There are two general mechanisms by which microorganisms acquire iron within an iron-poor environment. The first is direct utilization of host iron compounds; alternatively, microorganisms synthesize reductants or chelators to dissociate the iron from host complexes (Payne, 1988; Ratledge et al., 1982; Sritharan and Ratledge, 1988; Kammler *et al.*, 1993).

1.5.1. **Utilization of host iron compounds**

Direct utilization of host iron complexes should enhance the growth of microorganisms (Hartmann and Braun, 1980). For example, *Neisseria gonorrhoeae* and *N. meningitis* can utilize transferrin-bound iron (Archibald and DeVoe, 1979; Mickelsen and Sparling, 1981). In addition, both pathogenic *Neisseria* species have been shown to utilize lactoferrin as an iron source (McKenna *et al.*, 1988; Dyer *et al.*, 1987). Among nonpathogenic *Neisseria* species fewer strains are able to utilize transferrin or lactoferrin than among pathogenic species (Mickelsen and Sparling, 1981; Mickelsen *et al.*, 1982; Simmonson *et al.*, 1982; Brocks *et al.*, 1991). Transferrin-binding proteins TBP1 (100 kDa) and TBP2 (85 kDa) have been identified; TBP1 is required for transferrin utilization (Lęgrain *et al.*, 1993; Cornelissen *et al.*, 1992). *Haemophilus influenzae* is also able to utilize transferrin, but not lactoferrin, as a source of iron, while the nonpathogenic *H. parainfluenzae*

is unable to utilize this iron source (Morton and Williams, 1989; Herrington and Sparling, 1985; Pidcock *et al.*, 1988; Holland *et al.*, 1992). *Bordetella pertussis* is able to utilize transferrin, ovotransferrin or lactoferrin (Redhead *et al.*, 1987). Many *Bacteroides* species are also able to utilize transferrin (Verweij Van Vught *et al.*, 1988) and one virulent strain of the fish pathogen *Aeromonas salmonicida* was reported to be able to utilize transferrin or lactoferrin (Chart and Trust, 1983). It has been observed that pyoverdin, a siderophore from *Pseudomonas aeruginosa* acquires iron from transferrin (Wolz *et al.*, 1994). *Staphylococcus aureus* is capable of binding both human and bovine lactoferrins, while bovine mastitis isolates of coagulase-negative staphylococci also bind bovine lactoferrin. A 42 kDa cell wall protein in staphylococci has been identified as the receptor for human transferrin. Expression of this protein is partially iron regulated in *S. epidermidis*, but not in *S. aureus* (Konetschny-Rapp *et al.*, 1991; Modun *et al.*, 1994; Hoog *et al.*, 1994).

Haemoglobin and haem have been widely reported to serve as sole sources of iron for bacteria. *E. coli* (Griffiths, 1987a) and *Yersinia* species (Perry and Brubaker, 1979) have been shown to utilize haem as an iron source. *Neisseria meningitidis, N. gonorrhoeae* (Dyer *et al.*, 1987), *Haemophilus influenzae* (Pidcock *et al.*, 1988; Lee, 1992) and *Vibrio cholerae* (Stoubner and Payne, 1988) utilize both haem and haemoglobin. Recently, Lewis and Dyer (1995) observed that Hpu (haemoglobin-haptoglobin utilization), the 85 kDa iron-regulated outer membrane protein of *N. meningitidis* is the receptor for haemoglobin and haemoglobin-haptoglobin complex.

Because levels of free haem in normal serum are low (Morgan, 1981) and would not support the growth of most pathogens (Griffiths, 1987b), some pathogenic

microorganisms are able to increase the availibility of free haemoglobin in blood by secreting haemolysins, toxins capable of erythrocyte lysis (Griffith and Bullen, 1987). Haemolytic *E. coli* strains were more frequently isolated from patients with urinary tract infection than from healthy humans (Brooks *et al.*, 1980). Minshew *et al.*(1978) showed a correlation between haemolysin production and a variety of extraintestinal infections including septicaemias. Law and Kelly (1995) reported that *E. coli* O157 isolates produced enterohaemolysin and were able to utilize both haem and haemoglobin. The incidence of these properties in the non-O157 groups was variable and occured at a significantly lower level than among the O157 isolates. Similarly, aerolysin, a haemolysin produced by *Aeromonas sobria*, has a role in systemic infection by this organism (Goebel *et al.*, 1988). Furthermore, the expression of haemolysin genes has been shown to be derepressed under conditions of iron - limitation in some strains of *E. coli* (Gruenig *et al.*, 1987).

1.5.2. **Obtaining iron by synthesizing reductants**

For many microorganisms, the ability to obtain iron from host iron complexes requires dissociation of the iron rather than direct utilization of these compounds. The secretion of reductants could effect the release of iron from transferrin or lactoferrin, which have a relatively low affinity for ferrous iron (Hantke, 1987). A soluble reductant in supernatants of *Listeria monocytogenes* cultures capable of removing iron from transferrin has been identified by Cowart and Foster (1985). The dental plaque organism *Streptococcus mutans* acquires ferrous iron via a membrane flavin reductase (Evans *et al.*, 1986). Similarly, the yeast *Saccharomyces cerevisiae* employs a cytoplasmic membrane reductase to reduce ferric iron (Lesuisse *et al.*, 1987).

1.5.3. Chelator-mediated iron transport

Many microorganisms either produce or scavenge iron chelators called siderophores for iron acquisition. Siderophores are low molecular weight compounds (500-1000 daltons) with an extremely high affinity for ferric iron, whose biosynthesis is regulated by iron levels. Although siderophores display considerable structural variation, they may in general be classified as either hydroxamates or phenolates (Neilands, 1984). They are formed generally by aerobic and facultatively anaerobic bacteria and fungi, but so far there have been no reports of siderophores in strictly anaerobic bacteria, in lactic acid bacteria or in *Saccharomyces* spp. (Neilands, 1984). Siderophores may also act as growth factors, antibiotics and bacterial virulence factors (Crosa and Hodges, 1981; Walter *et al.*, 1983). They are secreted into the extracellular medium where they complex available Fe^{3+}; the ferrisiderophore complexes are then assimilated by the cells via specific surface receptors and once inside the cells, the iron is released either by reduction of the iron to Fe^{2+}, for which the siderophores have low affinity, or by hydrolysis of the ferrisiderophores (OBrien, 1971; Bindereif and Neilands, 1985; Grewal *et al.*, 1982; Weinberg, 1989) (Figure 1.2).

Another iron chelator that can serve as an iron carrier is citrate. A citrate iron transport system has been identified in strains of *E. coli*, but is not present in *Salmonella typhimurium* or *Shigella flexneri*. Ferri-citrate transport in *E. coli* is induced by the presence of citrate in the external medium (Frost and Rosenberg, 1973).

Figure 1.2. Regulation of the iron uptake system in Gram-negative bacteria (from Neilands, 1982).

1.6. High affinity iron transport systems in *E. coli*

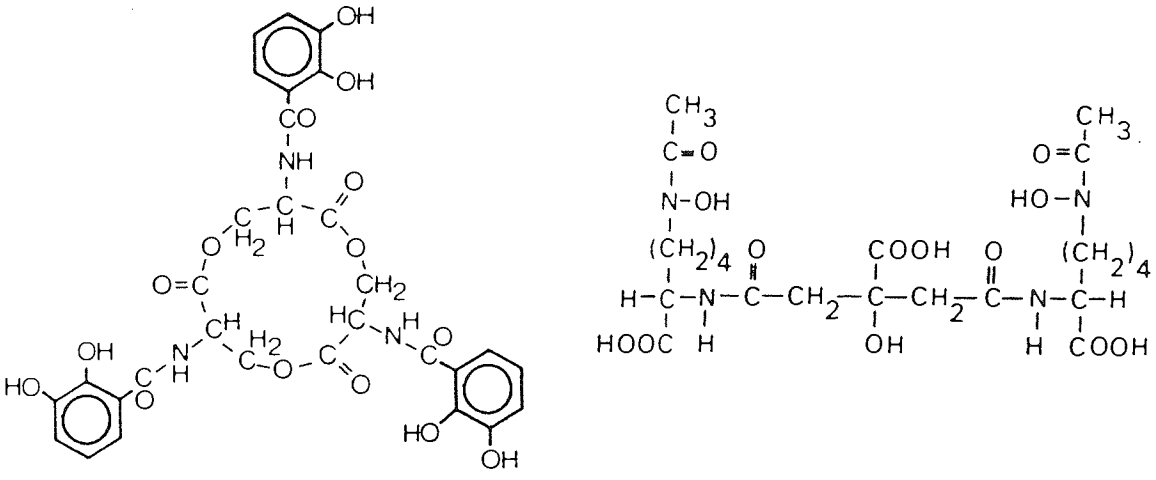
As outlined above, since most bacterial growth depends on the availibility of iron, an essential nutrient that participates in many biological processes, the possession of high affinity iron transport systems is crucial for bacteria to override iron limitation in the environment (Kammler *et al.*, 1993). Thus, most Gram-negative bacteria produce siderophores and specific outer membrane protein receptors (IROMPs, iron regulated outer membrane proteins) in response to iron starvation. Siderophore systems in *E. coli* have been studied most extensively; four high affinity iron transport systems have been described, involving enterobactin, aerobactin, ferrichrome and other hydroxamates, and citrate (Klebba *et al.*, 1982; Lambert, 1988b).

1.6.1. The enterobactin system

Enterobactin or enterochelin was first identified as an endogenous catechol siderophore of *E. coli* (O'Brien and Gibson, 1970) and *Salmonella typhimurium* (Pollock and Neilands,1970; Fernandez-Beros *et al.*, 1989). This siderophore is also produced by *K. pneumoniae* and *Shigella species* (Payne *et al.*, 1983; Perry and San-Clemente, 1979). The enterobactin receptor protein in *E. coli* is an 81 kDa outer membrane protein, which is expressed only in cells grown in iron-restricted medium (McIntosh and Earhart, 1976). The enterobactin genes have been cloned and studied in detail. They occupy approximately 26 kb of DNA on the *E. coli* chromosome and are organized into a number of transcriptional units (Fleming *et al.*, 1983 and 1985; Laird and Young, 1980; Pettis and McIntosh, 1987).

Genes *entCBA* encode enzymes required for the biosynthesis of catechol from the precursor chorismate. EntA, EntB and EntC are soluble enzymes which convert

chorismic acid to 2,3-dihydroxybenzoic acid. EntC is isochorismate synthetase (Crosa, 1989), EntB is 2,3-dihydro-2,3-dihydroxybenzoate synthetase (Nahlic *et al.*, 1989) and EntA is 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (Liu *et al.*, 1989).



(1) (2)

Figure 1.3. The structure of (1) enterobactin and (2) aerobactin

EntD, E, F and G are subunits of a synthetase that forms enterobactin from dihydroxybenzoic acid and L serine (Figure 1.6) (Nahlik *et al.*, 1987; Greenwood and Luke, 1980).

The protein products of genes *fepABCDEG* are specifically required for transport of the ferrienterobactin complex. FepA is the 81 kDa outer membrane protein receptor for ferrienterobactin, while enterobactin iron uptake across the periplasm and

cytoplasmic membrane requires the products of the other *fep* genes. FepB is a periplasmic binding protein, the FepC protein appears to reside in the cytoplasmic membrane and the other genes encode components that may act with FepC to form a cytoplasmic membrane permease (Ozenberger, 1987; Pierce and Earhart, 1986; Pierce *et al.*, 1983). The *fepD* and *fepG* genes have recently been sequenced and shown to encode very hydrophobic proteins with extensive homology to other integral membrane proteins involved in cytoplasmic membrane transport of TonB-dependent transport systems (Chenault and Earhart, 1991; Shea and McIntosh, 1991). The *fes* gene product is a reductase whose role may be to release the iron from enterobactin (Holifield and Neilands, 1978).

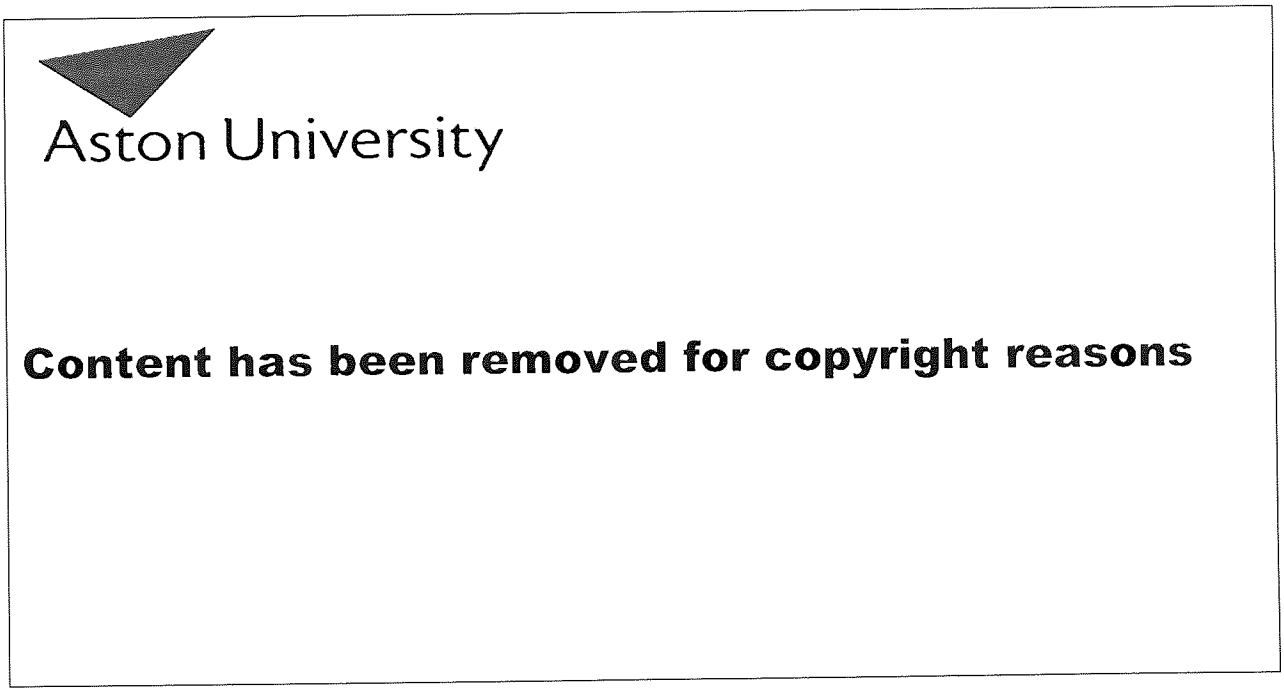


Figure 1.4: Synthesis of enterobactin (Silver and Walderhaug,1992).

1.6.2. **The aerobactin system.**

Aerobactin (Figure 1.3 no 2) is an endogenous hydroxamate siderophore which was first isolated from *Aerobacter aerogenes* strain 62-1 (Gibson and Magrath, 1969) and subsequently identified in other enteric bacterial species (Table 1.1).

Table 1.1: Location of the aerobactin genes among the *Enterobacteriaceae*.

Species	Genetic location
E. coli from human extraintestinal infections	Usually chromosomal
E. coli from animal extraintestinal infection	Plasmids (usually ColV)
Enteroinvasive *E. coli*	Chromosome
Enteropathogenic *E. coli*	Plasmids (not ColV) or chromosome
Shigella spp.(not dysenteriae type I)	Chromosome
Salmonella spp. from gastro-enteritis	Chromosome
Salmonella spp. from extra-intestinal infections	Plasmids
Klebsiella spp.	Plasmids
Enterobacter spp.	Plasmids

The aerobactin genes were first identified associated with plasmid ColVK30 (Williams 1979; Williams and Warner, 1980; Warner *et al.*, 1981; Valvano and Crosa, 1984), but are also present on other ColV plasmids (Gross *et al.*, 1984; Williams and George, 1979) and on non-ColV plasmids in *E. coli, Aerobacter (Enterobacter) aerogenes* and *Salmonella* species (McDougall and Neilands, 1984; Colonna *et al.*, 1985; Bindereif and Neilands, 1985; Roberts *et al.*, 1986; Fernandez Beros *et al.*, 1988; Gonzalo *et al.*, 1988; Loper *et al.*, 1993). Furthermore, aerobactin genes have been found on the chromosomes of *E. coli* K1 isolates (Carbonetti *et al.*, 1986; Valvano and Crosa, 1984; Valvano *et al.*, 1986), *Shigella* species (Lawlor and Payne, 1984), *Salmonella* species (McDougall and Neilands, 1984) and *K. pneumonie* (Nassif and Sansonnetti, 1986).

The aerobactin system of plasmid ColVK30 has been cloned (Bindereif and Neilands, 1983 and 1985) and found to comprise five genes arranged in an operon (Carbonetti and Williams, 1984). Four genes, *iucABCD* encode enzymes required for aerobactin biosynthesis from the precursors L-lysine and citrate. Biosynthesis begins with N-oxygenation of lysine by the *iucD* gene product (53 kDa), followed by N-acetylation of hydroxylysine by the *iucB* gene product (33 kDa). IucA and IucC proteins (63 kDa and 62 kDa respectively) then catalyze the sequential attachment of two acetylhydroxylysine sidechains to citrate (deLorenzo and Neilands, 1986; de Lorenzo *et al.*, 1986). The fifth gene, *iutA*, encodes the 74 kDa outer membrane aerobactin receptor protein (Figure 1.5) (Carbonetti and Williams, 1984; Bouchet, 1994).

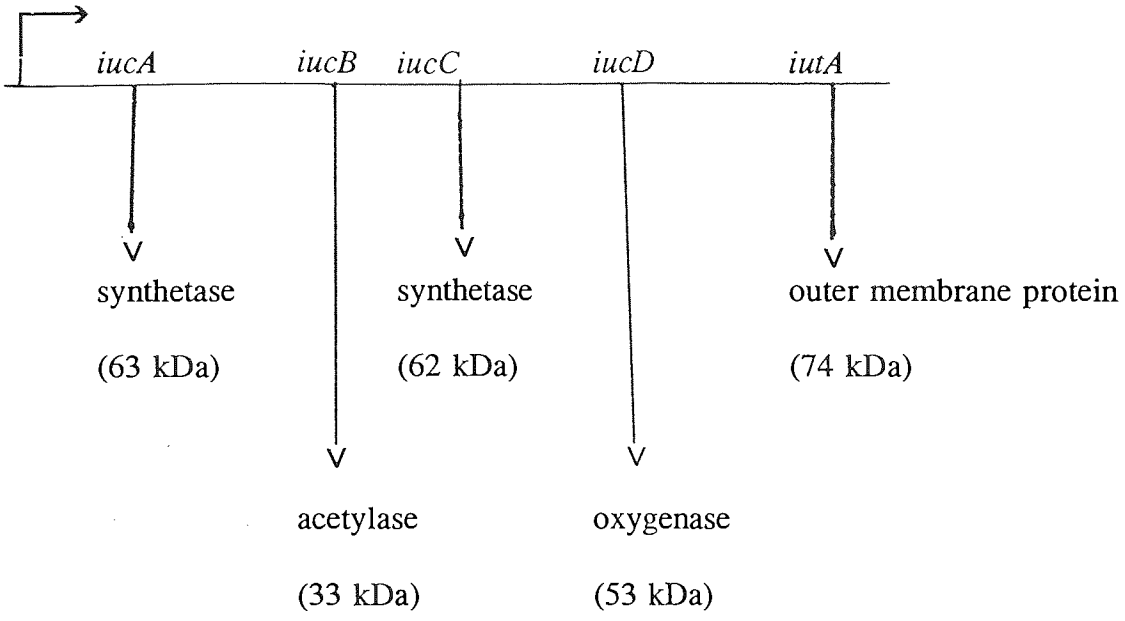


Figure 1.5: The five-gene cluster of the aerobactin system of plasmid ColV-K30 and the polypeptide products it encodes. the direction of its promoter.

A number of experiments have shown that aerobactin is more effective as a siderophore *in vivo* than enterobactin. At neutral pH enterochelin deferrates transferrin at a faster rate than aerobactin, but in the presence of serum albumin the relative rates are reversed (Konopka and Neilands, 1984). This may be due to the aromatic nature of enterobactin which tends to promote its adsorption to proteins; enterobactin molecules attached to serum proteins are presumably less effective as siderophores. Also, serum-bound enterobactin molecules may function as haptens, since the presence of antibodies against enterobactin has been detected in normal human serum (Moore *et al.*, 1980), and such antibodies inhibit enterobactin uptake (Moore and Earhart, 1981).

1.6.3. Exogenous siderophore systems

E. coli also expresses systems for the utilization of various exogenous siderophores as demonstrated by the range of outer membrane proteins expressed in iron limited conditions (Table 1.2). The outer membrane protein FhuE (76 kDa) is the receptor of coprogen and rhodotorulic acid, both linear hydroxamate siderophores of fungal origin (Hantke, 1983). Coprogen is produced by *Penicillium* and *Neurospora* species, rhodotorulic acid by *Rhodotorula*, *Sporobolomyces* and *Leucosporidium* species (Hider, 1984).

The *E. coli* outer membrane also contains the FhuA (78 kDa) protein, the receptor of ferrichrome, ferricrysin and ferricrocin. Ferrichrome is a cyclic trihydroxamate siderophore produced by many fungal species including *Ustilago sphaerogena* and all penicillia (Wayne and Neilands, 1975; Hider, 1984); ferricrysin and ferricrocin are produced by *Aspergillus* species (Hider, 1984). Hydroxamate--mediated uptake of iron across the periplasm and cytoplasmic membrane requires the products of the *fhuBCD* genes (Braun *et al.*, 1983; Hantke, 1983; Prody and Neilands, 1984; Wookey *et al.*, 1981). FhuB is a very hydrophobic cytoplasmic membrane protein, FhuC is a hydrophilic cytoplasmic membrane protein with homology to ATP binding proteins, and FhuD is a hydrophilic periplasmic protein (Koster and Braun, 1989).

FepA and IutA have been mentioned above. Fiu and Cir are reported to act as receptors for simple catechols such as 2,3-dihydroxybenzoylserine, a degradation product of enterochelin, and 2,3-dihydroxybenzoic acid, an intermediate in the synthesis of the siderophore (Nikaido and Rozenberg, 1990).

Table 1.2: Iron-regulated outer membrane proteins of *E. coli* K-12.

Protein	Size	Receptor for
Fiu	83 kDa	monomeric catechols
FepA	81 kDa	ferrienterobactin,colicins B and D
FecA	80.5 kDa	ferricitrate
FhuA	78 kDa	ferrichrome, ferricrysin, ferricrocin, colicin M, phage T1, T5, 80
FhuE	76 kDa	coprogen, rhodotorulic acid
IutA	74 kDa	ferriaerobactin, cloacin DF13
Cir	74 kDa	monomeric catechols, colicins Ia and Ib

1.6.4. The citrate system

As mentioned before, besides the iron uptake systems that involve siderophores, *E. coli* possesses another high affinity system using citrate as an iron carrier. The receptor is a 80.5 kDa outer membrane protein. Ferricitrate transport is induced by the presence of citrate in the external medium (Frost and Rosenberg, 1973; Hussain *et al.*, 1981). Note that citrate does not serve as a carbon source for *E. coli*, and thus it is extracellular, rather than intracellular, citrate that induces the system (Zimmermann *et al.*, 1984).

Ferricitrate requires the products of the *fecBCDE* genes for crossing the

periplasm and cytoplasmic membrane. FecB is the putative periplasmic binding protein. FecC and FecD are very hydrophobic polypeptides localized in the cytoplasmic membrane. The product of the *fecE* gene is a hydrophilic cytoplasmic membrane-associated protein containing regions of homology to ATP-binding proteins (Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989).

1.7. **TonB-dependence of siderophore uptake**

Uptake of all siderophores, both hydroxamates and phenolates, and of ferric dicitrate across the outer membrane of *E. coli* requires the participation of the cytoplasmic membrane protein TonB (Frost and Rosenberg, 1973; Hantke and Braun, 1975; Williams, 1979; Schoffler and Braun, 1989; Postle, 1990). The TonB protein is anchored in the cytoplasmic membrane by its N-terminal hydrophobic sequence while the remainder of the protein extends into the periplasmic space (Postle, 1990). The sequence of the *tonB* gene and the membrane topology of the TonB protein of *Salmonella typhimurium* are similar to those of *E. coli* (Hannavy *et al.*, 1990). It has been suggested that the TonB protein couples metabolic energy in the cytoplasmic membrane to the outer membrane receptor proteins. In the absence of TonB, receptors bind their substrates but do not carry out active transport (Hancock and Braun, 1976; Hantke and Braun, 1975). Vitamin B12 is also actively transported across the outer membrane of *E. coli* in a process that is dependent on the TonB protein (Kadner, 1990). In addition, it has been reported that the uptake of many colicins and bacteriophages in *E. coli* are also TonB-dependent (Davies and Reeves, 1975; Hantke and Braun, 1975; Hancock and Braun, 1976). Recently, it has been reported that the TonB protein is required for haem utilization *in vitro* and for

virulence of *H. influenzae* type b in an animal model (Jarosik, *et al.*, 1994); moreover, the uptake of iron from human transferrin by *H. influenzae* is a TonB-dependent process (Jarosik, *et al.*, 1995).

Outer membrane proteins which are dependent for activity on TonB contain a consensus peptide sequence called the "TonB box": A-Thr-X-X-Val-Y-Ala where A indicates an acidic residue, X is a non-polar residue and Y is Ser or Thr (Nau and Konisky, 1989). This sequence was also found in the PupA ferric pseudobactin receptor of *Pseudomonas putida* (Bitter *et al.*, 1991), in the FoxA ferrioxamine B receptor of *Yersinia enterocolitica* (Baumler and Hantke, 1992), in the FpvA ferripyoverdine receptor of *Pseudomonas aeruginosa* (Poole *at al.*, 1993) and in the TBP1 transferrin receptor of *Neisseria* (Cornelissen *et al.*, 1992). TonB interacts directly with the outer membrane transport proteins in a manner that recognizes the local conformation but not specific side chains within this conserved region (Gudmunsdottir *et al.*, 1989; Bell *et al.*, 1990). TonB-dependent uptake systems also require the involvement of the ExbB and ExbD proteins (Hantke and Zimmermann, 1981). The TonB protein is degraded by cellular proteases, a process which is inhibited by ExbB, which also stabilizes ExbD (Fisher *et al.*, 1989). The membrane topology of *Escherichia coli* ExbD and ExbB have been identified. Residues 1 to 22 of ExbD are located in the cytoplasm, a segment of residues 23 to 43 forms a transmembrane domain and residues 44 to 141 are located in the periplasm (figure 1.3) (Kampfenkel and Braun, 1992). The N-terminus of ExbB is located in the periplasm, followed by three transmembrane segments (residues 16 to 39, 128 to 155 and 162 to 194) a small periplasmic loop and two large portions in the cytoplasm (Figure 1.6 from Kampfenkel and Braun, 1993).

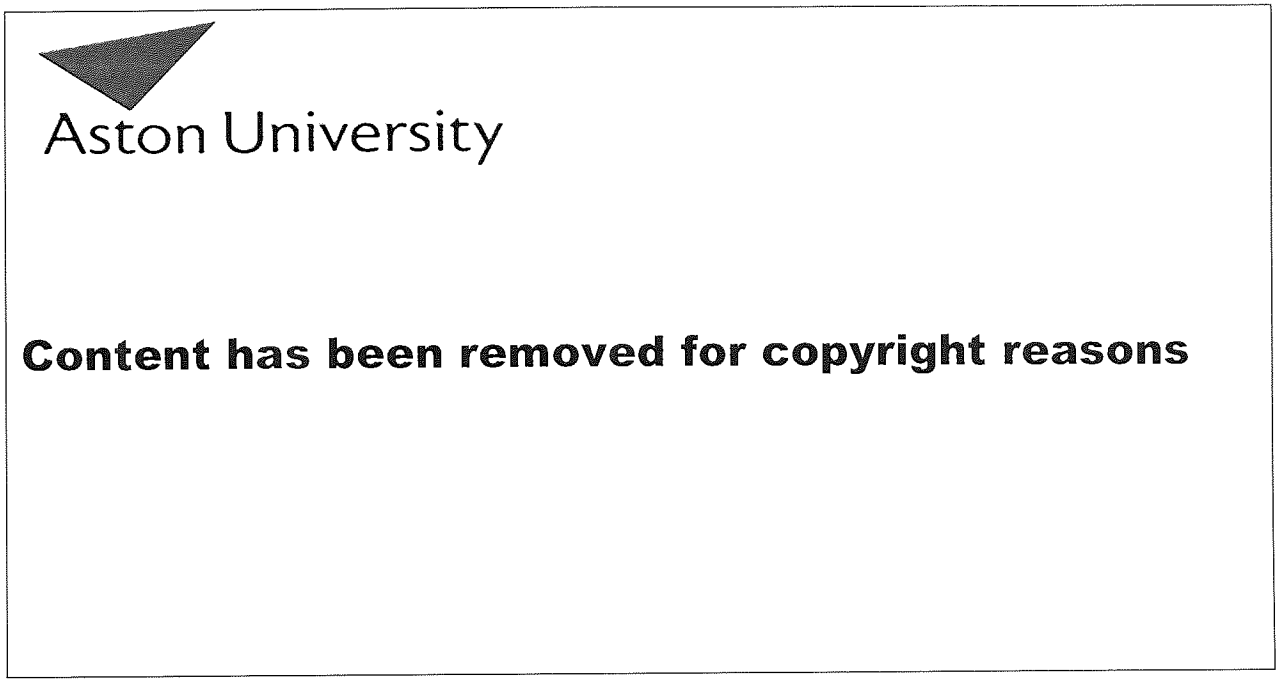


Figure 1.6: Location of TonB, ExbB and ExbD in the bacterial membrane (Roof *et al.*, 1991; Kampfenkel and Braun, 1992 and 1993).

1.8. Siderophore systems in *Klebsiella* and other bacteria

Siderophore-mediated iron uptake systems of *Klebsiella* and other enteric bacteria have not been studied as extensively as those of *E. coli*, but they are generally assumed to be similar. In addition to *Klebsiella*, the enterobactin system has also been found in *Salmonella* and *Shigella* species. Their enterobactin receptor proteins are apparently identical and these appear as a 81 kDa protein on SDS-PAGE. The

Perry and San Clemente, 1979; Pollock and Neilands, 1970; Schmitt and Payne, 1988; Williams *et al.*, 1987). The aerobactin iron uptake system is much more widespread among the *Enterobacteriaceae*, having been found in strains of *Klebsiella, Enterobacter, Salmonella, Shigella, Citrobacter, Proteus, Morganella, Yersinia, Serratia* and *Hafnia* (Colonna *et al.*, 1985; Crosa *et al.*, 1988; Martinez *et al.*, 1987).

There have not been any reports of aerobactin or enterochelin being synthesized by bacteria of families other than *Enterobacteriaceae*, but many non-enteric bacteria produce siderophores and they are also able to utilize some siderophores when available exogenously. The following paragraphs describe some of the best studied examples.

Pseudomonas aeruginosa synthesizes at least two siderophores in response to iron deprivation, pyochelin and pyoverdin (Cox and Adams,1985). It was observed that pyoverdin was more effective than pyochelin (Sriyosachati and Cox, 1986). In addition, it is capable of utilizing a number of heterologous siderophores, including pyoverdins produced by other pseudomonads, ferrioxamine B, aerobactin and enterochelin (Poole *et al.*, 1990). There are two receptors for ferripyochelin, a 14 kDa protein (Sokol and Woods, 1983) and a 75 kDa protein (Heinrichs *et al.*, 1991), both in the outer membrane. The receptor for ferripyoverdine in *P. aeruginosa* strain PAO1 has been identified as an 80 kDa by Meyer *et al.*, 1990 or a 90 kDa by Poole *et al.*, 1991. The ferric enterobactin receptor gene (*pfeA*) of *P. aeruginosa*, which also encodes an 80 kDa outer membrane protein, has recently been cloned (Dean and Poole, 1993). In addition, malleobactin, a hydroxamate-type of siderophore has been isolated from *P. pseudomallei* (Yang *et al.*, 1991).

Vibrio cholerae strains produce the catecholamine siderophore vibriobactin (Payne and Finkelstein, 1978; Sigel and Payne, 1982; Griffiths *et al.*, 1984), and the fish pathogenic species *V. anguillarum* produces the catecholic siderophore anguibactin. The ability to synthesize and utilize anguibactin may be associated with large plasmids, the prototype of which is pJM1 (Crosa, 1980). The opportunist pathogen *V. vulnificus* produces both catechol and hydroxamate siderophores (Simpson and Oliver,1983).

Study on *Klebsiella* isolates from human asymptomatic bacteriuria, cystitis and acute pyelonephritis, Podschum *et al.* (1993) reported that expression of siderophores was detected in all but 1 of the 146 isolates. Enterobactin production was a uniform attribute. In contrast to enterobactin expression, aerobactin was produced by few human urinary tract infections (UTI) isolates (3%). Apparently, aerobactin synthesis is rare in *Klebsiella* isolates compared with that in *E.coli*.

In response to iron deprivation, *K. pneumoniae* expressed up to six IROMPs in the 70 to 85 kDa range, the 74 and 76 kDa are aerobactin receptors and the 81 kDa is the enterobactin receptor (Williams *et al.*, 1987; Williams *et al.*, 1989). To date, the function(s) of the other proteins in *Klebsiella* have not been determined.

1.9. **The genetic regulation of iron uptake systems**

Regulation of a wide range of genes that respond to iron starvation is governed by the product of the *fur* (ferric uptake regulation) gene (Reeves, 1979; Griggs and Konisky, 1989), which was identified in a mutant of *E. coli* that expressed iron regulated genes constitutively (Hantke, 1981). The *fur* gene, which encodes a negative regulatory system, has been cloned (Hantke, 1984) and sequenced (Schaffer *et al.*,

1985). At low concentrations of intracellular ferrous ions, the Fur protein has a weak affinity for the operator of iron regulated genes and so, with the operator unoccupied, transcription occurs. At high internal concentration of ferrous iron, the Fur protein binds tightly to the operator DNA and transcription is blocked. As a DNA-binding repressor protein, Fur protein requires Fe^{2+} or certain other divalent metal ions (Mn, Cd, Cu, Zn and Co) as a corepressor to bind specific sequences at the promoter regions of iron-controlled genes (Bagg and Neilands, 1987). The promoter sequence involved in Fur binding was first identified in the promoter of the aerobactin operon of plasmid ColV-K30 (de Lorenzo *et al*, 1987); since then similar sequences have also been found in the *cir* gene promoter (Griggs and Konisky, 1989) and many other iron regulated genes. A consensus sequence for Fur binding has been identified, the "Fur box": GATAATGATAATCATTATC (Calderwood and Mekalanos, 1987; de Lorenzo *et al.*, 1987; Pressler *et al.*, 1988).

Similar iron responsive regulation systems are assumed to operate in other microorganisms. The *fur* regulatory gene has been cloned from *Yersinia pestis* (Stagg and Perry, 1991), *Pseudomonas aeruginosa* (Prince *et al.*, 1993) and *Campylobacter jejuni* (Wooldridge *et al.*, 1994).

In addition to genes related to acquiring iron, other genes are also regulated by the level of iron. Production of bacterial toxins, including the diphtheria toxin of *Corynebacterium diphtheriae* (Cryz *et al.*, 1983; Boyd *et al.*, 1990), *E. coli* Shiga-like toxins SLTI and SLTII (Calderwood and Mekalanos, 1987), the haemolysin of *Vibrio cholerae* (Stoebner and Payne, 1988) and *Pseudomonas aeruginosa* exotoxin A (Prince *et al.*, 1993) are all regulated by the amount of iron in the growth environment.

Fur influences the production of several pH-regulated gene products in *Salmonella typhimurium* (Foster, 1991; Foster and Hall, 1992; Prince *et al.*, 1993). Furthermore it has been reported that 36 proteins in *S. typhimurium* are affected by iron availability and most (34) of these are under the control of Fur. Although many of the Fur dependent proteins are under negative control, a significant proportion (15 of 34) appear to be under a form of positive control. Surprisingly, not all ironregulated proteins are controlled by Fur and not all Fur-dependent proteins are obviously regulated by iron status (Foster and Hall, 1992). The Fur protein, in the presence of a divalent metal such as iron, also represses the expression of manganese superoxide dismutase in *E. coli* K-12 (Niederhoffer *et al.*, 1990; Tardat and Touati, 1991; Compan and Touati, 1993).

1.10. The *tol* genes and cloacin DF13 susceptibility

It has been known for a long time that many outer membrane proteins, including ferric siderophore receptor proteins, can have more than one function. For example, the 78 kDa FhuA protein (formerly TonA) is the receptor for ferrichrome, but is also the receptor for bacteriophages T1, T5 and ϕ80, and for colicin M and the antibiotic albomycin (Bourdineaud *et al.*, 1989; Jackson *et al*, 1986; Konisky, 1979 and 1982; Pugsley, 1985; Killmann and Braun, 1992). The 81 kDa FepA protein is the receptor for ferric enterobactin as well as for colicin B (Neilands, 1982). The fact that the killing activity of cloacin DF13 was inhibited by aerobactin suggested that they bind to the same receptor in *Enterobacter cloacae* (Van Tiel-Menkveld *et al*, 1982).

Cloacin DF13 is produced by bacteriocinogenic strains of *Enterobacter cloacae* harbouring the plasmid CloDF13 (Stouthamer and Tieze, 1966; de Graaf *et al.*,

1969). It is characterized by its ability to kill cells of susceptible strains of *Enterobacter* and *Klebsiella* species (de Graaf *et al.*, 1969; Krone, 1985; Cooper and James, 1985). Cloacin DF13 is secreted as a protein complex comprising a 59 kDa activity protein and a 10 kDa immunity protein (de Graaf and Klaasen-Boor, 1977; Oudega *et al*, 1977, 1979a and 1979b; Van den Elzen *et al.*, 1983). Secretion requires the involvement of protein H encoded by plasmid CloDF13 (Oudega *et al.*, 1982). Three consecutive stages can be distinguished in the lethal action of cloacin DF13 on susceptible cells: (i) binding of cloacin DF13 to an outer membrane receptor protein, which is also involved in the uptake of the iron chelator aerobactin (Van Tiel-Menkveld *et al.*, 1982; Marolda *et al.*, 1991); (ii) transport of the cloacin or a cloacin fragment across the outer membrane (Krone *et al.*, 1986); (iii) upon entry into the cell cytoplasm, blockage of protein synthesis by cloacin-mediated cleavage of the 16S ribosomal RNA (de Graaf *et al.*, 1973; Oudega and de Graaf, 1976). Binding of cloacin molecules to the receptor was not affected by the removal of the immunity protein, but killing activity was strongly reduced (de Graaf and Klaasen-Boor, 1977). The involvement of the immunity protein in killing was also demonstrated by Gaastra *et al.* (1979) who found that cloacin molecules alone inactivated ribosomes *in vitro* but had no killing activity *in vivo*; this indicates that an interaction between immunity protein and cloacin is essential for the translocation of cloacin molecules across the cell envelope and for the killing of susceptible cells. Fragmentation of the cloacin and release of the immunity protein correlated with cloacin DF13 susceptibility (Krone *et al.*, 1986). Although both ferric aerobactin and cloacin DF13 interact with the receptor protein at the cell surface, the translocation of ferric aerobactin across the outer membrane is TonB dependent, whereas that of cloacin DF13 is not. Cloacin

DF13 has extensive homology in its protein sequence with the group A colicin E3 (Van den Elzen *et al.*, 1983). Because of this similarity, it was hypothesized that cloacin DF13 was indeed a group A colicin, and it was recently observed that *tolQ, tolR* and *tolA* were required for internalization of cloacin DF13. These genes are not, however, involved in the transport of ferric aerobactin (Thomas and Valvano, 1993).

Sun and Webster (1986) described *fii* mutants of *E. coli* which did not allow the male-specific filamentous bacteriophage f1 to infect bacteria harbouring the F plasmid and were tolerant to colicins E1, E2 and E3. They later reported that the *fii* locus was in fact a gene cluster designated *tolQRA* (Sun and Webster, 1987; Webster, 1991), whose products are required for the uptake of group A colicins, including cloacin DF13 (Thomas and Valvano, 1992; Thomas and Valvano, 1993). The sequence homology of *exbBD* and *tolQR* (Eick-Helmerich and Braun, 1989), and the partial functional replacement implying interaction of TolQR with TonB and of ExbBD with TolA (Braun and Herrmann, 1993), suggest a membrane topology of TolQ similar to that of ExbB and a membrane topology of TolR similar to that of ExbD (see Figure 1.6). Kampfenkel and Braun (1993) proposed that the N terminus of TolQ was located in the periplasm and that it contains three transmembrane segments, a small periplasmic loop and two large portions in the cytoplasm. This result was supported by the work of Vianney *et al* (1994). The N terminus of TolR was located in the cytoplasm followed by a transmembrane segment, and the remainder of the protein was located in the periplasm (Muller et al 1993). A mutation in the transmembrane segment of TolQ rendered *E.coli* cells resistant to group A colicins, indicating that the membrane-spanning regions play an important role in the activity of the protein. Furthermore, by analyzing various *tolQ* mutants, it was observed that only very small

amounts of TolQ protein were sufficient for phage and colicin import, but greater amounts of TolQ were necessary to maintain envelope integrity (Vianney *et al*, 1994). TolA appears to be capable of interacting with outer membrane components which in themselves are capable of multiple interactions. The carboxy-terminal domain of TolA interacts with components in the periplasm or on the inner surface of the outer membrane to function in maintaining integrity of this membrane. TolA is strictly required for the action of all group A colicins (Levengood-Freyermuth *et al.*, 1991 and 1993).

1.11. Transposons

1.11.1. Structure of bacterial transposons

Two specialized forms of recombination for restructuring the chromosome can be distinguished. Both are responsible for reassorting, adding or deleting genetic information. The site specific recombination system is responsible for single reciprocal crossover between short homologous segments. An example of site specific recombination is integration and excision of bacteriophage lambda and Tn554. The transpositional recombination system comprises specific DNA vector- transposable elements or transposons which can move from one genetic location to another (Kayser and Berger-Bachi, 1994).

Transpositional recombination is distinct from and independent of the conventional homologous recombination. The DNA sequences responsible for transposition are linear pieces of DNA that range in size from less than 1 to 23 kilobase pairs (kb). With rare exceptions, these segments contain nucleotide sequences repeated in inverse orientation at both ends. The inverted repeats (IRs) of different

transposons vary both in size, 8 to 40 base pairs (bp), and in the degree of conservation. They provide the recognition sites for the element's transposase(s), an enzyme necessary for the translocation process. The transposase also recognizes a short segment of the target DNA (3 to 13 bp). When a transposon integrates into this target, the recognition sequence is duplicated in direct orientation, i.e. direct repeats (DRs), as a consequence of the mechanism of insertion. Thus, after transposition, the transposed DNA is flanked by one copy each of the target sequence. Exceptions from this general structure do exist. In the laboratory, transposable elements are used as mutagens because they can inactivate genes that they transpose into, or possibly modulate the expression of a gene downstream from an insertion site, in both cases causing a phenotypic change indistinguishable from a mutation (Brown, 1993; Kayser and Berger-Bachi, 1994).

1.11.2. Classes of bacterial transposon

There are three classes of bacterial transposons: Class I transposons comprise the Insertion Sequences (IS) and the composite transposons. Insertion sequence elements are normal constituents of bacterial chromosomes and plasmids. They are the simplest class of transposable DNA, since their genetic functions are concerned only with the ability to translocate. Insertion Sequence elements have a size range of 750-1600 bp. They have one or two open reading frames that code for transposase, which act only in cis on the transposon ends on the same DNA-molecule as the transposase gene. The most frequent transpositional event seen with an IS element is a simple insertion. A DNA element flanked by two IS modules in opposite orientation forms a composite transposon. Often a divergence is observed between the right and

the left IS elements, and only one element remains functional. This is sufficient for transposition of the entire composite element. The central region carries markers unconnected with transposition, for instance drug resistance markers. Because composite transposons usually have four IS ends that can be used in the recombination, the interaction with a target sequence allows a wide possibility of genetic rearrangements.

Class II transposons form the family of Tn3-like elements and contain two genes necessary for transposition: *tnp*A encoding a transposase and *tnp*R encoding a resolvase, which is a site-specific recombinase. Both gene products are able to act in trans, in contrast to the IS transposase. In addition, class II transposons contain an internal cointegrate resolution site called *res*, and often, antibiotic resistance determinants as well. Class II transposons are flanked by terminal inverted repeats of 35-40 bp and duplicate a target sequence in direct repeats upon integration. Tn3-like elements display a cis acting transposition immunity.

Class III transposons comprise an increasing number of genetic elements that fit neither into class I nor II. Belonging to this call is Tn554, a site-specific transposable element of staphylococcal origin which carries resistance to the macrolide-lincosamide-streptogramin B antibiotics and to spectinomycin. Tn554 has asymmetric ends, without inverted or direct terminal repeats and there is no target duplication upon transposition. Tn554 is strictly site-specific, integrating into the staphylococcal chromosome exclusively at a site called att554; is shows a high frequency of transposition. Thus, Tn554 resembles integrative bacteriophages (Kayser and Berger-Bachi, 1994).

1.11.3. Transposition model

Figure 1.7. Transposition model (Brown, 1993).

1.11.4. Tn*phoA*

Several studies in the creation of mutant genes, especially of genes producing outer membrane proteins and periplasmic proteins have used transposon Tn*phoA*. This transposon has also been widely used in the study of the evolution of antibiotic resistant bacteria. Tn*phoA* is a type II transposon that can fuse the alkaline phosphatase gene lacking its signal peptide to the amino terminal sequences of proteins into whose genes it inserts. Alkaline phosphatase is not active unless it is exported from the cytoplasm. Therefore, for a fusion protein resulting from Tn*phoA* insertion into a gene to be enzymetically active, the product of that gene must contribute sequences that compensate for the missing signal peptide to promote export. Thus, Tn*phoA* acts as a probe for export signals in proteins into whose genes it inserts (Manoil and Beckwith, 1985).

Hybrid proteins generated by Tn*phoA* insertion contain around 17 amino acid

residues at their fusion joints resulting from translation of Tn5 and linker sequences. The use of transposon Tn*phoA* combines the advantages of working with hybrid proteins able to be secreted with the versatility of Tn5 transposition in generating the hybrids. For example, fusing an exported protein to alkaline phosphatase provides a simple way to monitor the expression and localization of the protein, using the sensitive indicator media and enzyme assay available for alkaline phosphatase. By random insertion of Tn*phoA* into the chromosome, it should be possible to identify new genes encoding transmembrane and periplasmic proteins simply by their ability to produce hybrid proteins with alkaline phosphatase activity (Manoil and Beckwith, 1985; Pugsley, 1993; Rich, 1991). Furthermore, fusion of alkaline phosphatase to complex transmembrane proteins can generate active hybrids. Since alkaline phosphatase appears to be active only when it is in the periplasm, the position of fusion joints of such active hybrids may help to identify the region of the membrane protein facing the periplasm. The use of Tn*phoA* may aid in determination of the detailed transmembrane topology of such a protein and may also help to identify export signals in these complex transmembrane proteins. Tn*phoA* is likely to function in a number of bacteria other than *E. coli*, since its parent transposon , Tn5, shows a broad host range for transposition (Manoil and Beckwith, 1985). The hybrid proteins expressed by such gene fusion display *phoA* activity only if the target genes encode a membrane, periplasmic, outer membrane, or extracellular protein (Taylor *et al.* 1989; Hensel and Holden, 1996; Goldberg *et al*, 1990; Bosch, 1986; Spratt, 1994).

In general, two possibilities can occur when a transposon integrates into a gene. Firstly, transposon may inactivate the gene that it transposes into, and secondly

it may modulate the expression of a gene downstream from the insertion site (Figure 1.8). In both cases causing a phenotypic change indistinguishable from mutation (Brown, 1993).

Figure 1.8. Possible consequences of transposition (Brown, 1993).

1.12. Aims and objectives of the study

Previous work has investigated the uptake of catecholic cephalosporins via IROMPs in *E. coli*, and *P. aeruginosa*. Investigations in *E.coli* by several groups (Curtis *et al.*,1988; Nikaido *et al.*,1990; Critchley *et al.*, 1991) revealed that mutants which are defective in either *cir* or *cir/fiu* or *fiu, fhuE, fecA, fepA* or *fiu, fhuE, fecA, fepA, cir* or *fiu, fhuE, fecA, fepA, cir, fhuA* or *ton*B exhibit resistance towards catecholic cephalosporins. Studies on *P. aeruginosa* by Hazumi *et al.* (1992) revealed that strains showing specific resistance to the catecholic cephem, BO-1341, increased production of a 55 kDa protein in the periplasmic space and a 84 kDa outer membrane protein.

The catecholic cephalosporin BRL 41897A was designed by SmithKline Beecham Pharmaceuticals to be resistant to β-lactamase and to be taken up by bacteria via the iron transport system. This antibiotic thus has the potential to be effective towards multi drug resistant bacteria. Originally, Critchley *et al.* (1991) studied its uptake by *E. coli*, followed by Gensberg *et al* (1994) who investigated its uptake by *P. aeruginosa*. To date, no such study has been made with *K. pneumoniae*. Since *K. pneumoniae* has an important role as a multi resistant nosocomial pathogen, it is important to study this organism in relation to its iron-dependent uptake of antibiotics. One approach is to study *K. pneumoniae* resistant mutants which are defective either in their outer membrane proteins, including IROMPs, or periplasmic proteins by use of Tn*pho*A transposon mutagenesis. Examination of the phenotypes of catechol-cephalosporin resistant mutants generated by Tn*pho*A mutagenesis should indicate which components are involved in their uptake. These studies will enable us firstly to elucidate the relationship between the uptake of BRL 41897A and iron transport by *K. pneumoniae*. Secondly, it is important to be able to predict the types of resistant mutants that might emerge during therapy. Thirdly, the studies may be useful in designing new antibiotics which are more effective against resistant bacteria.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains

Klebsiella pneumoniae

M10 is a strain lacking capsular polysaccharide but containing an O2 type lipopolysaccharide (LPS). It was derived from strain NCTC 5055 (capsular type K2, LPS type O2) by chemical mutagenesis (Poxton and Sutherland, 1976) and has been characterised in terms of siderophore and IROMP production (Williams *et al.*, 1983).

KN4401 (*ent*$^-$, *aer*$^-$) produces dihydrobenzoic acid but not enterobactin or aerobactin. It is sensitive to cloacin and was used as a control in the cloacin test to identify mutants lacking IROMPs which function as cloacin receptors.

Yo1, Yo2 and Yo3 are clinical isolates from the Sardjito Hospital, Yogyakarta, Indonesia.

Escherichia coli

The K12 colV/VK30 (*ent*$^+$, *aer*$^+$) strain, which produces enterobactin and aerobactin and is sensitive to cloacin, was used as a control in the cloacin test, and in the Western Blot analysis of OMPs.

SM10/pRT291 carrying Km^R, Tc^R and Tn*phoA*, was used as the donor of Tn*phoA*.

SM10/pHIJ14 carries Gm^R, Tra$^+$ and Sp^R on a plasmid of the same incomptability group as pRT291.

Other *E. coli* strains used were:

AN1937 (*ent*$^-$, *fep*$^-$) used as an indicator for enterobactin production.

LG1522 (*ent*$^-$, *aer*$^+$, *fep*A), a *iuc* mutant containing the ColV-K30 plasmid (specifying normal aerobactin uptake but unable to produce aerobactin) in a *fepA*$^-$ genetic background. This was used as an indicator strain for aerobactin production.

Enterobacter cloacae

E. cloacae DF13 was used for the production of cloacin DF13 (Williams *et al.*, 1989).

E7, E12, E13 and E16 are clinical isolates from Dudley Road Hospital, Birmingham which produce β-lactamase constitutively

2.2. Media and buffers

2.2.1. Media

Casamino acids (CAA) medium:

This low-iron content growth medium has been used extensively for iron-restricted growth of *Pseudomonas* species. It comprises 0.5% w/v vitamin-free casamino acids (Difco), 1 mM $MgSO_4$ and 1 mM MOPS, pH 7.3 prepared in acid-washed, EDTA-treated glassware with double glass-distilled water. To reduce the iron availability further, 300 μM of 2,2-dipyridyl (Sigma) was added (referred to as Fe-CAA).

In some experiments 4-10 μM $FeCl_3$ was added to CAA (referred to as Fe+CAA) to supress siderophore and IROMP expression or determine the effect of iron upon antibiotic activity.

Luria Broth (LB) media contained Tryptone (Oxoid) 10 g/l, yeast extract (Oxoid) 5 g/l and NaCl 5 g/l.

Antibiotic media

A variety of media containing combinations of antibiotics was used to select mutants. These were prepared from nutrient broth (Oxoid) or Luria broth and their respective solidified media (1.5% w/v technical agar) to which filter sterilised antibiotic solutions were added aseptically. Abbreviations used in the text refer to nutrient broth or agar containing rifampicin or tetracycline as Rif or Tet medium; similar medium containing both antibiotics is referred to as Rif Tet medium etc. Where Luria broth or agar was used it is prefixed by L., e.g. Luria Broth containing tetracycline is referred to as L.tet etc.

2.2.2. Buffers

TBS (Tris buffered saline) contained 12.1 g of Tris (Sigma), 85 g NaCl, and distilled water to 10 l, pH adjusted to 7.4 with HCl.

TTBS (Tris Tween buffered saline): 30 ml Tween 20 (Sigma) was added to 10 l TBS.

Transblot solution: 15 g Tris (Sigma), 72 g glycine, 1 l methanol and distilled water to 5 l.

TBE (1x): 10.8 g Tris base, 5.5 g boric acid, 0.93 g $Na_2EDTA.H_2O$), pH8.3 and distilled water to 1 l.

2.3. Antibiotics

BRL 41897A and BRL 42948A were provided by Dr. I. Chopra, SmithKline Beecham Pharmaceuticals, Brockham Park, Surrey, UK.

Mitomycin, Rifampicin, Gentamicin, Kanamycin and Tetracycline were obtained from Sigma. Nitrocefin was obtained from Oxoid.

2.4. Chemical reagents and enzymes

Antibiotics were used in media at the following concentrations: gentamicin, 30 μg/ml; kanamycin, 50 μg/ml; rifampicin, 50 μg/ml, tetracycline, 15 μg/ml, and mitomycin, 0.5 μg/ml.

The chromogenic alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl-phosphate (XP) (Sigma) was incorporated into the agar media at a final concentration of 100 μg/ml.

2.5. Antibodies to outer membrane proteins

The following antibody preparations were provided by Dr. E. Griffiths, National Institute for Biological Standards and Control, Potters Bar, Herts, UK :

(1) Monoclonal antibody to the 81 kDa FepA outer membrane protein of *E.coli* 0111.

The preparation provided has been purified from ascites fluid by ammonium sulphate precipitation and FPLC. It was supplied as a 1 mg/ml solution in 0.1 M phosphate buffer (pH 7.2).

(2) Polyclonal rabbit antibody to the 74 kDa Cir outer membrane protein from *E.coli* 0111 purified by electroelution from SDS-PAGE gels.

2.6. Determination of the minimum inhibitory concentration (MIC) of antibiotics

Aliquots of double strength medium (2.5 ml CAA, CAA+Fe or nutrient broth) were dispensed into sterile test tubes. Amounts of sterile antibiotic solutions and water were added to give the desired concentrations in a 5 ml volume and finally a 0.1ml of a 1:100 dilution from an overnight culture was added. The tubes were

vortexed and incubated at 37°C for 18 h. The tubes were examined for growth and the MIC defined as the lowest concentration inhibiting growth.

2.7. Cloacin susceptibility

A crude preparation of cloacin was prepared by exposing a growing culture of *E. cloacae* DF13 (in nutrient broth) to mitomycin C (0.5 μg/ml) followed by overnight incubation at 37°C with aeration. Cells were removed by centrifugation and the cloacin-containing supernatant sterilised by filtration. *K. pneumoniae* strains were tested for cloacin sensitivity after overnight growth in Fe-CAA by spreading approximately 10^6 organisms on Fe-CAA agar plates of the same medium supplemented with 200 μM ethylenediamine-di-(o-hydroxyphenylacetic acid) (EDDA). Samples (10 μl) of the crude cloacin preparation were added to wells cut in the agar. Cloacin sensitivity was indicated by a clear zone around the well.

2.8. Electrophoretic analysis of proteins

2.8.1. Preparation of outer membrane

Cells from overnight 50 ml cultures were harvested by centrifugation (12,000 g for 10 min) and resuspended in 10 ml of distilled water. The suspension was sonicated on ice with 5 x 30 sec bursts (MSE Soniprep, maximum power, 2 cm diameter probe) and 30 sec, intervening cooling periods, then centrifuged (12,000 g for 5 min) to remove any remaining whole cells. The cytoplasmic membrane was selectively solubilised by addition of 2% w/v sodium-lauroyl sarcosinate (Sigma) and the insoluble outer membranes (OMs) were pelleted by centrifugation (20,000 g for 60 min).

2.8.2. Preparation of periplasmic proteins

The method was based on that of Smith and Payne (1992). Cells from mid log phase cultures were harvested by centrifugation at 10,000 g for 10 minutes, washed three times with 0.01 M TrisHCl buffer, pH 7.3 containing 0.03 M NaCl at 4°C and suspended in 20 pellet volumes (0.5 ml for a 50 ml culture) of 0.033 M TrisHCl buffer pH 7.3 at 23°C. An equal volume of 0.33 M TrisHCl containing 40% w/v sucrose and 0.4 mM EDTA was added to the suspension, and swirled gently for 5 minutes. The suspension was centrifuged at 20,000 g for 20 minutes at 4°C. The pellet was dispersed rapidly in 40 volumes of cold 0.5 mM $MgCl_2$ and swirled for 10 minutes followed by centrifugation at 20,000 g for 20 minutes. The supernatant, containing shock-released periplasmic proteins, was concentrated by freeze drying before analysis by SDS-PAGE.

2.8.3. Polyacrylamide gel electrophoresis for analysis of proteins (SDS-PAGE)

The running gel was prepared as detailed in Table 2.1 and polymerisation of the running gel was initiated by the addition of TEMED. The gel solution was poured between glass plates, separated by 0.25 mm plastic spacers, to within 1 cm of the top and allowed to set for about 30 minutes. A spray of electrode buffer was used on top of the gel to ensure that the surface would be level. After polymerisation, the excess electrode buffer was removed and the stacking gel was cast. A teflon comb was inserted into the stacking gel to create wells for sample application.

Either OM preparation or periplasmic protein was denatured by mixing with an equal volume of sample buffer (5 ml 0.5 M TrisHCl, pH 6.8, 10 ml 10% w/v SDS, 5 ml glycerol, 0.5 ml β-mercaptoethanol, 10 ml distilled water and 0.4 ml 5%

w/v bromophenol blue, a tracking dye) and heating to 100°C for 10 min and 2-10 μl/lane was loaded onto the mini-gels.

Electrophoresis was carried out in the Bio-Rad Protean or Mini Protean systems, at room temperature at a voltage of 200 V until the tracking dye front had run to the end of the gel (Lugtenberg *et al.*, 1975).

Table 2.1. Composition of running gel and stacking gel for SDS-PAGE.

Constituents	Running gel(12%)	Stacking gel	Sample buffer
Stock 1	5 ml	-	-
Stock 2	-	2.5 ml	-
SDS10% w/v	0.5 ml	0.15 ml	5 ml
1.5 M Tris, pH 8.8	6 ml	-	-
0.5 M Tris, pH 6.8	-	3.75 ml	2.5 ml
Dist. water	8 ml	8 ml	5 ml
TEMED	50 μl	40 μl	-
AMPS 10% w/v	70 μl	50 μl	-
Glycerol	-	-	2.5 ml
2-mercaptoethanol	-	-	0.25 ml
5% Bromophenol blue	-	-	0.2 ml

Stock 1: 44% w/v acrylamide and 0.8% N,N'-methylene-bis-acrylamide (Bis).

Stock 2: 30% w/v acrylamide and 0.8% w/v N,N'-methylene-bis-acrylamide (Bis).

Final acrylamide concentration: 12% w/v in the running gel and 3% w/v in the stacking gel.

2.8.4. Visualisation of protein

After electrophoresis, gels were removed from the glass plates, and proteins either stained directly or transferred to nitrocellulose for subsequent probing with antisera (immunoblotting).

Directly stained gels were soaked with gentle agitation in 0.1% w/v Coomassie Brilliant Blue R-250 w/v in 50% methanol/10% acetic acid for 30-60 mins. Gels were then destained in 10% methanol/20% acetic acid until the background stain was removed. Gels could then be dried onto filter paper.

2.9. Immunoblotting (Western blot)

Following separation by SDS-PAGE, cell components were transferred onto nitrocellulose membrane (0.45 μm pore size, Bio-Rad) according to the Western blotting method of Towbin *et al* (1979). Transfer was carried out in a Mini Trans-Blot cell (Bio-Rad) using transblot buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3), at 100 V for 45 min (4°C). Transfer efficiency was determined by Coomassie staining the gels after transfer, or by staining the nitrocellulose with naphthol blue black (0.7% w/v in acetic acid).

Nitrocellulose membranes containing transferred proteins were first washed for one hour with gentle agitation in TBS containing 1% bovine serum albumin (BSA). This blocked unbound sites on the nitrocellulose (1 hr, 5°C). Following this, blots were probed for at least three hours at 4°C with appropriate antisera (section 2.5) diluted in TBS. Blots were then rinsed thoroughly in TBS three times, and soaked with gentle agitation in TBS/1% w/v BSA containing 0.25 μg/ml protein-A conjugated to horse radish peroxidase (Sigma) for 3 hours at 4°C. This solution was removed and the blots rinsed again in TBS. Blots were visualised with freshly-prepared developing solutions containing 0.01% v/v H_2O_2 and 4-chloro-1-naphthol 25 μg/ml in 10 mM TrisHCl, pH 7.4. This solution was warmed to 25°C to speed the reaction. Colour development was stopped with distilled water.

2.10. β-lactamase production

Single bacterial colonies taken from a plate were suspended in 20 μl of distilled water and placed in the well of a microtitre plate. 20 μl of nitrocefin solution was then added. The nitrocefin solution was made as follows: 1 mg of lyophilised nitrocefin SR112 (Oxoid) was reconstituted by adding the contents (2 ml) of a vial of rehydration fluid SR112A containing 1.9 ml of 0.1 M phosphate buffer; pH 7.0 and 0.1 ml of dimethyl sulphoxide. β-lactamase activity was detected visually by a colour change from yellow to pink/red which occured within 5 to 30 minutes at room temperature.

2.11. Detection and characterization of siderophores

2.11.1. Chemical detection of catechols

Catechols in culture medium were detected by the method of Rioux *et al* (1983). The following were added to a small glass tube: 1.15 ml water, 0.1 ml 20% w/v H_2SO_4 and 0.5 ml of test solution (i.e. culture supernatant). Sequential additions were made of 50 μl 1% w/v ferric ammonium citrate in 0.09 M H_2SO_4, 0.2 ml of 2 M NH_4F, 0.2 ml 1% w/v 1,10-phenanthroline monochloride monohydrate and 0.3 ml of 3 M hexamethylenetetramine. The test tube was vortexed, incubated at 60 °C for 1 h, cooled to room temperature, vortexed, and the absorbance measured at 510 nm. The assay procedure was repeated with fresh medium and with blank samples to compensate for the slight turbidity due to precipitation of ferrous salts. Water replaced the test solution in the former mixtures and 1,10-phenanthroline in the latter. All reagents were freshly prepared on the day of use.

2.11.2. Chemical detection of hydroxamates

The method of Arnold and Viswanatha (1983) was performed as follows: 1.25 ml of 80% w/v mercaptoacetic acid was diluted to 50 ml water, the pH adjusted with 2.2 ml of concentrated (0.88 w/v) NH_3 solution and further diluted to 100 ml with water. To 25.5 ml of this solution was added 440 ml of 30 mg/ml $Fe(NO_3)_3.9H_2O$ which was subsequently diluted to 50 ml with 0.5 M NH_4HCO_3/NH_4OH, pH 8.5. 0.35 ml of this final solution was vortexed with 0.7 ml of test solution (culture supernatant), incubated in the dark for 5 min, vortexed and the absorbance at 532 nm measured. A blank assay was performed using sterile media as the test solution. Reagents containing mercaptoacetic acid were prepared on the day of use and stored

in the dark.

2.11.3. Biological assays for siderophores

Biological assay plates composed of CAA agar containing 300 μM 2,2-dipyridyl were surface seeded with approximately 10^6 indicator bacteria per plate (standard Petri dish). The presence of siderophore in culture supernatant or production of siderophores by strains *in situ* were determined by placing 10-20 μl of culture supernatant or a streak of bacteria on the surface of the agar. Plates were inverted and incubated at 37°C The presence of a particular ferri-siderophore was indicated by a halo of growth developing after 12-48 hours incubation. Ferric enterobactin was detected using the indicator strain *E. coli* AN1937, and ferric aerobactin was detected using *E. coli* LG1522.

2.12. Sensitivity test to antibiotics

Determination of the antibiotic sensitivity of *K. pneumoniae* KSL mutants used "Neosensitab" Antibiotic discs.

Stationary phase of the strains grown in Fe+CAA were harvested and resuspended in Fe+CAA to give a suspension of approximately 10^5 organisms/ml. Well dried Nutrient agar plates were flooded with 2.0 ml of this suspension and excess liquid removed with a pasteur pipette. Certain different antibiotic discs were dispensed onto the plates (maximum of 6 discs per plate). Each plate was allowed to stand at room temperature for 1 hour to enable the antibiotic to diffuse into the agar, and then incubated for 18 hours at 37°C.

2.13. Uptake of ferric-antibiotic complex

Overnight cultures of *K. pneumoniae* mutants were diluted 1 in 20 in Fe-CAA media (and Fe+CAA for the M10 parent strain) and incubated at 37°C for 4 hours in an orbital shaker to reach log phase. The cells were harvested at room temperature by centrifugation at 10,000 g for 15 min. The cell pellet was washed twice with 0.1 mM MOPS buffer solution pH7, and then suspended in the same buffer to an optical density at 470 nm of 2.0. The cell suspension and a separate solution of 30 μM BRL 41897A, 100 nM $^{55}FeCl_3$ (0.477 GBq/mg, Amersham) and MOPS buffer (^{55}Fe-BRL 41897A complex) were incubated in a water bath at 37°C for 30 min. An equal volume of the ^{55}Fe-BRL 41897A complex was added to the cell suspension and 0.2 ml portions of the suspension were deposited on membrane filters (diameter 25 mm; pore size 0.2 μm, cellulose nitrate membrane filters, Whatman) at appropriate intervals using a syringe and Swinnex filter holder. The filters were washed immediately with two 10 ml portion of 0.5 M HCl, air dried, and the radioactivity was determined with a 1600 TR Packard Tri-Carb liquid scintillation analysier, using ^{3}H window settings, and a count time of 1.00 min with 10.0 min background (Smith *et al.*, 1994).

2.14. Plasmid DNA preparation

DNA was prepared by the method of Sambrook *et al.* (1989). Cells from 1.5 ml of an overnight nutrient broth culture containing appropriate antibiotics were harvested by centrifugation at 10,000 x g, resuspended in 200 μl of 50 mM glucose, 25 mM TrisHCl, pH 8.0, 10 mM EDTA and incubated at room temperature for 5 min. 400 μl of 0.2 M NaOH, 1%w/v SDS were added, and the mixture was

incubated on ice for 5 min. 300 μl of 3M sodium acetate, pH 5.0 were added and the mixture was incubated for a further 10 min on ice. The mixture was centrifuged at 10,000 x g for 10 min and the supernatant was removed to a fresh tube. 500 μl of propan-2-ol was added and the mixture was incubated at room temperature for 10 min; it was then centrifuged at 10,000 x g for 10 min. The supernatant was aspirated and the pellet dried and redissolved in 100 μl of water to which 200 μl of 4.4 M LiCl was added. The mixture was incubated for 10 min on ice. The precipitate was pelleted by centrifugation at 10,000 x g for 10 min, the supernatant was removed and DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate, pH 5.0 and 2 volumes of absolute ethanol, and incubation on ice for 15 min. The precipitate was deposited by centrifugation at 10,000 x g for 10 min. The final pellet was resuspended in 50 μl of sterile distilled water containing 0.1 mg/ml RNAse A (Sigma). A large scale DNA preparation was essentially a scaled up version of the above method except that, after RNAse A treatment for 10 min at 37°C, the solution was extracted twice with phenol and once with chloroform before ethanol precipitation, washing in 70% ethanol, vacuum drying and resuspension in 200 μl of TE buffer containing 10 mM TrisHCl, pH 8.0 and 1 mM EDTA.

2.15. Plasmid purification

The bacterial pellet from 2 ml of LB culture was resuspended in 0.3 ml of buffer P1 (see list at the end of section for buffer solution). 0.3 ml of buffer P2 was added to the suspension, wich was mixed gently and incubated at room temperature for 5 minutes. After adding 0.3 ml of chilled buffer P3 and mixing immediately but gently, the suspension was incubated on ice for 10 minutes. The tube was centrifuged

at 10,000 rpm for 15 minutes in a microfuge and the supernatant was removed promptly. A QIAGEN-tip 20 column (Qiagen) was equilibrated by applying 1 ml of QBT buffer and the column was allowed to empty by gravity flow, the above supernatant was applied to the QIAGEN-tip 20 column and was allowed to enter the resin by gravity flow. The column was washed with 4 x 1 ml of QC buffer.

DNA was eluted with 0.8 ml of QF buffer into clean 1.5 microcentrifuge tubes, and precipitated with 0.7 volumes of isopropanol, previously equilibrated at room temperature. The tube was centrifuged immediately at 10.000 rpm in a microfuge for 30 minutes and the supernatant was removed carefully. Finally, DNA was washed with 1 ml of cold 70% ethanol, air dried for 5 minutes and redissolved in a suitable volume of buffer.

Buffer P1 contained 100 μg/ml RNase A, 50 mM TrisHCl, 10 mM EDTA, pH 8.0.

Buffer P2 contained 200 mM NaOH and 1% SDS.

Buffer P3 contained 3.0 M K acetate, pH 5.5.

Buffer QBT consisted of 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0 and 0.15% Triton X-100.

Buffer QC consisted of 1.0 M NaCl, 50 mM MOPS and 15 % ethanol, pH 7.0.

Buffer QF contained 1.25 M NaCl, 50 mM TrisHCl and 15 % ethanol, pH 8.5.

STE contained 100 mM NaCl, 10 mM TrisHCl and 1 mM EDTA, pH 8.0.

TE buffer contained 10 mM TrisHCl, pH 8.0 and 1 mM EDTA.

2.16. Spectrometric determination of DNA concentration

For quantitation of DNA, readings were taken at 260 nm and 280 nm. An OD of 1 at 260 nm corresponds to a solution of 50 μg/ml for double stranded DNA, 40 μg/ml for single stranded DNA and 20 μg/ml for single stranded oligonucleotides. Contaminating phenol and protein absorbs at 280 nm. Nucleic acid solutions with a 260 nm:280 nm absorbance ratio less than 1.8 were considered too impure for this method of quantification.

2.17. Chromosomal DNA preparation

Bacterial strains were grown overnight in LB containing 0.5% w/v glucose. 10 ml of this culture were transferred into glass McCartney bottles and cells were harvested by centrifugation at 5000 rpm for 5 min. Cells were resuspended in 4 ml of buffer (15% sucrose, 50 mM TrisHCl; pH 7.5, 50 mM EDTA) containing 10 mg of solid lysozyme (egg white, Sigma) and incubated for 5 min at room temperature. 2 ml of TE containing 10% w/v of SDS was added, mixed gently and incubated for 15 min at room temperature. An equal volume of phenol/chloroform mixture (Sigma) was added and mixed gently by hand for 5 min, followed by centrifugation at 5000 rpm for 10 min. The clear top layer was removed with a disposable plastic Pasteur pipette (large diameter end) into a new glass McCartney bottle and extracted with phenol/chloroform again and spun as above. The clear top layer was removed into a plastic 30 ml Universal tube and 0.1 vol of 3 M sodium acetate and 2 vol of ethanol were added. This mixture was mixed gently and immediately strands of DNA were picked out (using a glass Pasteur pipette whose tip had been formed into a hook) into 70% ethanol and rinsed gently. The DNA was again picked out, redissolved gently

in 0.5 ml of TE buffer, extracted with phenol/chloroform followed by ethanol precipitation as described above, and redissolved in 300 μl of TE.

2.18. Digestion of DNA with restriction endonucleases.

DNA restrictions were performed using Stratagene's restriction enzymes and buffer, in accordance with the manufacturer's instruction. For complete plasmid digestion, reaction mixtures were allowed to incubate at 37°C for 1 hour. Complete chromosomal digests were incubated for at least 4 hours.

2.19. Ligation of DNA fragments into plasmid vectors

pUC18 is a high copy number plasmid carrying ampicillin resistance (Yanisch-Perron *et al.*, 1985). The poly linker is present in the N-terminal portion of a *lacZ* gene fragment, such that strains containing plasmids with no inserts will grow as blue colonies on X-Gal agar, whereas plasmids with inserts give white colonies, due to disruption of the coding region of the *lacZ* gene fragment. The plasmid vector was linearised by restriction at a single site within the vector polylinker and recovered from agarose gels as described in section 2.21. Fragments to be subcloned were similarly recovered to ensure purity. The DNA samples were mixed together, with a two to three fold molar excess of insert DNA over vector, and made up to 44 μl with water. To this was added 5 μl 10 x ligase buffer, 0.5 μl 10 mM ATP and 0.5 μl (2 units) T4 DNA ligase. The mixture was incubated at 37°C for one hour in the case of sticky end ligations, or 12-20°C overnight for blunt end ligations.

2.20. Preparation of horizontal agarose gels

Agarose gels were cast and run in Bio-Rad DNA sub-cells. Ultrapure agarose (Bethesda Research Laboratories) was dissolved in 0.5 x TBE buffer at 100 °C. The solution was allowed to cool to approximately 60°C, and ethidium bromide was added to 0.5 μg/ml. The gel was poured and allowed to set at room temperature for 45 minutes. Once set, gels were submersed in 0.5 x TBE. DNA samples were mixed with 0.2 vol 6 x DNA loading buffer and electrophoresis was carried out at 70-200 V until the tracking dye reached the end of the gel. Molecular weight markers were used where appropriate (appendix). DNA bands were visualised on a UV light box. Photographs were taken using Polaroid 655 film in a Polaroid MP4 camera.

2.21. Extraction and purification of DNA from agarose gels

The QIAquick gel extraction kit was supplied by Qiagen, the procedure was as follows: DNA fragments from agarose gels were excised with a clean, sharp scalpel. The gel was added to 3 volumes of QX1 buffer (for example to 100 mg of gel add 300 μl of QX1 buffer). The gel was dissolved by incubation at 50°C for 10 minutes with mixing and inverting the tube 2 - 3 times. The sample was loaded onto a QIAquick spin column, placed on a 2 ml collection tube, and was then centrifuged for 60 seconds at maximum speed in a microfuge. The QIAquick column was washed with 0.75 ml of PE buffer and centrifuged for 60 seconds. The column was centrifuged for an additional 60 seconds in order to remove residual PE buffer from the tube. DNA was then eluted into a clean 1.5 ml microcentrifuge tube by adding 50 μl of 10 mM TrisHCl, pH 8.5 to the column which was centrifuged for 60 seconds.

80 ml of ethanol (96-100%) was added to PE buffer before use.

All spins are at top speed in a conventional, table-top microcentrifuge (13,500 rpm).

QX1 contained chaotropic salt.

2.22. Southern blot

Digested DNA was transferred from agarose gels to positively charged nylon membranes (Boehringer Mannheim) as described by Southern (1975). Briefly, the DNA was depurinated in 0.25 M HCl for 7 minutes and denatured with 0.6 M NaCl, 0.2 M NaOH for 30 minutes. Neutral pH was restored with 1.5 M NaCl, 0.5 M TrisHCl, pH 7.0 for 30 minutes. Fragments were then blotted by capillary transfer of 20 x SSC buffer (3 M NaCl; 0.3 M Na-citrate, pH 7.0 at 20°C) overnight (Southern, 1975). Subsequent hybridization and detection procedures are described in section 2.24.

2.23. Colony blot

Using sterile, blunt-ended forceps, a sterile positively charged nylon filter (Boehringer Mannheim) was laid onto the surface of a day-old LB agar plate containing the appropriate antibiotic. White colonies (hybrid colonies) were streaked on to the filter and on to LB agar plates containing the appropriate antibiotic without any filter as a master plate, in the same pattern, with sterile wooden tooth picks. The plates were incubated in an inverted position at 37°C overnight.

The filter was peeled-off using blunt-ended forceps and placed, colony side up, on to

3MM paper impregnated with 10% w/v SDS for 3 minutes, and then tranferred to a second sheet of 3MM paper for 5 minutes, which had been saturated with denaturing solution (0.6 M NaCl, 0.2M NaOH). Subsequently, the paper was transferred to a third sheet of 3MM paper, which had been saturated with neutralizing solution (1.5 M NaCl, 0.5 M TrisHCl, pH 7.0) for 5 minutes. Following this, the filter was transferred to the last sheet of 3MM paper, which had been saturated with 2 x SSC for 5 minutes, and the filter was then laid, colony side up, onto a sheet of dry 3MM paper and allowed to dry at room temperature for at least 30 minutes. The filter was exposed to a UV transilluminated device (face down) for 3 minutes. Prior to hybridization, the filter was floated on the surface of a tray of 2 x SSC until it became thoroughly wetted from beneath. The filter was submerged for 5 minutes and then transferred to a dish containing at least 200 ml of prewashing solution containing 5 x SSC, 0.5% SDS and 1 mM EDTA, pH 8.0, covered with Saran Wrap and transferred to a rotating platform in an incubator. In this and all subsequent steps, the filters were slowly agitated to prevent them from sticking to one another. The filters were incubated for 30 minutes at 50°C. The bacterial debris was scraped gently from the surface of the filters using Kimwipes soaked in prewashing solution, and the filters were tranferred to 150 ml of hybridization solution as in section 2.24.

2.24. Digoxigenin (DIG) nucleic acid labelling and detection

2.24.1. Labelling DNA with digoxigenin-11-dUTP

The following were placed in a microfuge tube on ice: 10 ng - 3 μg of linearized purified DNA, which had been denatured for 10 min at 95°C and chilled quickly on ice/NaCl for 3 min, 2 μl hexanucleotide mixture, 2 μl dNTP labeling

mixture, sterile water to 19 μl and finally add 1 μl Klenow enzyme (2 units). All reagents were supplied by the manufacturers (Boehringer Mannheim).

The tube was centrifuged briefly to mix the reagents and incubated for 60 minutes at 37°C, (for some probes longer incubation up to 20 hours was used to increase the yield of labelled DNA). Then 2 μl of 0.2 M EDTA solution, pH 8.0 was added to stop the reaction, and the DNA was precipitated by adding 2.5 μl 4 M LiCl and 75 μl of prechilled ethanol (-20°C). The tube was left for overnight at -70°C.

Subsequently the tube was centrifuged at 12,000 g for 10 min and the pellet was washed with 50 μl cold ethanol (70% v/v), centrifuged and the pellet dried under vacuum and dissolved in 50 μl of 10 mM TrisHCl, 1mM EDTA, pH 8.0 at 37°C.

2.24.2. Hybridization with DIG-DNA probes

Nylon membranes from colony blots or Southern blots were UV crossed-linking with a transillumination device for 3 minutes. Filters were prehybridized in a sealed box of suitable size with at least 20 ml of hybridization solution per 100 cm^2 of filter at 68°C for 1 hour with gentle shaking. Following the prehybridization the solution was discarded and replaced with hybridization solution containing freshly denatured labelled probe DNA (about 2.5 ml solution per 100 cm^2 of filter). The filters were incubated for at least 6 hours at 68°C with gentle shaking. Filters were washed 2 x 5 minutes at room temperature with at least 50 ml of 20 x SSC, 0.1% w/v SDS, per 100 cm^2 filter and 2 x 15 minutes at 68°C with 0.1 x SSC, 0.1% w/v SDS. Filters were used directly for detection of hybridized DNA or were stored air-dried for later detection.

20 x SSC: 3M NaCl; 0.3 M Na-citrate, pH 7.0 (20°C).

Hybridization solution: 5 x SSC, 0.1% (w/v) N-laurylsarcosinate, Na-salt (Sigma), 0.02% (w/v) SDS. Add to the freshly prepared solution 1% (w/v) blocking reagent (Boehringer Mannheim) prepared 1 hour in advance by dissolving at 50-70°C.

2.24.3. Detection of probe-target DNA hybrids

The hybridized filters were washed briefly for 1 minute in buffer 1 and incubated for 30 minutes with about 100 ml of buffer 2. The filters were then incubated for 30 minutes with about 20 ml of diluted anti-DIG-conjugate solution (the antibody-conjugate supplied by Boehringer Mannheim) which was diluted to 150 mU/ml (1 : 5000) in buffer 2). Unbound antibody-conjugate was removed by washing 2 x 15 min with 100 ml of buffer 1 and the membrane was equilibrated for 2 minutes with 20 ml of buffer 3. Finally the filters were incubated in the dark with about 10 ml of freshly prepared colour-substrate solution sealed in a plastic bag. The colour started to form within a few minutes and the reaction was usually complete after 12 h-16 h. The filters were then dried at room temperature and stored.

Buffer 1 contained 100 mM TrisHCl and 150 mM NaCl, pH7.5 (20°C).

Buffer 2 contained 15% (w/v) blocking reagent in buffer 1, it was prepared 1 hour in advance by dissolving at 50-70°C.

Buffer 3 contained 100 mM TrisHCl, 100 mM NaCl; 50 mM $MgCl_2$, pH 9.5 (20°C).

Colour solution contained 45 μl nitroblue tetrazolium salt (NBT) solution and 35 μl X-phosphate-solution were added to 10 ml buffer 3.

All the incubations were performed at room temperature with gentle shaking except for the colour reaction.

2.25. Transformation

Plasmid DNA was introduced into *E. coli* recipient cells by the method of Hanahan (1983). Recipient cells were grown in LB at 37°C with aeration until the cultures reached an optical density at 600 nm of 0.4, and chilled on ice. Cells were harvested from 1 ml aliquots by centrifugation at 10,000 x g for 2 min. Cells were resuspended in 1 ml of 10 mM MOPS (3-[N-morpholino] propanesulfonic acid), pH 7.0, 10 mM RbCl and harvested by centrifugation as above. Then cells were resuspended in 1 ml of 100 mM MOPS, pH 6.5, 50 mM $CaCl_2$, 10 mM RbCl and incubated on ice for 15 min. Cells were harvested as above and resuspended in 200 μl of 100 mM MOPS, pH 6.5, 50 mM CaCl2, 10 mM RbCl to which up to 1 μg of DNA in up to 10 μl of solution was added and the mixture was incubated on ice for 30 min. The cells were heat shocked at 44°C for 45 sec and returned to ice. 1 ml of LB was added to the mixture, which was then incubated at 37°C for 60 min before plating out dilutions on selective media. Colonies appearing after incubation at 37°C overnight were recovered for further analysis.

Transformation of plasmid DNA into *K. pneumoniae* cells used the method of Merrick *et al* (1987) whereby the mixture was incubated on ice for 2 h prior to heat shock.

2.26. Cycle sequencing

Cycle sequencing was carried out according to the manufacturer's instructions, using a TAQuence cycle-sequencing kit supplied by United States Biochemical (USB).

Step 1. Termination mix preparation

Four tubes (labeled A, C, G, T) were loaded with 4 μl of the appropriate termination mixes (tube A contained ddATP, and C, G and T tubes contained ddCTP, ddGTP and ddTTP respectively).

Step 2. Labelled primer

The following reagents were added to a micro centrifuge tube: 1 μl primer (10 pmol), 3.75 μl of γ-^{32}P-ATP (12.5 pmol of 3000 Ci/mmol), 8 μl of 5 x Polynucleotide Kinase buffer, 26.75 μl distilled water and finally 0.5μl PNK (Polynucleotide Kinase enzyme). This mixture was incubated at 37°C for 10 minutes.

Step 3. Central mix

The following reagents were placed in a separate tube: 10 μl reaction buffer, 18.75 μl Taq dilution buffer and 47.5 μl distilled water.

Step 4. To the central mix tube (step 3) was added the following: 5 μl labelled primer (from step 2) and 1.25 μl Taq enzyme.

Step 5. To a separate tube containing 1 μl of the chromosomal DNA template preparation was added 16.5 μl of the central mix containing labelled primer and Taq enzyme (step 4).

Step 6. While the PCR thermal cycling apparatus was preheated to 95°C, 4 μl of the mixture from the tube above (step 5) was put into each of the termination mix (A, G, C, T) tubes (step 1). The tube contents were mixed, centrifuged and placed in the thermal cycler (PCR machine) for 35 cycles (94°C for 1 minute, 54°C for 1 minute

and 72°C for 1 minute). After the last cycle, the tubes were cooled to 11°C and 4 μl stop solution was added into each tube. Finally the tubes were heated to 95°C for 5 minutes prior to loading. 4 μl of each sample was loaded onto the sequencing gel.

2.27. Sequencing gel electrophoresis

The sequencing gel was prepared by combining the following: 5.7 gm/0.3 gm Acrylamide/Bis-acrylamide, 42-50 gm Urea, 10 ml 10 x TBE Buffer and 40 ml distilled water. After dissolving, the volume was adjusted to 100 ml with distilled water, filtered and degassed. To the acrylamide solution was added 1 ml of 10% w/v ammonium persulphate and 25 μl TEMED prior to pouring into the glass plates (Bio-Rad Sequi-Gen system: 21 x 40 cm x 0.04 cm). The gel was prepared 2 - 20 hours prior to use, and pre run for 15-60 minutes at 1900 volts until the temperature reached 50°C. Samples (4 μl) were loaded and the gel run at 1900 volts for 1 - 3 hours. Once run, the apparatus was disassembled and the plates prised apart. The gel was then soaked in 5% acetic acid and 15% methanol to remove the urea for 15 minutes and was then dried onto 3MM Whatman paper at 80°C for 2 hours under vacuum (Bio Rad model 583 gel dryer). The dried gel was exposed to autoradiography film (Kodak X-OMAT AR) for 24-48 hours at room temperature in a light-proof cassette. After exposure, film was developed according to the manufacturer's protocol.

2.28. Automated Sequencing

Automated Sequencing was carried out using Applied Biosystems equipment with fluorescent detection (Alta Bio Science, University of Birmingham).

2.29. Synthesis of primers

Oligonucleotides were prepared by solid phase synthesis using phosphoramidite technology on a Beckman 1000 M DNA synthesiser.

2.30. Analysis of nucleotide sequence

DNA sequence analysis was performed using the GCG (University of Wisconsin Genetics Computer Group) package at the SERC Seqnet computing facility at Daresbury, UK. GenBank database searching was performed using the FASTA and TFASTA software, multiple sequence alignments were performed using the CLUSTALV and ALIGN programs

2.31. Polymerase chain reaction (PCR)

2.31.1. Inverse PCR

5-10 μg of chromosomal DNA was digested to completion with a selected restriction enzyme. After incubation at 37°C overnight, the enzyme was removed by phenol/chloroform extraction and DNA was precipitated with ethanol and resuspended in 10 μl TE buffer. The concentration of the DNA was estimated by comparing the fluorescence of an aliquot mixed with ethidium bromide with a series of aliquots of known DNA concentration. Digested chromosomal DNA was diluted to about 0.3 μg /ml and recircularised by ligation (the reaction contained 0.1 μg DNA in a total volume of 200 μl with ligase at 0.5 u/μl and was carried out at 20°C overnight). The DNA was extracted with phenol/chloroform, precipitated with ethanol and the concentration was estimated as before. Subsequently, 25 ng of template and 20 pmoles of each primer per 50 μl reaction volume was amplified by PCR.

2.31.2. Procedure for PCR

Into each of 0.5 ml tubes the following additions were made:

4 μl of $MgCl_2$, 5 μl of 10 x PCR reaction buffer, 5 μl of T Mac (Tetramethyl ammonium chloride), 8 μl of 10 x dNTP's, 1 μl of Primer 1 (20 pmole), 1 μl of Primer 2 (20 pmole), 1 μl of template (between 0.25-25 ng), distilled water to 49.75 μl and finally 0.25 μl of Taq DNA polymerase. The samples was then heated in the PCR machine at 94°C for 4 minutes prior to cycling; 30 cycles of 94°C for 1.0 minutes, 54°C for 1 minutes and 72°C for 1 minutes were used (Ausubel *et al.*, 1991).

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10 x Reaction Buffer consists of 100 mM of TrisHCl, pH 8.3, 15 mM of $MgCl_2$, 500 mM of KCl and 1 mg/ml of gelatine.

10 x dNTP's contains 2 μl of each of 100 mM dATP, dCTP, dCTP, and dTTP and distilled water to 100 μl.

2.32. Purification of PCR products

The PCR product purification kit was supplied by Qiagen and the manufacturer's procedure was followed 5 volumes of Buffer PB was added to 1 volume of the PCR reaction and mixed. The sample was loaded onto a QIAquick spin column, and centrifuged for 60 seconds at maximum speed in a microcentrifuge (13,500 rpm) discarding the eluate. The column was washed with 0.75 ml of PE buffer and centrifuged for 60 seconds. After removal of the PE buffer, the column

was centrifuged for an additional 60 seconds. The column was then placed on a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 μl 10 mM TrisHCl, pH 8.5 and centrifuging for 60 seconds (QIAGEN protocol, December 1993).

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44 ml of ethanol (96 - 100%) was added to Buffer PE (50 preparation kit) before use.

The entire procedure was conducted at room temperature.

All centrifugations were at 13.500 rpm in a table-top microcentrifuge.

Buffer PB contains a chaotropic salt.

CHAPTER 3

ISOLATION OF *KLEBSIELLA PNEUMONIAE* MUTANTS RESISTANT TO THE CATECHOLIC CEPHALOSPORIN BRL 41897A

3.1. Introduction

β-lactam antibiotics which incorporate a catechol moiety show excellent antibacterial activity against Gram negative bacteria, including *K. pneumoniae* (Basker *et al.*,1984, 1989). This potent activity results from the ability of such antibiotics to cross the outer membrane via the *tonB*-dependent high affinity iron-transport systems which are expressed when bacteria are grown under conditions of iron-limitation (Watanabe *et al.*,1987; Critchley *et al.*, 1991).

In previous studies most mutants resistant to catecholic β-lactams lack one or more of the IROMPs. It has been shown that the catecholic β-lactams are transported into *E. coli* by the Fiu (83 kDa) and Cir (74 kDa) proteins, but not by the enterobactin receptor FepA (81 kDa) (Critchley *et al.*,1991; Nikaido and Rosenberg, 1990; Curtis *et al.*,1988). Studies with *P. aeruginosa* by Hazumi *et al.* (1992) revealed that strains showing specific resistance to the catecholic cephem, BO-1341, increased production of a 55 kDa protein in the periplasmic space and a 84 kDa outer membrane protein. So far, the uptake of catecholic β-lactams has not been studied in *K. pneumoniae*.

To study which outer membrane or/and periplasmic proteins are involved in the uptake of catecholic cephalosporins in *K. pneumoniae*, it is necessary to develop a system for the isolation of defined mutations in genes involved in the production of outer membrane or/and periplasmic proteins. To accomplish this objective, a broad

host-range system for the efficient delivery and subsequent transposition into the chromosome of the described Tn*phoA* fusion vector has been devised. The Tn*phoA* gene fusion approach is based on the fact that the enzyme alkaline phosphatase must be localized extracytoplasmically (usually in the periplasm) to express activity in whole cells. Signal sequence mutations that block its transport across the cytoplasmic membrane render the enzyme inactive. Thus, export of the enzyme is essential for high levels of alkaline phosphatase activity to be expressed in whole cells. It has been shown that export and activity can be concomitantly restored by fusing a restriction fragment containing a truncated *phoA* gene (lacking secretion signals) to portions of genes encoding signal sequences of heterologous proteins such as OmpF and LamB (Taylor *et al.*,1989).

Manoil and Beckwith (1985) have extended the utility of this approach by inserting a similar *phoA* restriction fragment near one end of Tn5 to create a transposon, designated Tn*phoA*, that can randomly generate gene fusion to phoA upon insertion into a cloned target gene or the chromosome. The hybrid proteins expressed by such gene fusion display PhoA activity only if the target gene encodes a membrane, periplasmic, outer membrane, or extracellular protein. Because such exported proteins represent the most frequent classes of proteins recognized to be involved in the uptake of drugs by bacteria, the use of Tn*phoA* provides a strong enrichment for insertion mutations in transport genes. Plasmids carrying Tn*phoA* were introduced into *K. pneumoniae* by conjugation as described below.

3.2. Isolation of BRL 41897A-resistant mutants

Figure 3.1 depicts the method for delivery and selection of transposition of

Tn*phoA* from *E. coli* to *K. pneumoniae*. In order to carry out the conjugation, a rifampicin resistant mutant from the M10 strain (designated M10R) was produced by isolating resistant colonies from Rif medium. The M10 strain of *K. pneumoniae* which lacks the normal capsular polysaccharide (Poxton and Sutherland, 1976) was chosen to aid genetic manipulation.

Step 1: The transposon donor strain *E. coli* SM10/pRT291 and recipient *K. pneumoniae* M10R were grown overnight in L.tet and L.rif respectively. They were diluted 1 in 10 in LB medium without antibiotics and then grown at 37°C for 2 hours. One ml of each culture was filtered through a sterile nitrocellulose filter, which was placed on the surface of NA and incubated for 3-4 hours at 37°C. The filter was then placed in 4 ml of sterile PBS solution and vortexed for 1 minute. The suspension of bacteria was plated on Rif Tet Kan media, since all transconjugants should acquire both tetracycline and kanamycin resistance (Tcr and Kmr) due to the plasmid carrying Tn*phoA*. Colonies which grew on the media were *K. pneumoniae* M10R/pRT291 (designated KA5).

Step2: conjugation of KA5 with the *E. coli* donor SM10/pHIJ14 was carried out using the same procedure as above, except the *E. coli* was grown in Gen media and the suspension was plated on agar containing gentamicin, kanamycin and rifampicin, thereby simultaneously selecting for pHIJ14 and retention of Tn*phoA*. All of the resulting colonies were Tc sensitive, due to loss of the pRT291 vector, and kanamycin resistant, due to transposition of Tn*phoA* into the *K. pneumoniae* chromosome. The chromogenic phosphatase indicator XP was included in the agar to select for those transpositions which resulted in active *phoA* gene fusions. Insertion of the transposon into the chromosome produced dark blue colonies.

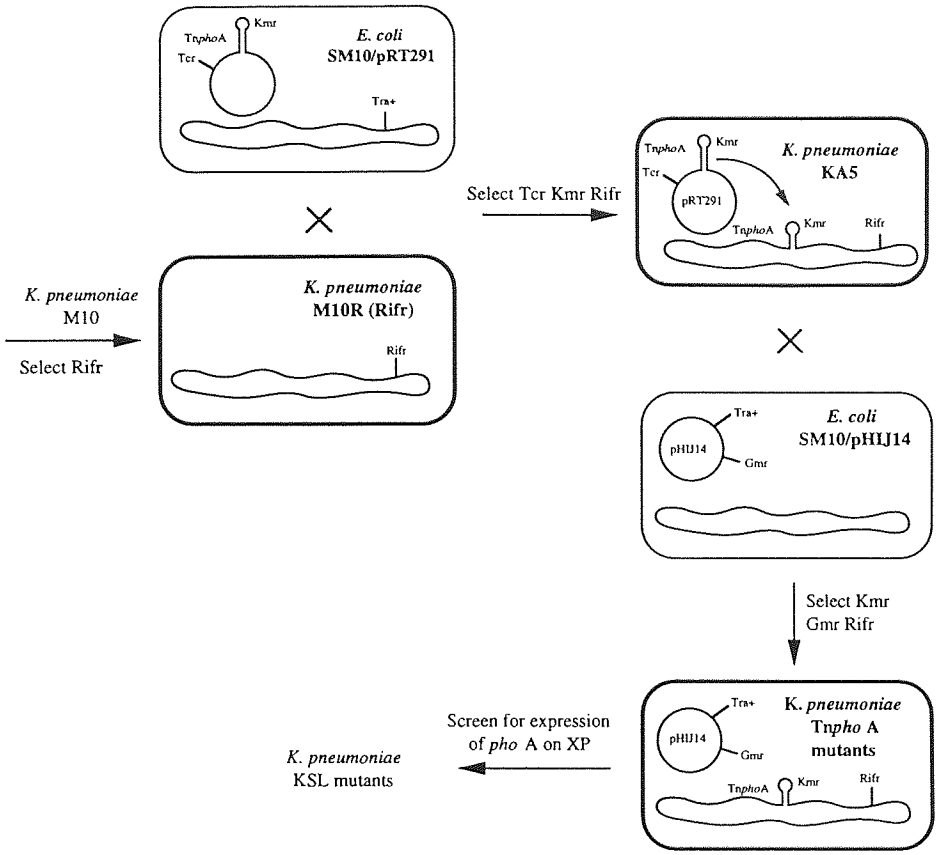


Figure 3.1. Method for delivery and selection of transposition of Tn*phoA* from *E. coli* to *K. pneumoniae*.

More than 300 resistant mutants were isolated, these all gave dark blue colonies over a pale blue colony background after the plates were stored on the bench for 2-3 days. Individual dark blue colonies were streaked onto NA plates containing increasing concentrations of BRL 41897A and resulting colonies were selected.

50 of the dark blue colonies showed resistance to BRL 41897A and MICs were determined for 33 of these mutants (Table 3.1).

Table 3.1. The MIC of BRL 41897A in Fe-CAA against the mutants of *K. pneumoniae* obtained by transposon mutagenesis

No.	Strain	MIC (μg/ml)	No.	Strain	MIC (μg/ml)
	M10	0.19			
1.	KSL1	0.38	18.	KSL52	1.52
2.	KSL3	0.76	19.	KSL57	0.76
3.	KSL13	0.38	20.	KSL58	0.76
4.	KSL15	0.38	21.	KSL59	0.76
5.	KSL16	0.38	22.	KSL62	0.76
6.	KSL19	1.52	23.	KSL86	0.38
7.	KSL27	0.38	24.	KSL87	0.38
8.	KSL31	0.38	25.	KSL105	0.38
9.	KSL32	0.38	26.	KSL238	0.38
10.	KSL33	0.38	27.	KSL270	0.38
11.	KSL35	0.38	28.	KSL301	0.38
12.	KSL37	0.38	29.	KSL304	0.38
13.	KSL38	1.52	30.	KSL305	0.38
14.	KSL39	0.76	31.	KSL309	0.76
15.	KSL40	0.38	32.	KSL311	0.38
16.	KSL41	0.76	33.	KSL318	0.38
17.	KSL51	0.76			

Mutants with the highest MIC values were further examined to determine their phenotypes such as their sensitivity towards cloacin, their outer membrane proteins, including IROMPs, periplasmic proteins and their Fe-BRL 41897A uptake characteristics (Chapter 4).

3.3. Ultra violet mutagenesis (Miller, 1972)

Two of the Tn*phoA* mutants, KSL19 and KSL38, were treated with UV light in the expectation that further mutation might increase their MIC to BRL 41897A by further disrupting one or more of the IROMPs and/or periplasmic proteins involved in its uptake.

Overnight cultures of KSL19 and KSL38 were subcultured by 1 in 10 dilutions into 2 flasks each containing 50 ml of LB media. After these had grown to a density of 2-3 x 10^8 cells/ml, the cells were harvested by centrifugation at 10,000 g for 10 min, suspended in 40 ml of 0.1 M $MgSO_4$ and placed on ice for about 5 minutes to prevent further growth. At this point the culture was titered by plating 10^{-5} and 10^{-6} dilutions (0.1ml) onto LB plates. Then, 5 ml samples of each culture were exposed to UV light for successively longer times in an open glass petri dish. The UV lamp was warmed up for at least 30 minutes prior to use. The lamp was set at a distance of 24-30 cms from the bacterial suspension. The aliquots were exposed to the UV light for 15, 30, 60, 80, 100, 120, and 150 seconds. Each irradiated suspension was titred immediately and then centrifuged and resuspended in 10 ml of LB and grown overnight in foil-covered tubes, along with an untreated control. A killing curve was constructed and a UV dose producing 0.1-1% survival was selected for mutagenesis. The MICs of colonies which showed increased resistance to BRL 41897A were

determined (Table 3.2) prior to other analysis (i.e. SDS-PAGE for IROMPs).

Table 3.2. MIC of BRL 41897A against "UV-second generation" mutants of *K. pneumoniae* produced by exposure of KSL19 and KSL38 to UV light.

Strain	MIC (μg/ml) in Fe-CAA media
M10	0.19
KSL19	0.76
KSL38	0.76
UV-second generation mutants derived from KSL19	
KSL408	6.25
KSL409	6.25
KSL410	6.25
UV-second generation mutants derived from KSL38	
KSL327	6.25
KSL328	6.25
KSL329	6.25
KSL330	6.25
KSL337	6.25
KSL338	6.25

The results in Table 3.2 show that the UV-mutants were approximately 8 times

more resistant than their respective parent strains (KSL19 and 38). Chapter 4 describes the characterization of the mutant strains.

CHAPTER 4

CHARACTERIZATION OF BRL 41897A RESISTANT MUTANTS

4.1. Effect of iron upon MIC of BRL 41897A towards the mutant strains

The MIC of BRL 41897A towards the resistant mutants in Fe-CAA media ranged between 2-8 times the MIC of the parent strain, M10 (Table 3.1).

As expected, when these mutants were grown in CAA medium containing 4 μM $FeSO_4$ (Fe+CAA) the MICs were not increased, whereas the MIC of the parent strain in the Fe+CAA increased about 4-fold compared to that obtained in Fe-CAA (Table 4.1).

Table 4.1. The influence of Fe on the antimicrobial activity (MIC) of BRL 41897A and BRL 42948A.

	MIC of BRL 41897A (μg/ml)		MIC of BRL 42948A (μg/ml)	
Strain	Fe-CAA	Fe+CAA (4μM)	Fe-CAA	Fe+CAA (4μM)
M10	0.25	1.0	1.0	1.0
KSL19	0.50	0.50	1.0	1.0
KSL38	1.0	1.0	2.0	2.0
KSL41	0.50	0.50	0.50	0.50
KSL51	1.0	1.0	2.0	2.0
KSL59	1.0	1.0	2.0	2.0

These results indicate that the sensitivity of M10 to BRL 41897A is iron dependent and that the uptake of the antibiotic is enhanced by a functional iron uptake system. The lack of an effect of added iron upon the sensitivity of the mutants suggests that they are altered in some aspect of iron uptake.

BRL 41897A has greater antibacterial activity than BRL 42948A, presumably due to the ability of BRL 41897A to bind iron and utilise a siderophore transport pathway to enhance its uptake by cells. The catechol substituent of BRL 41897A mimics the dihydroxybenzoyl groups of enterobactin, whereas the single p-hydroxyphenyl substituent of BRL 42948A is unable to bind iron in the same manner. The results in Table 4.1 support this hypothesis. The MIC of BRL 42948A against M10 did not increase when 4 μM $FeSO_4$ was added to CAA whereas the MIC of BRL 41897A increased four-fold.

Table 4.2 reveals that UV-mutants derived from the "first generation" mutants, KSL19 and KSL38, have about 8 times higher MICs than those of the "first generation" mutants. The sensitivity of these "second generation" mutants was influenced by iron. Following the addition of 4 μM $FeSO_4$ to the CAA medium their MICs were increased 2-4 times, whereas for the parent strains (KSL19 and KSL38) the MICs did not increase.

Table 4.2. The influence of Fe on the MIC of BRL 41897A against the mutants obtained by UV irradiation.

Strain	MIC of BRL 41897A (μg/ml) Fe-CAA	Fe+CAA (4 μM)
M10	0.19	0.76
KSL19	0.76	0.76
KSL38	0.76	0.76
UV-mutants derived from KSL19:		
KSL408	6.25	12.5
KSL409	6.25	12.5
KSL410	6.25	12.5
UV-mutants derived from KSL38:		
KSL337	6.25	25.0
KSL338	6.25	25.0

4.2.a.Outer membrane proteins of KSL mutants in Fe+CAA media.

SDS-PAGE of sarkosyl OM preparations from cells grown on Fe+CAA media are shown in Figure 4.1 and Figure 4.2. KSL19 produced a decreased amount of a 49 kDa OMP and the others, KSL38, KSL52, KSL58 and KSL59 produced decreased amounts of a 22 kDa protein compared with the parent strain M10.

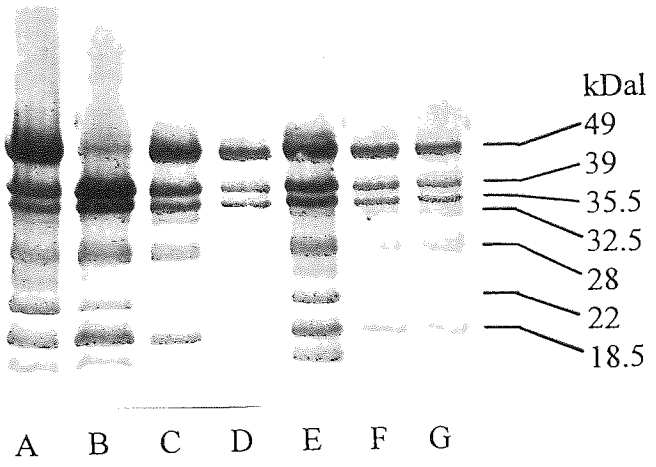


Figure 4.1. SDS-PAGE of sarkosyl OM of *K. pneumoniae* KSL first generation mutants. A: M10, B: KSL19, C: KSL38, D: KSL52, E: KSL57, F: KSL58, G: KSL59.

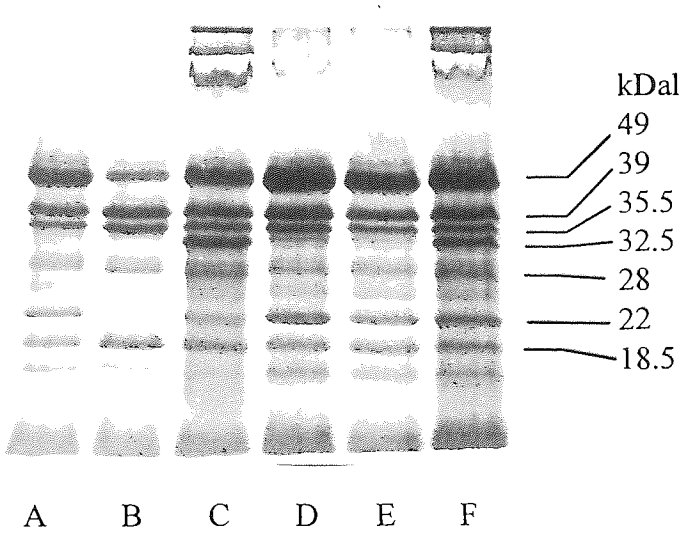


Figure 4.2. SDS-PAGE of sarkosyl OM of *K. pneumoniae* UV-second generation mutants. A: M10, B: KSL19, C: KSL38, D: KSL62, E: KSL338, F: KSL409.

4.2.b. The IROMPs of the resistant mutants

SDS-PAGE of sarkosyl OM preparations from cells grown in Fe-CAA showed that none of the mutants tested, except KSL19, KSL38 and KSL59 had significant alterations in their IROMPs compared to the parent strain, M10 (Figures 4.3 and Figure 4.4).

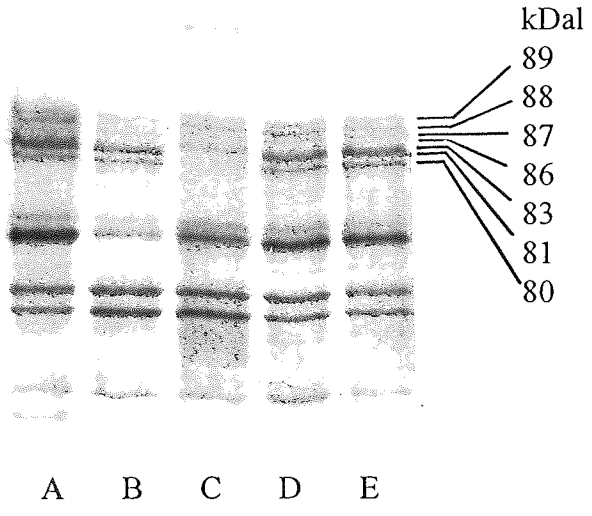


Figure 4.3. SDS-PAGE of sarkosyl OMs of KSL19, KSL38, KSL39 and KSL41 grown in Fe-CAA.

A: M10; B: KSL19; C: KSL38; D: KSL39; E: KSL41

In the case of KSL19 the 88 kDa IROMP was produced in very low levels and KSL38 produced very low levels of the 80 kDa IROMP. Figure 4.3 and Figure 4.4 show that mutants KSL38 and KSL59 were defective in the synthesis of the 80 kDa IROMP.

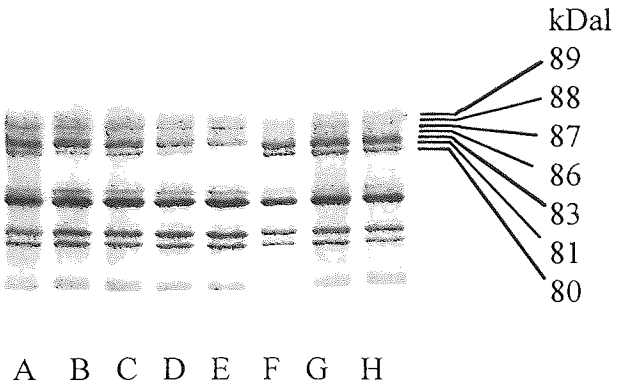


Figure 4.4. SDS-PAGE of sarkosyl OMs of *K. pneumoniae* KSL mutants.

A: M10; B: KSL52; C: KSL57; D: KSL58; E: KSL59;

F: KSL31; G: KSL32; H: KSL33

Figure 4.5 also shows the SDS-PAGE profiles of sarkosyl OM preparations of "UV-second generation" mutants derived from KSL19 and KSL38 grown in Fe-CAA. The mutants showed only minor differencies in expression of IROMPs compared with their respective parent strains.

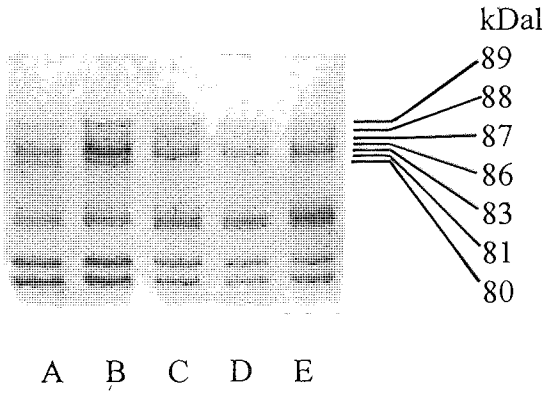


Figure 4.5. SDS-PAGE of sarkosyl OMs of *K. pneumoniae* UV-second generation mutants.

A: KSL409; B: KSL410; C: KSL38; D: KSL338; E: KSL337

4.3. Periplasmic proteins of the mutants

Most of the resistant mutants showed different periplasmic protein profiles by SDS-PAGE analysis. Figure 4.6 shows that there were no apparent differences in the periplasmic proteins of M10 grown in Fe-CAA and in Fe+CAA media. Mutants KSL19, KSL38, KSL51 and KSL59 showed increased expression of a 70 kDa periplasmic protein compared to M10. In addition, KSL38 produced increased amounts of a 23 kDa protein and slightly increased amounts of a 48 kDa protein, whereas it produced less of a 22 kDa and a 44 kDa protein (results are summarised in Table 4.3).

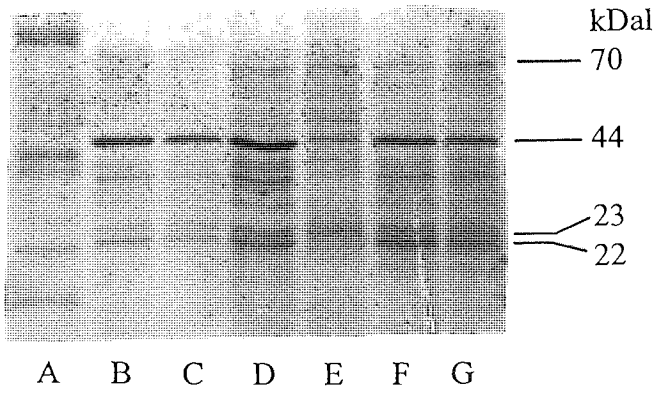


Figure 4.6. SDS-PAGE of periplasmic proteins of *K. pneumoniae* KSL mutants.

A: *P. aeruginosa* PAO1 OMs (as mol wt markers); B: M10 (Fe-CAA); C: M10 (Fe+CAA); D: KSL19; E: KSL38; F: KSL51; G: KSL59 (D, E, F and G all grown in Fe-CAA media)

Figure 4.7 shows that KSL13, KSL15, KSL16 produced increased amounts of a 70kDa periplasmic protein compared to the parent strain, M10.

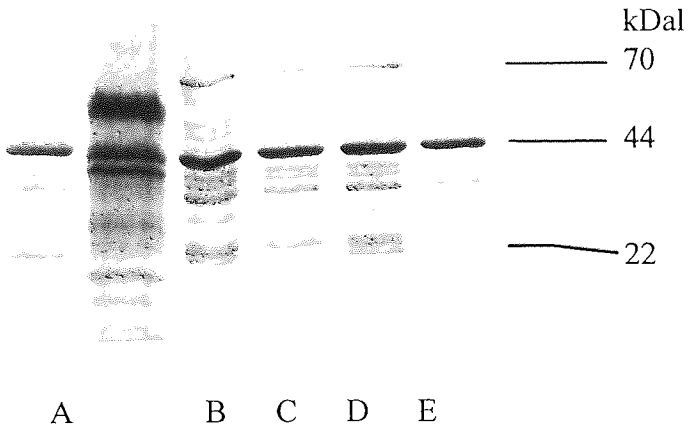


Figure 4.7. SDS-PAGE of periplasmic proteins of KSL13, KSL15, KSL16 and KSL27. A: M10; B: KSL13; C: KSL15; D: KSL16; E: KSL27 (all grown in Fe-CAA)

Similarly, mutants KSL52, KSL59, KSL62, KSL58 (Figure 4.6) and KSL83 (Figure 4.8) also produced increased amounts of the 70kDa periplasmic protein.

KSL62 and KSL58 also produced periplasmic proteins of 82kDa, 72kDa, 48kDa, 41kDa, 35kDa, but produced smaller amounts of a 22 kDa protein. The difference between KSL62 and KSL58 is that KSL58 was defective or failed to synthesise a 33kDa protein (Table 4.3).

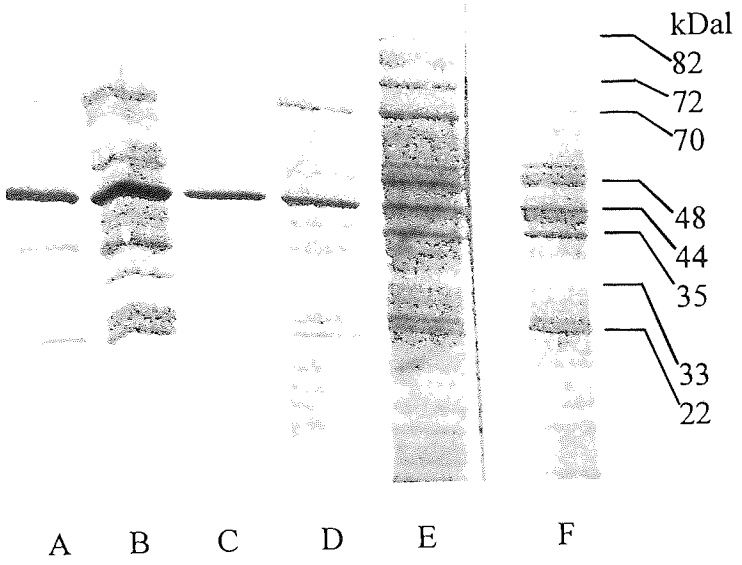


Figure 4.8. SDS-PAGE of periplasmic proteins of KSL52, KSL58 and KSL62. A: M10; B: KSL52; C: KSL57; D: KSL59; E: KSL62; F: KSL58 (all grown in Fe-CAA)

Three mutants, KSL83, KSL85 and KSL86 showed increased synthesis of a 41 kDa protein. KSL52 showed increased expression of 48, 33 and 23 kDa proteins.

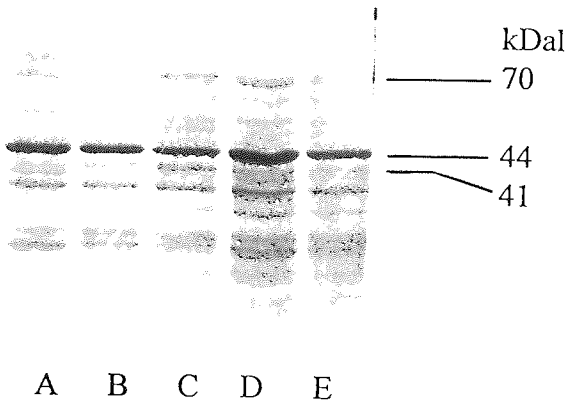


Figure 4.9. SDS-PAGE of periplasmic proteins of KSL83, 85 and 86.

A: M10 (Fe-CAA); B: M10 (Fe+CAA); C: KSL83; D:KSL85;

E: KSL86 (all Fe-CAA)

Table 4.3. Summary of the periplasmic protein compositions of mutants compared to the wild type (M10) grown in Fe-CAA

Mutants	Periplasmic proteins kDa									
	82	72	70	48	44	41	35	33	23	22
KSL13	=	=	↑	=	=	=	=	=	=	=
KSL15	=	=	↑	=	=	=	=	=	=	=
KSL16	=	=	↑	=	=	=	=	=	=	=
KSL19	=	=	↑	=	=	=	=	=	=	=
KSL38	=	=	↑	↑	↓	=	=	+/-	↑	↓
KSL39	=	=	↑	=	=	↑	=	=	=	=
KSL51	=	=	↑	=	=	=	=	=	=	=
KSL52	=	=	↑	↑	=	=	=	↑	↑	=
KSL58	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑
KSL59	=	=	↑	=	=	=	=	=	=	=
KSL62	↑	↑	↑	↑	↓	↑	↑	=	↑	↓
KSL83	=	=	↑	=	=	↑	=	=	=	=
KSL85	=	=	=	=	=	↑	=	=	=	=
KSL86	=	=	=	=	=	↑	=	=	=	=

key: = unchanged, ↑ increase, ↓ decrease , +/- variable

4.4. Cloacin susceptibility of the mutants

Table 4.4. Results of the cloacin sensitivity test.

Strains	Siderophore produced		Sensitivity to cloacin
	catechol	hydroxamate	
E. coli (pColV-K30)	+	+	S
KN4401	-	-	S
M10	+	+	S
KSL19	+	+	R
KSL38	+	+	R
KSL39	+	+	R
KSL41	+	+	R
KSL51	+	+	R
KSL52	+	+	R
KSL57	+	+	R
KSL58	+	+	R
KSL59	+	+	R
KSL62	+	+	R
KSL86	+	+	R
KSL87	+	+	R

Key: R resistant, S sensitive

Table 4.4 shows that all of the BRL 41897A resistant mutants were also resistant to cloacin, whereas the wild type M10 was sensitive. This result indicates that none of the mutants tested synthesized functional aerobactin receptors, or they were all defective in some other aspect of the cloacin uptake system.

4.5. Western blot analysis using antiserum raised against the *E. coli* enterobactin receptor (81 kDA)

Western blotting revealed no reaction with any of the first generation mutants KSL19, KSL38 KSL51, KSL52 (Figure 4.10), KSL57, KSL58, KSL59 (Figure not included), or UV-second generation mutants (Figure 4.11) or the parent strain M10 using antiserum raised against the *E.coli* enterobactin receptor (an 81kDa IROMP). A control using *E. coli* 14018 and *E. coli* colV showed positive reactions with an 81kDa protein. These results show that *K. pneumoniae* does not produce an enterobactin receptor which cross-reacts with the *E. coli* receptor.

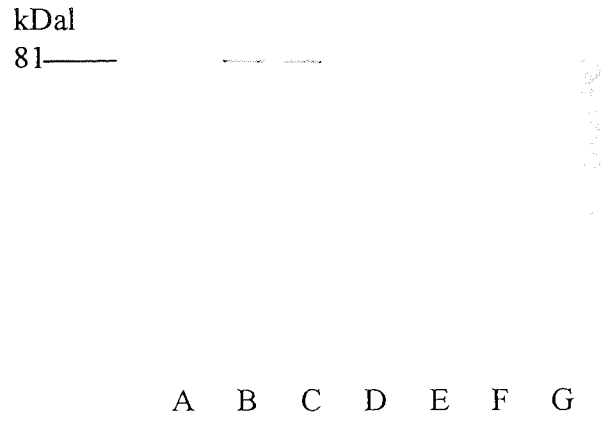


Figure 4.10. Immunoblot of the OM proteins of *K. pneumoniae* strains, *E. coli* 14018 and colV grown in Fe-CAA showing the reaction towards a monoclonal antiserum raised against the *E. coli* 81kDa IROMP. A: M10; B: *E.coli* ColV; C: *E. coli* 14018; D: KSL19; E: KSL38; F: KSL51; G:KSL52

Figure 4.11. Immunoblot of the OM proteins of *K. pneumoniae* strains and *E. coli* colV grown in Fe-CAA showing the reaction of a monoclonal antiserum raised against the *E. coli* 81 kDa enterobactin-receptor IROMP. A: *E. coli* colV; B M10; C: KSL330; D: KSL337; E: KSL338; F: KSL408; G: KSL409; H: KSL410.

4.6. Production of β-lactamase

Production of β-lactamase was examined using nitrocefin assays (see section 2.10).

Table 4.5. The production of β-lactamase by strains detected with nitrocefin.

Strains	β-lactamase
Enterobacter cloaceae (as a positive control)	
E7	+
E12	++
E13	+
E16	+
K. pneumoniae	
M10	-
Yo1	+
Yo2	++
Yo3	+
all 50 KSL mutant strains	-

The results of nitrocefin assays on whole cells and on broken cell suspensions revealed that neither the M10 parent strain nor any of the mutants produced significant amounts of β-lactamase (Table 4.5). Production of β-lactamase was therefore not responsible for the resistance of the mutants. This conclusion was

supported by studying 3 β-lactamase producing *K. pneumoniae* strains and 4 β lactamase producing *E. coli* strains (Table 4.5). All were sensitive to BRL 41897A but produced significant levels of β-lactamase. The presence of the formamido substituent at the C(7)α position of the cephalosporin ring makes the antibiotic highly resistant to β-lactamase.

4.7. Antibiotic susceptibility test

Table.4.6. Antibiotic resistance of *K. pneumoniae* KSL mutants to novobiocin and chloramphenicol.

Strains	Novobiocin	Chloramphenicol
M10	s	s
KSL19	R/s	
KSL38	R	R
KSL39	R	R
KSL52	R	R
KSL57	R	R
KSL58	R	R
KSL59	R	R
KSL62	R	R

key: R resistant, s sensitive

In addition to the resistance to gentamicin and rifampicin carried by the precursor, and to kanamycin which is carried by the transposon, some *K. pneumoniae* KSL mutant strains were also resistant to novobiocin and chloramphenicol (Table 4.6). No other differences in sensitivity were found between the parent strain and any of the mutants for the other antibiotics tested (trimethoprim, bacitracin, lincomycin, spectinomycin, ampicillin, streptomycin, erythromycin, amikacin, nalidixic acid, ciprofloxacin, carbenicillin and tetracycline).

4.8. Detection of siderophores

4.8.1. Biological assays

The following strains were used as indicators of siderophore production by mutants and M10:

1. *E.coli* LG1522 (*ent*$^+$, *aer*$^-$, *fepA*$^-$) produces enterobactin but cannot transport it due lack of the FepA receptor OMP. Conversely it does not synthesise aerobactin but can transport and utilise it for growth on Fe-medium when it is supplied by another organism. Therefore only used culture media from bacteria which produce aerobactin will promote growth of LG1522 on Fe-medium.

2. *E. coli AN*193 (*ent*$^-$, *aer*$^+$) produces neither aerobactin nor enterobactin but possesses the receptor and transport systems and is able to utilise both siderophores. Any donor bacteria producing aerobactin and/or enterobactin will promote growth of AN193 on Fe-medium.

3. *K. pneumoniae* KN4401 (*ent*$^-$, *aer*$^+$) is an equivalent strain to *E. coli* AN193.

Unfortunately neither of the *E. coli* indicator strains grew in the presence of Fe-culture supernatant from the wild type M10. However, *K. pneumoniae* KN4401

(*aer*$^-$, *ent*$^-$) grew in the presence of culture supernatants from both the wild type M10 and all of the mutants (Table 4.7). This result showed that both wild type and mutants synthesize siderophores (aerobactin and/or enterobactin) but it was not possible to determine whether any of the strains were defective in production of either one of these individual siderophores.

Table 4.7. Growth of *K. pneumoniae* KN4401 in the presence of culture supernatants from strains grown in Fe-CAA.

Strains	growth of *K. pneumoniae* KN4401
E .coli ColV	+
K. pneumoniae	
M10 (parent strain)	+
KSL19	+
KSL38	+
KSL39	+
KSL41	+
KSL51	+
KSL52	+
KSL57	+
KSL58	+
KSL59	+
KSL62	+

4.8.2. Chemical detections

Another approach to determine which mutants were defective in production of aerobactin and/or enterobactin was the chemical detection of siderophores in culture supernatants using specific colorimetric assays for catechol and hydroxamates.

Table 4.8. Detection of catechol and hydroxamates in Fe-CAA culture supernatans of *K. pneumoniae* KSL mutant strains.

Strains	Catechol (510 nm)	Hydroxamates (532 nm)
M10	0.301	0.105
KSL13	0.352	0.117
KSL15	0.361	0.097
KSL19	0.312	0.127
KSL38	0.364	0.141
KSL39	0.124	0.016
KSL41	0.345	0.135
KSL51	0.330	0.135
KSL52	0.387	0.145
KSL57	0.304	0.129
KSL58	0.305	0.113
KSL59	0.276	0.109
KSL62	0.338	0.121

The results showed that all strains produced detectable levels of catechols and hydroxamates and that there were no significant differencies between wild type and the mutants (Table 4.8).

4.9. Uptake of ^{55}Fe-BRL 41897A by whole cells

Study on the uptake of the ^{55}Fe-BRL 41897A complex in the wild type strain M10 showed that there was a significant increase in uptake by cells grown in Fe-CAA compared with cells grown in Fe+CAA medium (Figure 4.12). This indicated that the uptake was dependent upon iron-restriction and a functional siderophore uptake system and that the uptake assay was therefore suitable for investigation of the mutants.

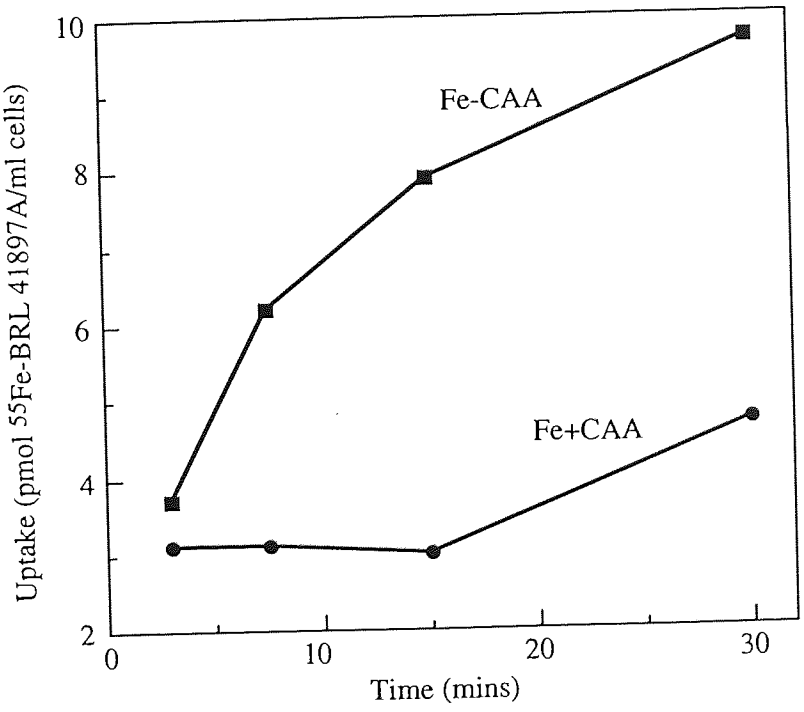


Figure 4.12. The time course for the uptake of ^{55}Fe-BRL 41897A by cells of strain M10 following growth in Fe-CAA and Fe+CAA.

Further experiments revealed that the apparent uptake of the ^{55}Fe-BRL 41897A complex in Fe-CAA by all mutants was greater than that of wild type (Figure 4.12).

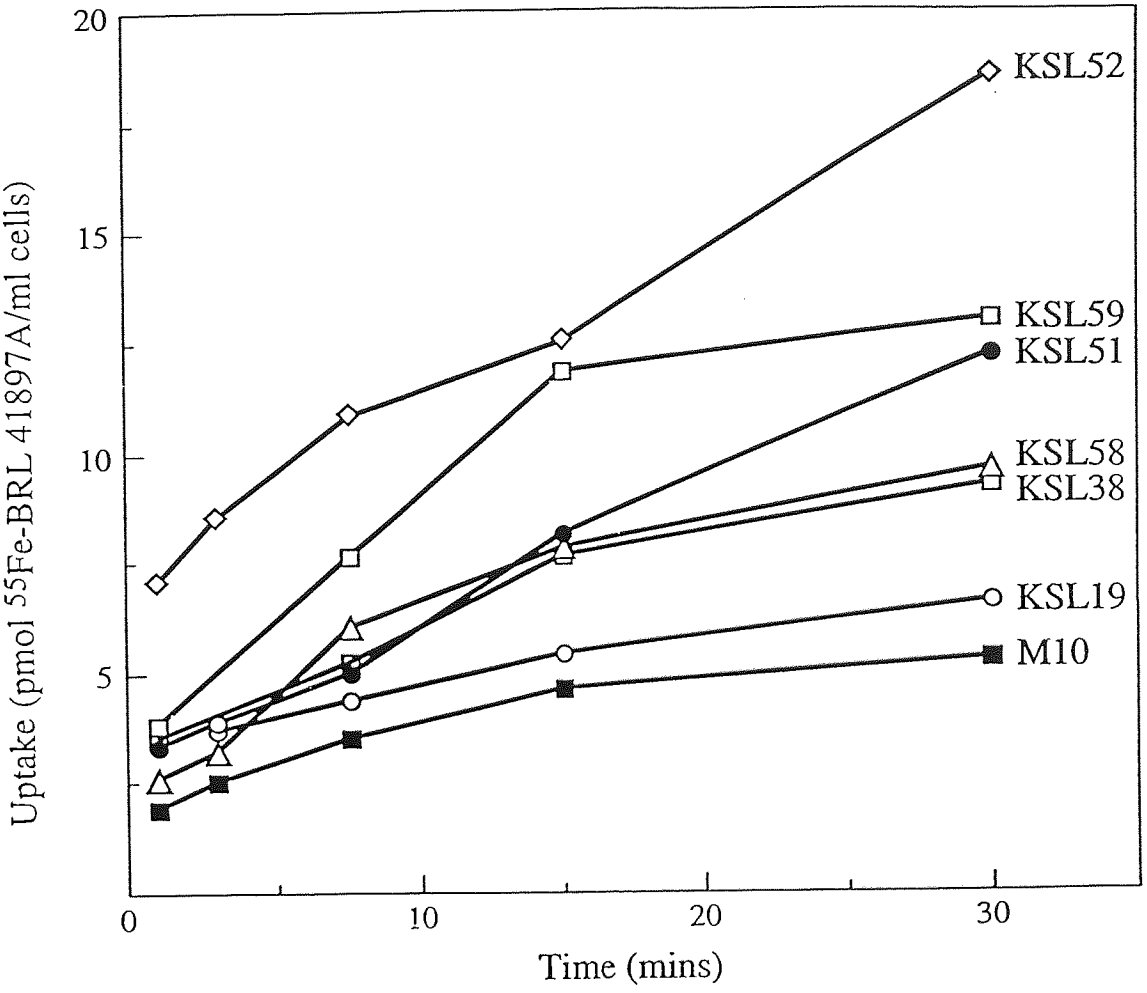


Figure 4.13. The time course for the uptake of ^{55}Fe-BRL 41897A by cells of strain M10, KSL19, KSL38, KSL51, KSL52, KSL58 and KSL59 growth in Fe-CAA

It had been expected that the uptake of ^{55}Fe-BRL 41897A by the mutants would be less than that of the wild type because it was assumed that the resistance of the mutants towards BRL 41897A was due to a defect in the receptors for the Fe-siderophore complex in the outer membrane (IROMPs). The enhanced uptake by the mutants suggested that the defects in iron uptake caused by the insertion of the transposon were compensated by the cells, either by producing new receptors, or by increasing the expression of existing receptors and/or related periplasmic proteins. It seems likely that it would be advantageous for *K. pneumoniae* to carry a number of "silent" genes for Fe-siderophore receptor proteins on its chromosome. These receptors are essential for bacteria to acquire iron in the environment. In order to survive when one or more receptors are defective, the bacteria will activate the silent genes (Pugsley and Schnaitman, 1978).

Evidence supporting this hypothesis is illustrated by KSL19, which had lost one IROMP (88kDa) but over produced another IROMP (86kDa). The increased expression of periplasmic proteins by most of the mutants may be a further illustration of the activation of silent genes. The possible alterations in the iron uptake systems are discussed further in the concluding discussion after the sequencing information on the disrupted genes presented in Chapter 5.

CHAPTER 5

CLONING OF GENES CARRYING THE Tn*phoA* INSERT

5.1. General Strategy

Tn*phoA* mutagenesis provides an efficient method of selecting mutants which are defective in genes controlling expression of membrane, periplasmic and outer membrane proteins. The strategy is outlined in Figure 5.1.

The dark blue colour of the transposon-generated mutants on XP medium indicates the insertion one or more of the *phoA* (alkaline phosphatase) genes into the chromosome by transposition. The parent strain exhibited a pale blue colour on XP medium due to basal expression of the endogenous *phoA* gene.

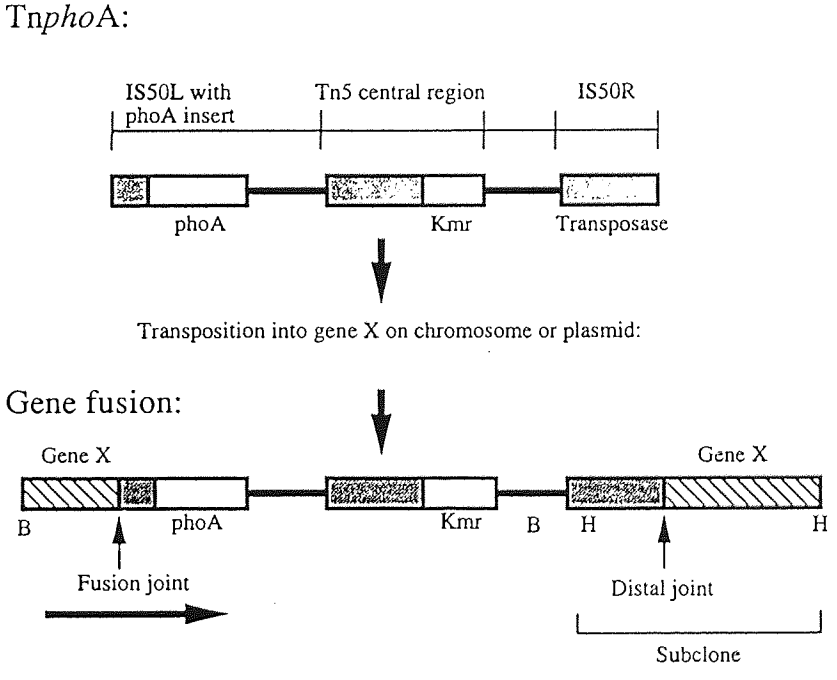


Figure 5.1. Events leading to the formation of an active *phoA* gene fusion by using Tn*phoA*. kmr: kanamycin resistant gene

Using Southern Blot analysis it should be possible to determine how many copies of Tn*pho*A were inserted into the chromosome and the size of the fragments containing the inserted gene(s).

Based on analysis of restriction sites within Tn*pho*A, it should then be possible to clone fragments carrying either the whole of Tn*pho*A (for example using *Kpn*I), the left hand side only (using *BamH*I, *Hind*III or *Xho*I) or the right hand side only (using *Hind*III, *Xho*I or *BamH*I) (Figure 5.2).

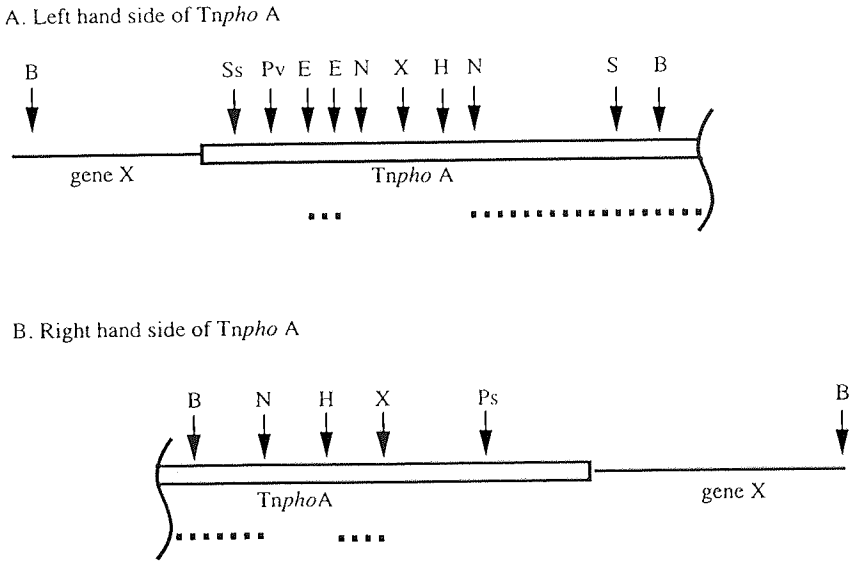


Figure 5.2 : Restriction sites in the Tn*phoA* fragment

B: *BamH*I; E: *EcoR*I; H: *Hind*III; X: *Xho*I; Ps: *Pst*I; Pv: *Pvu*II

S: *Sal*I; N: *Nco*I; Ss: *Ssp*I.

Restriction enzymes such as *Xba*I, *Kpn*I and *EcoR*V do not cut within Tn*pho*A.

Dotted lines show fragments used as probes.

In order to clone the chromosomal fragment carrying the gene with the inserted Tn*phoA*, the mutant chromosome was cut with the enzyme used in the Southern blot analysis, ligated with cut plasmid vector, and transformed into an *E.coli* host cell. By use of colony dot blot hybridization (see Chapter 2) it should be possible to detect transformants reacting with an oligonucleotide probe (e.g. a *Nco*I fragment from Tn*phoA*). Colonies giving a positive reaction with the probe would be isolated, and the plasmid DNA prepared for confirmation of reaction of the inserted DNA with the probe. Pure plasmid DNA would then be cycle sequenced using a primer based on the Tn*phoA* sequence. It should be possible to clone fragments of the wild type M10 chromosome carrying the Tn*phoA* inserted gene by using the mutant clone as a probe in the Southern blot analysis. The same procedure to clone the mutant fragment could then be applied to the M10 fragment which gave a positive reaction with the probe. By sequencing the DNA of the mutant fragment it would be possible to sequence the rest of the M10 clone by use of primers derived from the sequence. Eventually, it should be possible to elucidate the whole of the Tn*phoA* inserted gene.

5.2. Southern blot analysis

Southern blot analysis of plasmid DNA of 10 mutants (KSL19, 38, 39, 40, 51, 52, 57, 58, 59, 62) cut with *Kpn*I revealed that only KSL19 and KSL62 carried a single Tn*phoA* insert in their genome. The size of the *Kpn*I fragment in these mutants was 21 kilobases. The rest of the mutants carried more than one insert, most of them within 21 and 17 kilobase *Kpn*I fragments (Figure 5.3).

Figure 5.3. Southern Blot analysis of the mutant chromosomal DNA cut with *Kpn*I using a *Nco*I fragment as a probe (see Figure 5.2).

A: KSL62; B:KSL57; C: KSL52; D: KSL51; E:KSL41; F: KSL39; G: KSL38; H: KSL19; I: M10; K: λ cut with *Hind* III.

In a separate experiment using *Kpn*I it was shown that KSL19 had one Tn*phoA* insert, whereas KSL38 and KSL52 had two inserts (Figure 5.4).

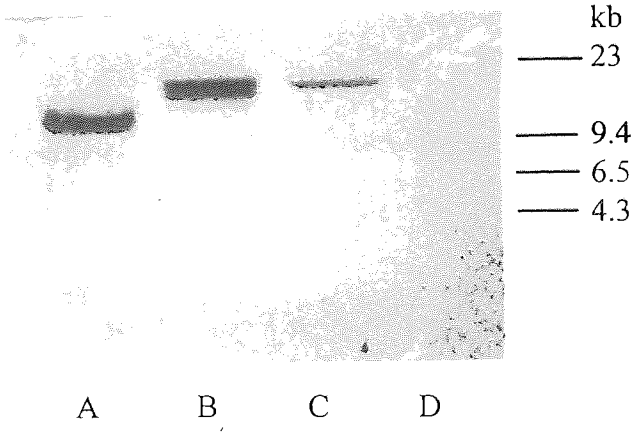


Figure 5.4. Southern Blot analysis of KSL19, KSL38 and KSL52 chromosome cut with *Kpn*I using a *Nco*I fragment as a probe.

A: KSL52; B: KSL38; C: KSL19; D: M10

Using *BamHI*, which cuts once in the middle of Tn*phoA*, it was confirmed that KSL19 carried only one insert, giving two fragments of 9kb and 12kb in the *BamHI* digest. KSL38 gave 4 fragments with *BamHI* (20, 14, 9.5 and 4.5 kb) and KSL52 gave 4 fragments (9.5, 9.2, 4.4 and 2 kb) confirming that both these mutants have two inserts of the transposon in their chromosome (Figure 5.5).

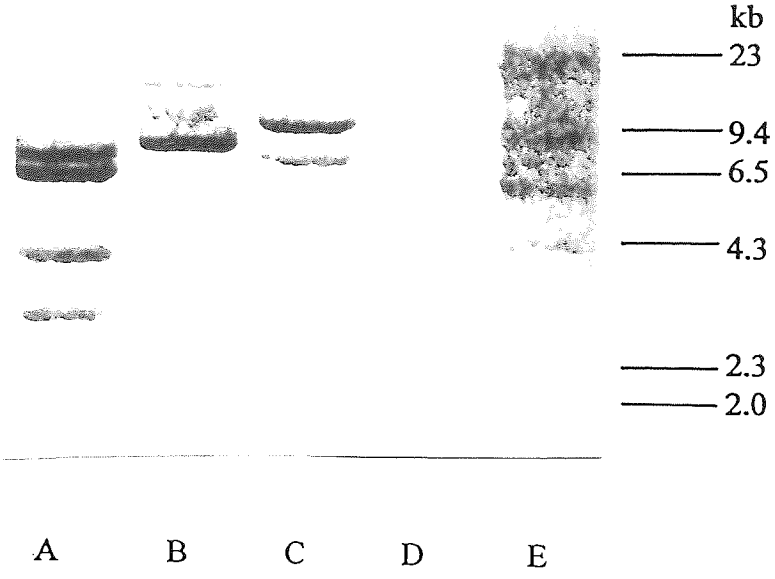


Figure 5.5. Southern blot analysis of mutant chromosome cut with *BamHI* using an EcoRI fragment as a probe (Figure 5.2). A: KSL52; B: KSL38; C: KSL19; D: M10; E: λ cut with *Hind*III.

5.3. Cloning of Tn*phoA* inserted genes from the *K. pneumoniae* BRL 41897A mutants

5.3.1. Attempts to clone chromosome fragments carrying the whole of Tn*phoA* with the disrupted gene

Since Southern blot analysis showed that only one fragment in a *Kpn*I chromosomal digest of mutants KSL19 and KSL62 reacted with the *Nco*I fragment

probe (Figures 5.3 and 5.4), it should be possible to clone *Kpn*I cut chromosomal DNA fragments (about 21 kb) into the Bluescript (pKS+) vector. However, no clones were obtained with this enzyme from KSL19 or from one of the two KSL38 fragments (20 kb).

5.3.2. Attempts to clone chromosome fragments containing the left hand side of Tn*phoA*

(1). *BamH*I fragments

No positive clones were obtained using *BamH*I fragments from KSL19 and KSL38 probed with the *Nco*I fragment (Figure 5.2).

(2). *Xho*I fragments

Using *Xho*I digested chromosome, it was thought that positive clones would be obtained because the fragments are only about 4 kb in size. Although several colonies reacted with the *EcoR*I fragment probe (Figure 5.2), unfortunately the colonies either carried no plasmid, or the plasmid was similar to Tn*phoA* in the vector (based on sequencing data).

(3). *Hind*III fragments

As with *Xho*I, the clones of *Hind*III digests either contained no plasmid or contained plasmid with Tn*phoA* (based on sequencing data).

5.3.3. Attempts to clone the right hand side of the Tn*phoA* fragment

The right hand side of the Tn*phoA* fragment was cloned using a *Hind*III chromosomal digest and a probe prepared from *Hind*III-*Xho*I fragment of Tn*phoA* (Figure 5.2). Southern blot analysis of chromosomal DNA of several mutants cut with *Hind*III reacted with the probe (Figure 5.6).

Clones of the *Hind*III fragments of KSL19, KSL62 and one fragment of KSL38 in pUC18 were obtained.

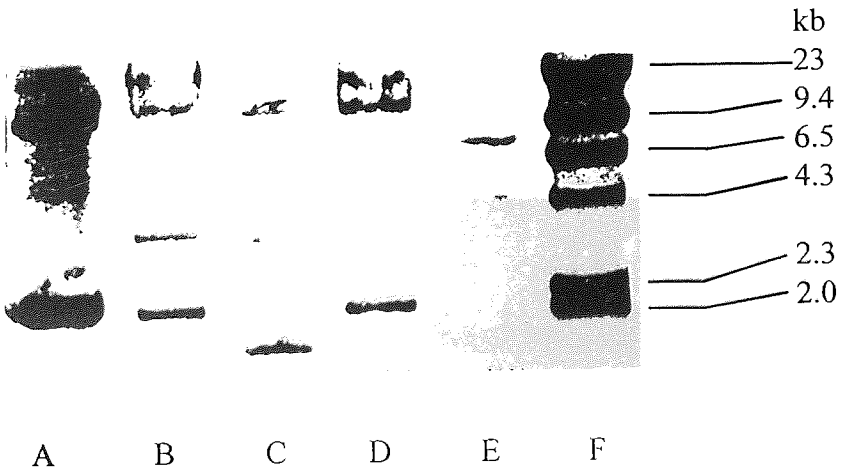


Figure 5.6. Southern blot analysis of mutant chromosomal DNA cut with *Hind*III using the right hand side *Hind*III-*Xho*I fragment of Tn*phoA* as a probe. A: KSL62; B: KSL52; C:KSL51; D: KSL38; E: KSL19; F: λ cut with *Hind*III.

(1). KSL62

Following the positive result of colony blotting of clones from *Hind*III cut KSL62 chromosome in pUC18, designated KIH62 (Figure 5.7), plasmid preparations of these clones were examined by electrophoresis (Figure 5.8) and Southern blot analysis using a *Hind*III-*Xho*I fragment of Tn*phoA* as a probe (Figure 5.9).

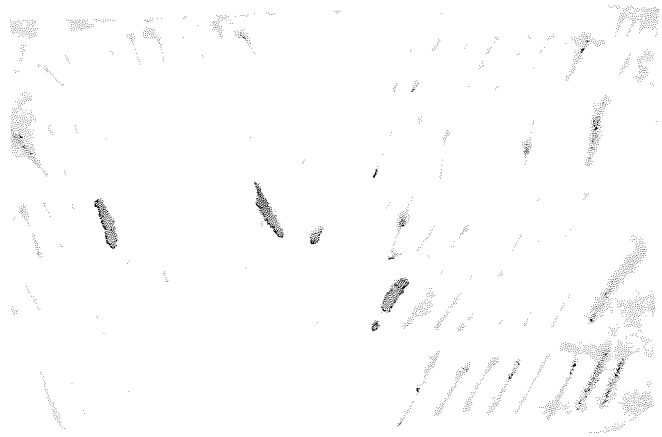


Figure 5.7. Colony blot of KIH62.

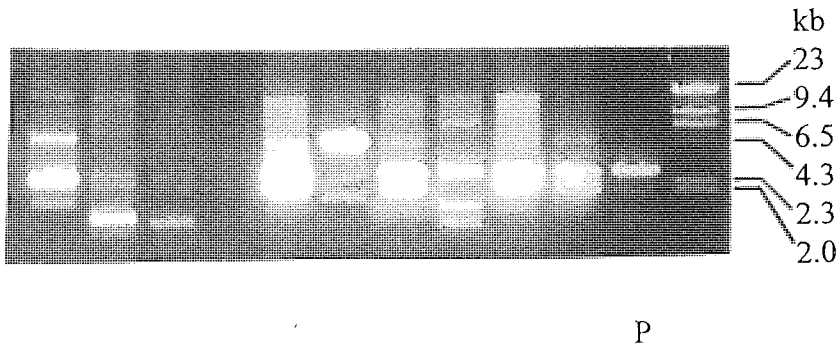


Figure 5.8: Electrophoresis of several KIH62 clones.

P: pUC18

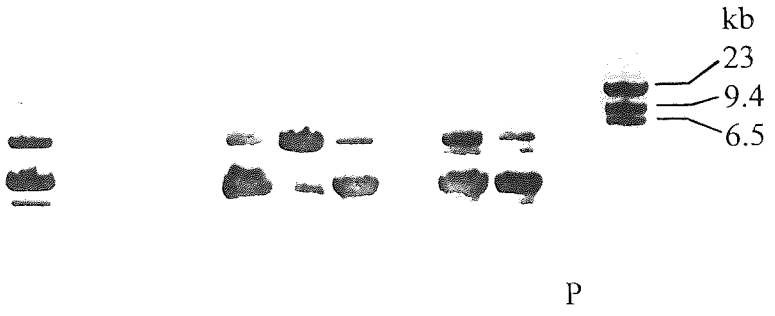


Figure 5.9 : Southern blot analysis of KIH62 clones using a *Hind*III-*Xho*I fragment of Tn*phoA* probe (see Figure 5.2). P: pUC18

The plasmid DNA from clone number 162 was sequenced using an outward-facing primer based on the right hand side of the inserted Tn*phoA* sequence DNA (Figure 5.10).

```
========-AGCAGGAAAC CCGCGAAAAC ATACAACGGC
31  AAGGCGCAGA GGTGAAAAAT GAGAATACTG AAATGGAAAA
71  GGACCATAAA ACAAAAGCAA ATGAGTTTAA AGGTGATTAT
111 AATGAAAGAA AGAGTAAGGT GAAATCTCTT ACCAGGAGCT
151 GACTCGCCTA CTGCGCTAGG GCAAGCGTCG TATTCAAAGG
191 ATTATCTTGA TGCTAAAGGT AAATGATGAT TAGCACAACT
231 ACTATTAGGA GTGAGACT
```

Figure 5.10 : The DNA sequence of KIH62. ==== Tn*phoA*

Using a fragment of the KIH62 plasmid cut with *Hind*III as a probe, the related fragment in the wild type *K. pneumoniae* (M10) chromosome was detected by Southern blot analysis (Figure 5.11).

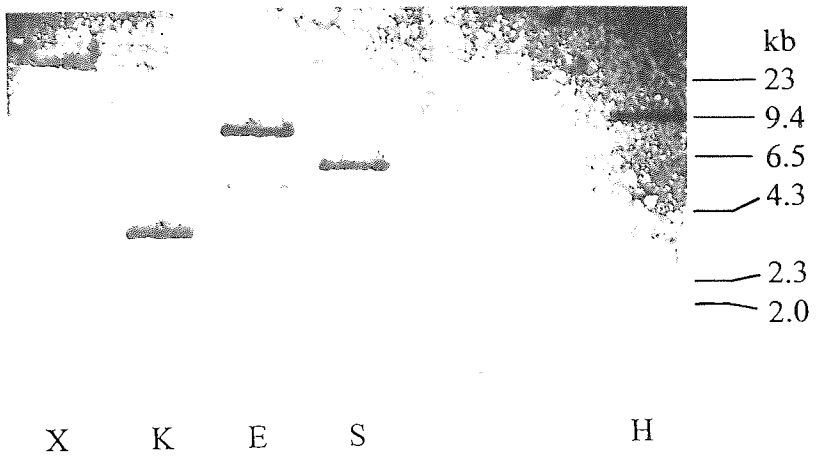


Figure 5.11 : Southern blot analysis of M10 chromosome cut with several enzymes using a *Hind*III cut KIH62 fragment as a probe.
X: *Xba*I; K: *Kpn*I; E: *EcoR*I; S: *Ssp*I; H: *Hind*III

(2). KSL38

The analysis procedure used to check the clone of the 2kb *Hind*III fragment of the KSL38 chromosome in pUC18 (designated KIH38) was similar to that used for KIH62 (Figure 5.12).

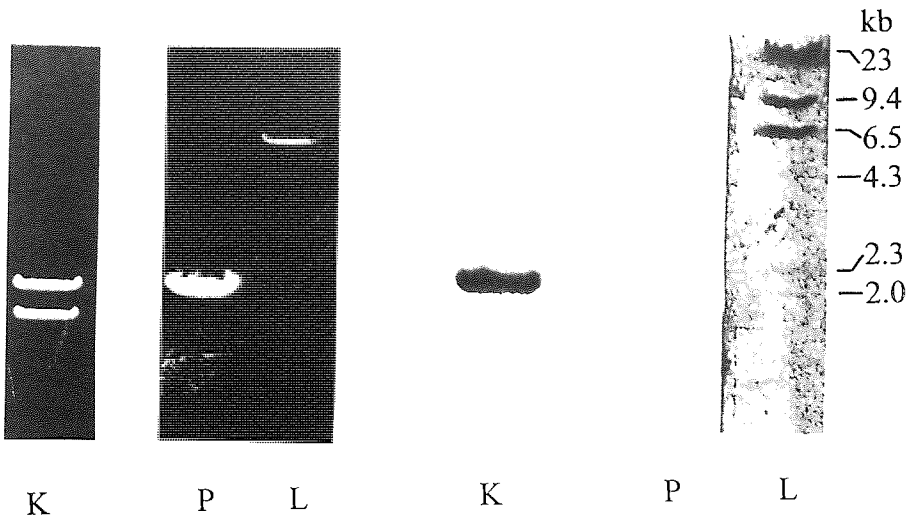


Figure 5.12: Southern blot analysis of KIH38 clone with *Hind*III using *Hind*III-*Xho*I fragment of Tn*phoA* as a probe.

K: KIH38; P: pUC18; L:λ cut with *Hind*III as a marker.

The result of sequencing the KIH38 clone using the outward-facing Tn*phoA* right hand side primer is shown in Figure 5.13 below.

```
        ========-AGGCCGTAAG TCCAGACTGA CCTGAAATCA
31      ACCACTCCAG CTGCCGGGCA TCCTGATAAA TCATCACCCG
71      TGCAAATGAG CAGGGAATTA AGTCCTGTGG TCATCTACAG
111     CGCATAAAAC GGAGTCTCTC ATGGGAATAA TCAGAACAGT
151     GGTATACGTG GGTATCATTG GAGGCATCTT TGCTATAATT
191     CTTCAACACA CCTTCGAAGC AGCCATATTC TATCAATCTG
231     ACTATCATAA CGATCTCGT
```

Figure 5.13: The DNA sequence of KIH38. ==== Tn*phoA*

(3). KSL19

The analysis procedure used to check the KIH19 clone was similar to that used for KIH62 (Figure 5.14).

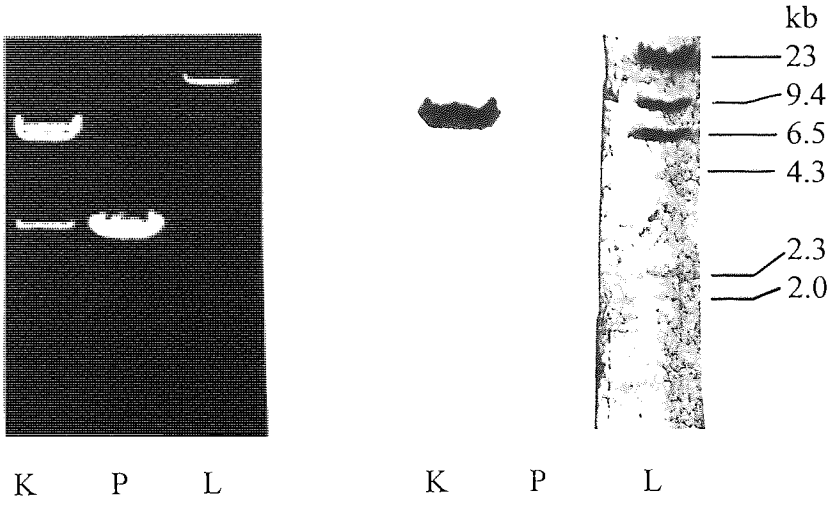


Figure 5.14: KIH19 clone.

A: Electrophoresis.

B: Southern blot using a *Hind*III-*Xho*I fragment of Tn*phoA* as a probe.

K: KIH19; P: pUC18; L: λ cut with *Hind*III as a marker.

The plasmid DNA of KIH19, carrying the right hand side of Tn*phoA*, was sequenced using the cycle sequencing method, and the result is shown below (Figure 5.15)

========-CTCTTAATCA GCATAGAGGG AACGACCATG

31 CAGGCTATTC TTAGTTTCTT AACGGAAATA TTCAGTCAAC

71 CCGAATTTTT AATGGGGCTT ATCGCCTTTA TTGGTTAGT

111 GGCGCTGCGC TCCCCTGGCA ATAAACTGCT TACCGGCACA

151 TTGAAGCCGA TTTTAGGCTA TTTGATGTTG AGCGCTGGGA

191 AGGCGTTATC GTTGCATCTC ATC

Figure 5.15. The DNA sequence of KIH19. ==== Tn*phoA*

The plasmid containing the cloned fragment of KIH19 (clone number 40) cut with *Hind*III was used as a probe to detect the related fragment in the *K. pneumoniae* M10 chromosome (Figure 5.16)

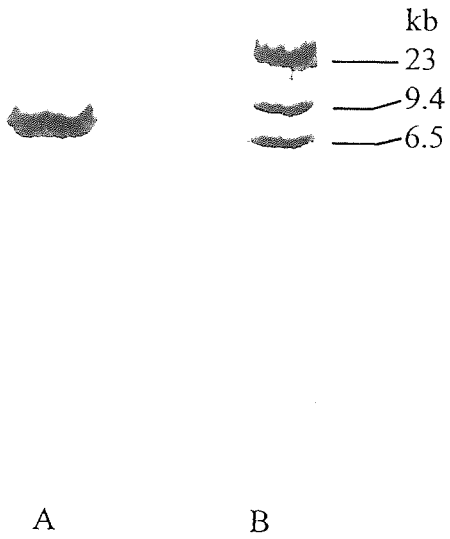


Figure 5.16. Southern blot of M10 chromosome cut with *Hind*III using a *Hind*III fragment of the KIH19 as a probe

5.4. Cloning the intact gene from *K. pneumoniae* M10

The procedure used to clone the M10 chromosome fragment into pUC18 was the same as for KIH19, KIH38 and KIH62. *Hind*III chromosome fragments were cloned into pUC18 and recombinants analysed by gel electrophoresis and Southern blotting using a *Hind*III fragment from KIH19 as a probe (Figure 5.17). The clones were designated M19.

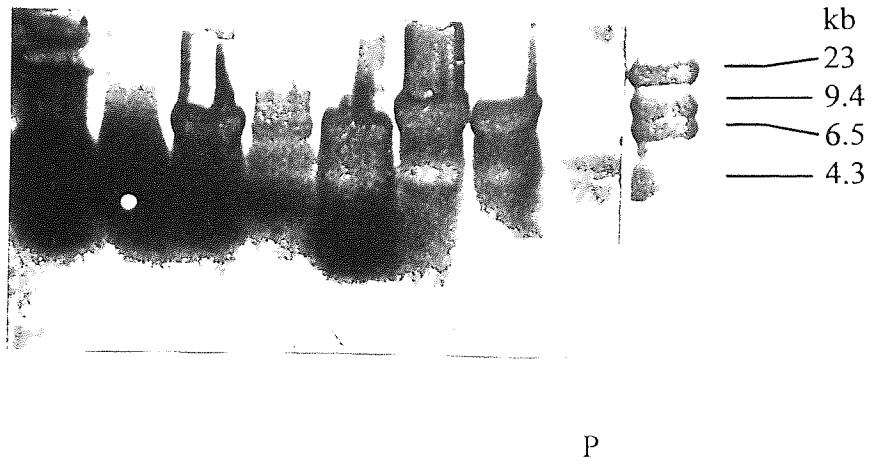


Figure 5.17: Southern blot analysis of the M19 clones cut with *Hind*III using a *Hind*III fragment from KIH19 as a probe. P: pUC18 cut with *Hind*III

The plasmid DNA of the positive clone was isolated, analysed by Southern blotting, and then sequenced using primers based on sequencing data from KIH19. For this part of the work automated sequencing facilities were employed

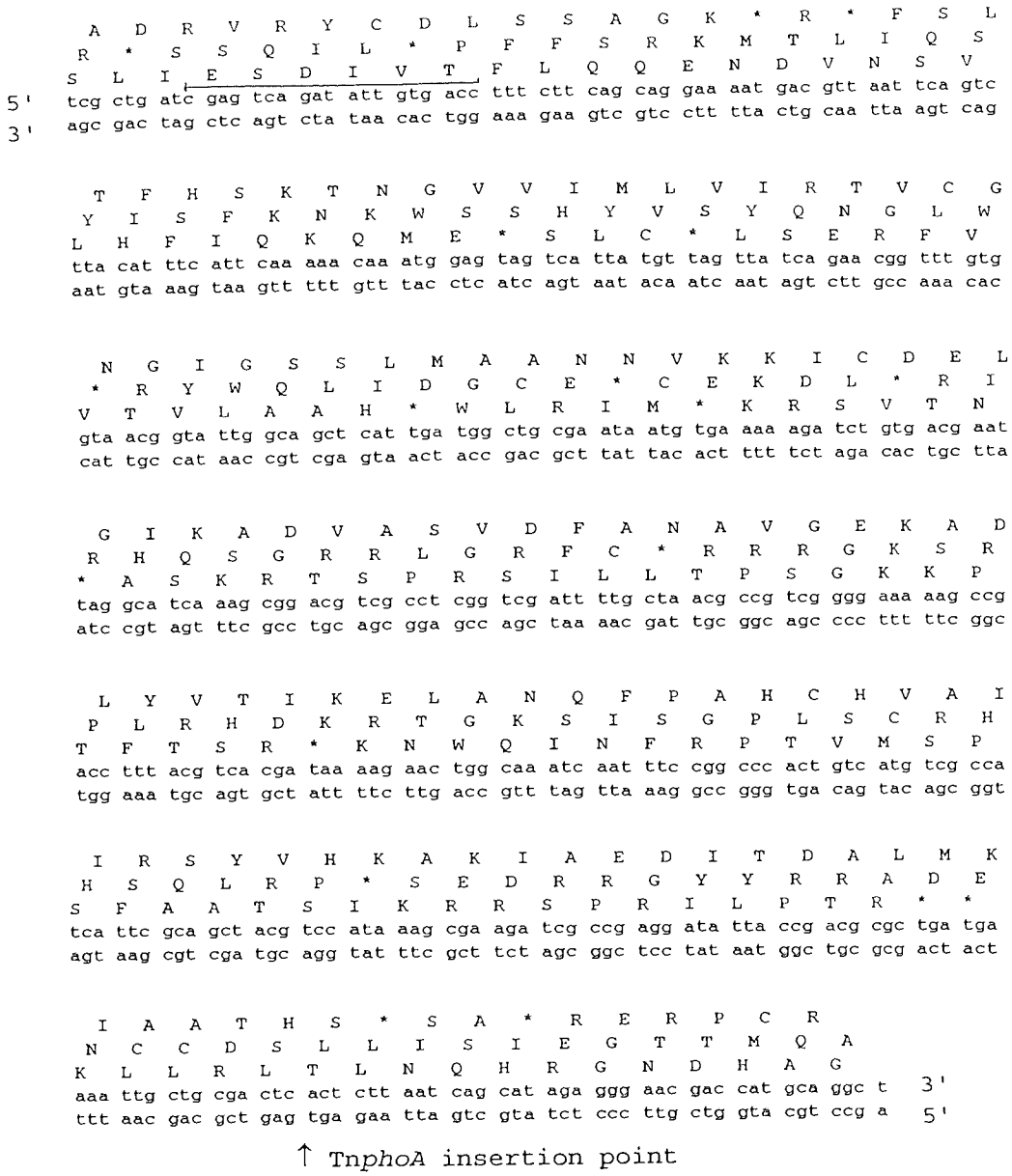


Figure 5.18: The DNA sequence of M19 around the point of insertion of Tn*phoA* in the KSL19 chromosome.

⟵⟶ denotes one primer used for PCR in Figure 5.19

Using primers based on the above sequencing data, the fragment carrying the left hand side of Tn*phoA* from KSL19 was amplified by PCR, and the result is shown below (Figure 5.19). The primers were: CGAGTCAGATATTGTGACC (indicated in Figure 5.18) and AAAAGGCGGGTTGAC (located 66bp downstream of the ORF).

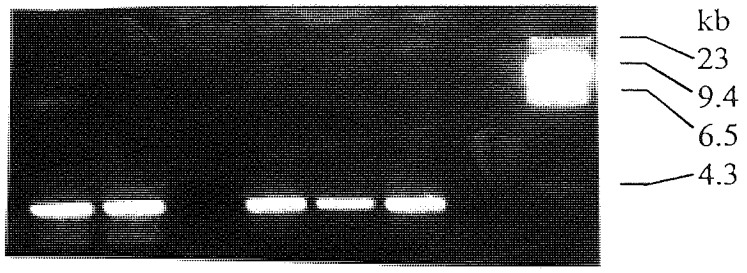


Figure 5.19. PCR product of the left hand side of Tn*phoA* within the KSL19 chromosome. A: uncut chromosome, B: chromosome cut with *Hind*III (H), *Xho*I(X) or *Eco*RI(E) and religate, C: No chromosomal DNA, as a negative control.

The sequence DNA of the PCR product is shown in Figure 5.20.

=======-TGA TTA AGA GTC GCA GCA ATT TTC

ACT AAT TCT CAG CGT CGT TAA AAG

ATC AGC GCG TCG GTA ATA TCC TCG GCG AT...

TAG TCG CGC AGC CAT TAT AGG AGC CGC TA...

Figure 5.20. The DNA sequence of the left hand side of the Tn*phoA* insert in the KSL19 chromosome. ===== Tn*phoA*

The sequence data from KIH19 and the PCR product of KSL19 showed that there is a direct repeat sequence on each end of the Tn*phoA* (Figure 5.21).

C TCT TAA TCA ========== C TCT TAA TCA

Figure 5.21. Direct repeat sequence in KSL19. ===== Tn*phoA*

5.5. Identification of ORF with deduced amino acid sequence of the *kslA* gene

Sequencing data of KIH19 confirmed the sequence of M19 and the point of insertion of the transposon. The addition of the left hand side (upstream) sequencing data revealed a single open reading frame, designated *kslA*, including the start and stop codons, the signal sequence and putative ribosome binding site region (Heidelberg, 1987) (Figure 5.22)

```
                                                    RBS
                                                  ____________
TAA TTC AGT CTT ACA TTT CAT TCA AAA ACA AAT GGA GTA GTC ATT
*   F   S   L   T   F   H   S   K   T   N   G   V   V   I
1

ATG TTA GTT ATC AGA GCA GTT TGT GGT AAC TGG ATT GGC AGC TCA
M   L   V   I   R   T   V   C   G   N   G   I   G   S   S
start_____________signalsequence_____________________________
46

TTG ATG GCT GCG AAT AAT GTG AAA AAG ATC TGT GAC GAA TTA GGC
L   M   A   A   N   N   V   K   K   I   C   D   E   L   G
_ sig.seq _____
92

CTA AAA GCG GAC GTC GCC TCG GTC GAT TTT GCT AAC GCC GTC GGG
I   K   A   D   V   A   S   V   D   F   A   N   A   V   G
136

GAA AAA GCC GAC CTT TAC GTC ACG ATA AAA GAA CTG GCA AAT
E   K   A   D   L   Y   V   T   I   K   E   L   A   N
179

CAA TTT CCG GCC CAC TGT CAT GTC GCC ATC ATT CGC AGC TAC GTC
Q   F   P   A   H   C   H   V   A   I   I   R   S   Y   V
225

CAT AAA GCG AAG ATC GCC GAG GAT ATT ACC GAC GCG CTG ATG
H   K   A   K   I   A   E   D   I   T   D   A   L   M
268

AAA ATT GCT GCG ACT CA----C TCT TAA TCA GCA TAG
K   I   A   A   T           S   *   S   A   *
                    TnphoA      stop
311
```

Figure 5.22. DNA sequence of *kslA* gene, carries putative ribosome binding site (RBS) regions, start and stop codons, deduced peptide signal sequence and Tn*phoA* insertion site

5.6. Signal sequence

The following key structural features of signal peptides have been identified by Inouye *et al.*(1977).

1. One to three positively charged amino acid residues occur in the amino-terminal region of the signal peptide (amino-terminal basic region). In every known prokaryotic signal peptide, there is without exception, one to five positively charged amino acid residues (lysine and/or arginine) located at the amino-terminal portion. There should be no glutamic acid or aspartic acid residue in signal peptides.

2. A long hydrophobic sequence consisting of 14-20 amino acid residues follows the amino-terminal basic region (hydrophobic domain). The most frequent residues are alanine, valine, leucine (less frequently isoleucine), phenylalanine, tyrosine, and methionine (tryptophan has not been found).

3. Within the central region of the hydrophobic domain there is very often one to two glycine or proline residues.

4. In the hydrophobic domain there is almost always a serine and/or a threonine residue. The positions of these residues are in general between -3 and -6.

5. An alanine or glycine residue occurs at the carboxy terminus of the signal peptide (cleavage site) at the position -1 (Duffaud *et al.*, 1985).

The amino acid sequence at the N-terminus of the disrupted gene in KSL19 (*kslA*) showed these characteristic features of a signal peptide structure (Figure 5.23):

1. There is no glutamic acid or aspartic acid residue. There is one arginine in the amino terminal region.

2. The length of the hydrophobic domain is 14 amino acids, containing valine, leucine, methionine and alanine.

3. There are three glycines within the hydrophobic domain.

4. There are two serines at position -5 and -6.

5. There is an alanine residue at position -1 (carboxy terminal).

M L V I R T V C G N G I G S S L M A A N N

↑

Amino terminal_____Hydrophobic domain____ Cleavage site

region

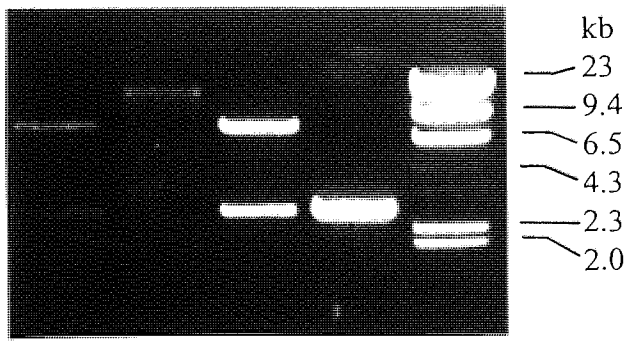
Figure 5.23 : Deduced signal peptide of the *kslA* gene in the KSL19 mutant.

5.7. Complementation

In order to characterise the M19 clone, further analysis was carried out

5.7.1. Complementation of KSL19 by transformation of the M19 plasmid.

The result of transformation of KSL19 with M19, designated T19, was checked by gel electrophoresis (Figure 5.24)



T K M P L

Figure 5.24: Gel electrophoresis of T19 transformants T: T19, K: KSL19, M: M19, P: pUC18, L: λ. All cut with *Hind*III.

5.7.2. MIC of BRL41897A towards transformant T19

The MIC of the transformant T19 showed that the plasmid from M19 restored the sensitivity of KSL19 to BRL 41897A to that of the wild type M10.

Table 5.1: MIC of BRL41897A towards T19 transformants grown in Fe-CAA.

Strain	MIC of BRL 41897A (μg/ml)
M10	0.5
KSL19	2.0
T19	1.0

CHAPTER 6

CONCLUDING DISCUSSION

Many new cephalosporins with broad spectrum antibacterial activity have been developed over the past few years. Several of these antibiotics such as the oxyimino cephalosporins (cefotaxime, ceftazidime, ceftriaxone, etc) are in clinical use for the treatment of a wide range of bacterial infections. However, widespread use of these antibiotics resulted in nosocomial outbreaks of resistance during the 1970s. Outbreaks caused especially by strains of *E. coli* and *K. pneumoniae* ESBL have been described since 1980s (see Introduction section on *K. pneumoniae*).

A recent study on the susceptibilities of ESBL-producing *K. pneumoniae* strains has shown that about 25% of them are resistant to cefoxitin, but the actual mechanism of this resistance has not been precisely defined (Martinez-Martinez *et al.*, 1996). It is important for the elucidation of the mode action of antibiotics and the design of new antibiotics to understand the mechanisms of resistance.

The mechanism of resistance of bacteria to β-lactam antibiotics are generally categorized into the following three types :

i. production of β-lactamase.

ii. alteration of PBPs.

iii. decrease in permeability of β-lactam antibiotics through the outer membrane, especially by reduced levels of porin proteins (Metcalfe and Holland, 1980).

Early study by Watanabe's group (1987) using a new cephalosporin E-0702 in *E. coli* revealed that resistant mutants did not exhibit increased production of β-

lactamase. No apparent differences between resistant mutants and the parental strains were observed in the affinity of E-0702 for PBPs. Furthermore, they reported that no significant reduction in, or loss of the OmpF and OmpC porin proteins in the outer membrane was observed. They hypothesised that the porin channels are not the main pathway of entry of E-0702, and this was supported at least by the fact that the molecular size of E-0702 (molecular weight, 770Da) is larger than that of compounds able to diffuse into the periplasmic space through the porin channels.

Since E-0702 resistance could not be explained by the three usual types of resistance mechanism described above, they investigated the genetic basic of the resistance. It was shown that resistance to E-0702 results from a chromosomal mutation localized in the *tonB* gene. The *tonB* mutants are defective in all known high-affinity iron transport systems including that of enterobactin. Its six phenolic hydroxyl groups act as ligands for iron forming a strong octahedral complex. Since the killing action of E-0702 is affected by the level of iron in the medium, it is likely that E-0702 coordinates iron via the vicinal hydroxyls on its side chain. These findings indicate that iron-chelated E-0702 is incorporated into *E. coli* cells through the outer membrane via the *tonB*-dependent transport system for iron with subsequent rapid expression of the killing action of E-0702 which exhibits high affinity for the essential PBPs (Watanabe *et al.*,1987). Whereas albomycin, tetracyclines, aminoglycosides and fosfomycin are antibiotics which are incorporated into bacterial cells by active transport system, no β-lactam antibiotics had been reported to penetrate the outer membrane via the active transport systems. Thus E-0702 was the first β-lactam to do so and its uptake is dependent on *tonB* function. They concluded that antimicrobial delivery via the iron transport system is an extremely fascinating

possibility. Following these results, several groups have developed new β-lactam antibiotics which show high levels of activity against *Enterobacteriaceae* and *P. aeruginosa* and which contain the catechol group in their molecules.

Curtis and co-workers (1988) used selected aminothiazolyl-oxime cephalosporin congeners substituted at the C-3' with a catechol moiety to probe the basis of the enhanced antibacterial activity against *E. coli* K12. Their findings indicated the involvement of both the Fiu and Cir IROMPs of *E. coli* K12 in the *tonB*-dependent antibacterial activity of this antibiotic. In addition to the products of the *fiu* and/or *cir* and *tonB* loci, those of the *exbB* and possibly *exbD* loci are also required for the full expression of antibacterial potency. In this respect, a close analogy exists between catechol-cephalosporin activity, the transport of siderophores and vitamins B12, and the action of certain colicins.

However, the uptake pathway appears relatively tolerant of chemical variability of the substrate, although unsubstituted vicinal hydroxyl groups on the catechol residue were a necessity for good activity. This contrasts with the constraints imposed on natural substrates transported by FepA, FecA, FhuA, and FhuE of *E. coli* K12. Interestingly, it was reported that a rifamycin derivative, CGP-4832, is apparently subject to *tonB*-dependent uptake by FhuA, even though it is structurally distinct from the natural substrate, ferrichrome. Conversely, coprogen and rhodotorulic acid, although being hydroxamic acid-based siderophores like ferrichrome, are recognized by the FhuE and not the FhuA receptor (Curtis *et al.*,1988). It is still difficult to understand, as potential mimetic substrates, why catechol-cephalosporins are transported via the Fiu and Cir receptors since there is a lack of information on the natural substrates transported by these receptors.

However, the enterobactin outer membrane receptor, FepA, is not involved in catechol-cephalosporin activity, even though the hexadentate iron chelation centre of enterobactin is provided by catechol residues amide linked to a triserine backbone.

Similarly, the uptake of some synthetic enterobactin analogs by *E. coli* K12 does not involve the FepA receptor.The *fiu* and *cir* genes of *E. coli* K12, in common with all those concerned with siderophore synthesis and capture, are subject to negative regulation by Fur, with Fe(II) as the corepressor. At basal level expression of the uptake receptors, significant rates of catechol-cephalosporin transport presumably occur since a reduction of receptor copy numbers to zero (*fiu, cir* mutants) causes the most pronounced increase in resistance. However, the rate of catechol-cephalosporin uptake facilitated at basal (or near basal) level receptor expression may still be a limiting factor in the overall antibacterial process, since susceptibility may be increased by mutation at *fur* (Curtis *et al.*,1988).

Similar work by Nikaido and Rosenberg (1990) has shown that β-lactam antibiotics containing catechol or 3-hydroxypyridone cross the outer membrane barrier mainly through the siderophore transport channels involving the Cir and Fiu receptors. They found that the Cir and/or Fiu systems transported unliganded siderophore as rapidly as, or perhaps more rapidly than they transported the ferric ion-siderophore complex. If these proteins can indeed transport unliganded catecholic compound efficiently, this may be beneficial to the cell for the uptake of 2,3-dihydroxybenzoic acid and its derivatives. Thus, the system may also function in recovering the products of the hydrolysis of enterobactin so that they can be reutilized in the synthesis of enterobactin. They also note that free Fe^{3+} is essentially unavailable in the tissues of animals because of its binding with high-affinity iron-

binding proteins; the catechol-containing β-lactams would not work well in such an environment if they had to be transported exclusively as Fe^{3+} complexes. They concluded that the compounds containing catechol or its analogs appear to utilize specific transport machineries of the cell for the uptake of drugs, apparently because they can utilize one of the two or more pathways present in the outer membrane; high levels of resistance to these compounds would require either an extremely rare double mutation or mutation in the *tonB* locus, which would essentially abolish the ability of the organism to transport iron and thus make it nonpathogenic (Nikaido and Rosenberg, 1990).

Following these two studies, Critchley *et al.* (1991) investigated a catecholic C(7)α-formamido-substituted cephalosporin, BRL 41897A, and found that it showed increased penetration into *E. coli* cells grown in an iron-deficient medium compared with cells grown in a medium supplemented with iron. In contrast, penetration of the corresponding monohydroxyphenyl analogue, BRL 42948A, was not influenced by iron concentration.

In this thesis using the same compounds as Critchley *et al.* a similar result was found in *K. pneumoniae*. The MIC of BRL 41897A was higher in the transposon mutants than the wild type M10, whereas no differences in MIC were found for the non-catecholic BRL 42948A. The results also showed that the MIC of *K. pneumoniae* towards BRL 41897A is increased under iron-rich conditions, suggesting that this antibiotic penetrates the outer membrane mainly via the iron transport system. Thus this antibiotic may be effective against severe infections where iron-limited conditions occur.

However, whereas the Fiu or Cir proteins are lost in the *E. coli* resistant

mutants, most of the *K. pneumoniae* mutants resistant to BRL 41897A had normal IROMPs (although KSL19 and KSL38 showed decreased expression of one of IROMPs). Mutant KSL19 lost an 88 kDa protein but increased expression or produced a novel protein of about 86kDa protein, KSL38 produced an 80kDa IROMP only weakly (Figure 4.1). Likewise, studies by Hazumi *et al* (1992) on *Pseudomonas aeruginosa* demonstrated that resistance of *P. aeruginosa* PAO2146 to the catecholic cephem antibiotic BO-1341 was accompanied by elevated expression of both an 84kDa outer membrane protein and a 55 kDa periplasmic protein instead of IROMPs. Similarly, in this thesis it was found that all of the mutants resistant to BRL 41897A showed increased expression of one or more periplasmic proteins (Figure 4.3).

Since all of the E-0702 resistant *E. coli* mutants tested by Watanabe *et al.* (1987) turned out to be defective in *tonB* gene function, with no defects in the receptor proteins in the outer membrane, it was concluded that various receptors for E-0702 are present in the outer membrane. E-0702 could penetrate the outer membrane as a result of the proper functioning of other receptors and could act on PBPs, even if one type of receptor was defective. Critchley *et al.* (1991) also assumed that catecholic β-lactams are capable of penetrating outer membranes using conventional porin pathways (Osborn *et al.*, 1972; Bavoil *et al.*, 1977; Nikaido and Rosenberg, 1979; Nikaido and Vaara, 1985; Pratt, 1996). Several *K. pneumoniae* KSL mutants in this study also showed reduced production of one OMP, presumed to act as a porin.

In this thesis, treatment of *K. pneumoniae* mutants KSL19 and KSL38 with UV light increased their MIC to levels higher than the first generation strains, however the results showed there to be no differences in the IROMPs or periplasmic

proteins compared with their parent strains, KSL19 and KSL38. It is assumed that the antibiotic is transported in *K. pneumoniae* through other receptors in addition to IROMPs receptors.

Interestingly, mutants KSL52, KSL57,KSL58, KSL59 and KSL62 were also resistant to chloramphenicol and mutants KSL19, KSL38, and KSL39 were resistant to both chloramphenicol and novobiocin. The molecular and genetic basis for this cross-resistance is unknown but possibly indicates an alteration in uptake.

Apparently, in certain mutant strains the decrease in major outer membrane proteins resulting from the mutation is compensated for by an increased level of other outer membrane proteins. This indicates that the cell has the capacity to maintain the total amount of the major outer membrane proteins. Schnaitman *et al.*(1975) showed that an *E. coli* strain lysogenised by phage PA2 contained a new major outer membrane protein and much less of proteins Ia and Ib. In a separate study, Schnaitman (1974) observed that the amount of protein II in strain AB1621, missing proteins Ia and Ib, was about equivalent to the sum of proteins Ia, Ib, and II*, present in the parental strain, AB1854. Later Ia protein was renamed OmpF and Ib protein OmpC protein (Braun and Hantke, 1981). Chai and Foulds (1977) also obtained similar results where the relative levels of the major outer membrane proteins of tol^{+} and tol^{F} strains were compared. The most dramatic alteration in the protein composition of the cell envelope components prepared from tol^{F} strains was the decreased level of protein Ia and the concomitant increase in proteins Ib and II*.

Another mutant strain of *E. coli* was isolated by Foulds and Chai (1978) containing a new outer membrane protein, protein E (referred to by others as protein Ic or e). This mutant contained neither major outer membrane protein Ia nor Ib. It

is known that at least three genetic loci determine the production of proteins Ia and Ib. Strains carrying mutations in the *par* (*meo*) locus lack protein Ib, and strains carrying mutations in the *tolF* (also termed *cmlB* or *cry*) locus lack protein Ia. Strains with mutation in the *ompB* locus fail to produce either protein Ia or Ib, as do strains carrying mutations in both *par* and *tolF*, such strains all produce protein E (Pugsley and Schnaitman, 1978).

Tomassen and Lugtenberg (1980) also isolated SDS-resistant pseudo revertants containing protein similar to protein E. A search for growth conditions that result in the induction of protein E in wild type cells of *E. coli* K12 resulted in the observation that the synthesis of this protein is derepressed by growth in limiting concentrations of inorganic phosphate. Phosphate limitation also results in derepression of the synthesis of several periplasmic proteins, designated as P1, P2, P3, and P4.

In earlier studies, Lugtenberg *et al.* (1976) used an improved gel electrophoresis system that allows the resolution of the major outer membrane protein of *E. coli* K12 into four bands, a, b, c, and d with apparent molecular weights of 40, 38.5, 38 and 36K. Band b is identical to Ia protein, band c is identical to Ib protein, and band d is identical to protein II*. Studies on the influence of growth conditions on the composition of the membrane proteins of *E. coli* K12 revealed that the growth medium exerts a dramatic influence, especially the ratio of b (Ia) over c (Ib) is dependant on the composition of the medium. The lack of protein c (Ib) in mutant strain CE1036 results in an increase in the amounts of protein d (II*) and b (Ia). Mutants that lack protein d (II*) possess increased amounts of protein c (Ib). Later studies showed that in *E. coli*, two major outer membrane proteins, Ia (OmpC) and Ib (OmpF), are differentially regulated in response to osmotic stress (Barbas *et*

al., 1993). OmpF is preferentially expressed under low osmolarity condition, while an increase in medium osmolarity results in repressed expression of OmpF and enhanced OmpC expression. OmpF and OmpC expression is controlled by the membrane receptor-cytoplasmic effector system consisting of EnvZ and OmpR. EnvZ is an integral inner membrane protein and OmpR is a transcriptional regulator that binds to the promoter regions of *ompF* and *ompC* and has both activator and repressor function (Jaffe *et al.*, 1982).

In this study, the results of SDS-PAGE analysis showed that the IROMPs of *K. pneumoniae* KSL19 vary compared with the parent strain, with a decrease of one protein and an over expression of another protein or production of a new IROMP. KSL19 and most of the other mutants also showed increased expression of periplasmic proteins. It is possible that new outer membrane proteins have greater affinity for the Fe-siderophore complex than the normal outer membrane proteins or IROMPs. However, in the mutant strains there might be some defects in the transport of the iron-siderophore complex therefore preventing access to the PBP target. This hypothesis was supported by the results of two experiments, the uptake of ^{55}Fe-BRL 41897A and the cloacin sensitivity test.

The results from the transport of ^{55}Fe-BRL41897A by *K. pneumoniae* showed that the uptake of this complex by the mutants was higher than that by the parent strain M10. Thus it is likely that the uptake of ^{55}Fe-BRL 41897A is mediated not only by normal IROMPs and/or outer membrane proteins but also by new outer membrane proteins and/or IROMPs. In the case of KSL19 uptake might be due to a new protein or to over expression of existing proteins. Based on the fact that all of the mutants tested were resistant to cloacin, whereas the parent strain M10 was sensitive, and that

most of the mutants had no defect either in outer membrane proteins or IROMPs, but had elevated levels of periplasmic proteins, there might be some alteration in the transport of the Fe-BRL 41897A complex at the periplasmic level.

The sequencing analysis showed that the Tn*phoA* was inserted within a newly-recognized gene of KSL19 (designated *kslA*) just before the stop codon. The sequence of the *kslA* gene shows some limited homology with *exbD* from *E. coli* (22% identity; 39.5% similarity of amino acid sequences, see Figure 6.1). This indicates a possible evolutionary relationship similar to that between the *tol* and *exb* genes which show approximately 25% identity and 75% similarity (Kampfenkel and Braun, 1993).

```
          .         .         .         .         .
 1 .....................................mlvirtvcgngigsslm 17
                                        ::|  .:.. ::  .|
 1 MAMHLNENLDDNGEMHDINVTPFIDVMLVLLIIFMVAAPLATVDVKVNLP 50
          .         .         .         .         .
18 aannvkkicde....lgikadvasvdfanavgekadlyvtikelanqfpa 63
   |......  .|    |::|||  .|: ::|..       || ..: ..: |
51 ASTSTPQPRPEKPVYLSVKAD.NSMFIGNDP.......VTDETMITALNA 92
          .         .         .         .         .
64 hchvaiirsyvhkakiaaditdalmkiaaths..................  95
    ...    ... :|. ..|. :.|||: .|
93 LTEGKKDTTIFFRADKTVDY.ETLMKVMDTLHQAGYLKIGLVGEETAKAK 141
                                  .
                                  .
```

Figure 6.1. Deduced peptide homology between *kslA* and *exbD* gene product.

It has been proposed by Kampfenkel and Braun (1993) that interaction between TonB, ExbB, and ExbD, and between TolA, TolQ and TolR, takes place in the

cytoplasmic membrane. Proper interaction is essential for creation of active complexes which induce conformational changes in the cognate receptor proteins so that adsorbed colicins, ferric siderophores, and vitamin B12 are released from the receptor proteins and translocated through the outer membrane.

It is highly likely that *K. pneumoniae* contains genes related to the *ton* and *tol* genes of *E. coli* and that *kslA* forms part of a group of genes involved in iron-dependent outer membrane transport. The sequence of the *K. pneumoniae tonB* gene has been determined (Bruske *et al.*, 1993). It is therefore likely that proteins equivalent to ExbB and ExbD in *E. coli* are also present in *K. pneumoniae*. The *kslA* gene could represent one of these TonB or TolC stabilising genes. Alternatively *kslA* may be a newly-identified locus which is involved in other TonB or Tol- dependent processes. Since TonB is involved in a range of iron-dependent transport systems and TolC appears to regulate outer membrane protein production, disruption of a gene which stabilizes these key proteins would have a significant effect upon a range of transport processes. Although it was not possible to identify the product of the *kslA* gene by SDS-PAGE of either outer membranes or periplasmic contents, the low molecular weight and a low cellular content would make detection difficult. The various changes noted in other outer membrane and periplasmic proteins presumably result indirectly from disruption of *kslA*.

The complementation studies showed that sensitivity to BRL 41897A was restored on transformation of a large (7 kb) fragment of DNA carrying the *kslA* gene. Future cloning studies should determine the nature of the genes in this fragment, their possible regulation by *kslA* and their dependance upon the KslA protein for functional stability.

Partial sequencing of the other mutants produced in this study, KSL38 and KSL62, indicated that additional genes of unknown function had been disrupted by the transposon. Future studies should be made on these mutants to identify the disrupted genes. This should give additional information upon the iron-dependent transport of catecholic-cephalosporins in *K. pneumoniae*.

CHAPTER 6

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APPENDIX

Amino acids

Amino acid.	Abbreviation.	Code Letter.	Mass.	Properties (hydrophilicity).
Alanine	Ala	A	89.09	Neutral, (-0.5)
Arginine	Arg	R	174.2	Basic, (3.0)
Asparagine	Asn	N	132.1	Neutral, (0.2)
Aspartic acid	Asp	D	133.1	Acidic, (3.0)
Cysteine	Cys	C	121.12	Neutral, (-1.0)
Glutamic acid	Glu	E	147.13	Acidic, (3.0)
Glutamine	Gln	Q	146.15	Neutral, (0.2)
Glycine	Gly	G	75.07	Neutral, (0.0)
Histidine	His	H	155.16	Basic, (-0.5)
Isoleucine	Ile	I	131.17	Neutral, (-1.8)
Leucine	Leu	L	131.17	Neutral, (-1.8)
Lysine	Lys	K	146.19	Basic, (3.0)
Methionine	Met	M	149.21	Neutral, (-1.3)
Phenylalanine	Phe	F	165.19	Neutral, (-2.5)
Proline	Pro	P	115.13	Neutral, (0.0)
Serine	Ser	S	105.09	Neutral, (0.3)
Threonine	Thr	T	119.12	Neutral, (-0.4)
Tryptophan	Trp	W	204.22	Neutral, (-3.4)
Tyrosine	Tyr	Y	181.19	Neutral, (-2.3)
Valine	Val	V	117.15	Neutral, (-1.5)

Hydrophilicity value according to Hopp and Woods (1981)