

MOLECULAR GENETIC ANALYSIS OF AEROBACTIN RECEPTORS

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Doctor of Philosophy
at the University of Leicester

by

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ABSTRACT

Some *Enterobacteriaceae* produce a low molecular weight compound, aerobactin, with a high affinity for ferric iron. The genes encoding the aerobactin system have been identified in plasmid or chromosomal DNA; they are arranged in an operon consisting of five genes; four genes encode enzymes responsible for the biosynthesis of aerobactin and the fifth encodes an outer membrane receptor protein specific for ferric aerobactin. The aerobactin receptor protein appears to be different in size (molecular weight) in different species. *Escherichia coli* (ColV-K30) express a 74 kDa protein, while *Shigella* spp. express a 76 kDa protein. The aerobactin receptor protein also acts as a receptor for the bacteriocin cloacin DF13. *Shigella* spp. expressing the aerobactin receptor protein were less sensitive to cloacin DF13 than *E. coli* (ColV-K30).

The aerobactin receptor genes of several *Shigella* spp. isolate have been cloned and the aerobactin receptor gene from *Shigella flexneri* ser.6 (*iutA_{Sh}*) has been sequenced. The restriction maps of *iutA_{Sh}* and *iutA* (aerobactin receptor gene from ColV-K30) were similar. The main difference was the existence of a *Bam*HI site in the middle of *iutA_{Sh}* but not in *iutA*. The predicted protein product of *iutA_{Sh}* consists of 732 amino acid residues, the same as the aerobactin receptor protein from ColV-K30 (*iutA*), and with 93% similarity.

Construction of *iutA::iutA_{Sh}* hybrid genes demonstrated that although various parts of *iutA* were able to increase the cloacin sensitivity function of *iutA_{Sh}*, the main function was located in the 3'-terminus of *iutA*. Constructs with the 3'-terminus of *iutA* expressed a 74 kDa, while constructs with 3'-terminus of *iutA_{Sh}* expressed a 76 kDa outer membrane protein. The aerobactin uptake of *E. coli* strains carrying constructs with the 3'-terminus of *iutA_{Sh}* was higher than that of *E. coli* strains carrying constructs with the 3'-terminus of the *iutA*. The highest aerobactin uptake was showed by *E. coli* strain carrying construct 2, which consist of the *iutA* fragment upstream of the *Cla*I site and the *iutA_{Sh}* fragment downstream of the *Cla*I site.

Bacteriophage B74K was capable of using the aerobactin receptor protein, but it was also capable of using other outer membrane proteins as receptors, and so could not be used for aerobactin receptor protein identification. In addition, the presence of aerobactin receptor protein increased the sensitivity of *E. coli* strains to phage BF23.

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Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Da	Dalton
DNA	Deoxyribonucleic acid
dATP	Deoxyriboadenosine triphosphate
dCTP	Deoxyribocytosine triphosphate
ddNTP	Dideoxyribonucleoside triphosphate
dGTP	Deoxyriboguanidine triphosphate
dNTP	Deoxyribonucleoside triphosphate
dTTP	Deoxyribothymidine triphosphate
DTT	Dithiothreitol
EDTA	Diaminoethanetetraacetic acid
IPTG	Isopropylthio- β -D-galactoside
kb	Kilobase
kDa	Kilodalton
MOPS	3-(N-morpholino)propane-sulphonic acid
PBS	Phosphate buffered saline
RNA	Ribo nucleic acid
SDS	Sodium dodecyl sulphate (Sodium lauryl sulphate)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBE	Tris-borate-EDTA
TE	Tris-EDTA
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

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CHAPTER 1

GENERAL INTRODUCTION

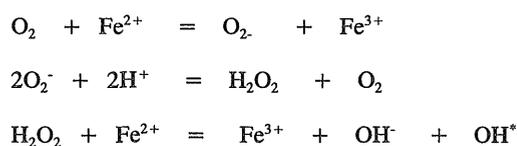
1.1 Iron in living organisms.

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 in the medium for maximum growth, while pseudomonads require four-fold higher levels (Neilands, 1974). Hartmann and Braun (1980) also found that *Escherichia coli* K-12 could grow aerobically at an iron concentration as low as 0.05 μM without any ionophores present and the growth rate increased between 0.05 and 2 μM iron. 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 Charlotheaux 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 *et al.*, 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) and ribonucleotide reductase, which is necessary for transformation of ribonucleoside 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):



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, 1987a).

1.2 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 $\text{Fe}(\text{OH})_3$, 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. 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).

1.2.1. Utilization of host iron compounds.

In order to survive in human body, bacteria should be able to utilize iron either from free iron or storage forms. Most of the intracellular iron is found as haem or stored in ferritin and hemosiderin. It has been estimated that 2.5 grams of the iron in an adult is found in haemoglobin (there are 3 to 4 grams of iron in the average adult human). The small quantities of iron present in the extracellular environment

of mammals are bound to the high-affinity iron-binding proteins transferrin and lactoferrin (Payne, 1988). Direct utilization of host iron complexes should enhance the growth of microorganisms. For example, *Neisseria gonorrhoeae* and *N. meningitidis* 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; Simonson *et al.*, 1982). Transferrin-binding proteins TBP1 (100 kDa) and TBP2 (85 kDa) have been identified; TBP1 is required for transferrin utilization (Legrain *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 *Haemophilus parainfluenzae* is unable to utilize this iron source (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 pyoverdinin, a siderophore from *Pseudomonas aeruginosa*, acquires iron from transferrin (Wolz *et al.*, 1994). *Staphylococcus aureus* are 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* (Modun *et al.*, 1994).

Haemoglobin and haem have been widely reported to serve as sole sources of iron for bacteria. *E. coli* (Griffiths, 1987b) 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), an 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 availability of free haemoglobin in blood by secreting haemolysins, toxins capable of erythrocyte lysis. Haemolytic *E. coli* strains were more frequently isolated from patients with urinary tract infection than from healthy humans (Brook *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 occurred 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).

1.2.2. 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. A soluble reductant in supernatants of *Listeria monocytogenes* cultures capable of removing iron from transferrin has been identified (Coward and Foster; 1985). The dental plaque organism *Streptococcus mutans* acquires ferrous iron via a membrane flavin reductase (Evans *et al.*, 1986). Similarly, *Legionella pneumophila* (Johnson *et al.*, 1991) and the yeasts *Saccharomyces cerevisiae* (Lesuisse *et al.*, 1987), and *Candida albicans* (Morissey *et al.*, 1996) employ cytoplasmic membrane reductases to reduce ferric iron.

1.2.3. 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 accompanied by release of the iron.

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; Hussein *et al.*, 1981).

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

As outlined above, since most bacterial growth depends on the availability of iron, an essential nutrient that participates in many biological processes, the possession of high affinity iron transport systems may be crucial for bacteria to override iron limitation in the environment. Thus, most bacteria produce siderophores and specific outer membrane protein receptors 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 enterochelin, aerobactin, ferrichrome and other hydroxamates, and citrate.

1.3.1. The enterochelin system.

Enterochelin was first identified as an endogenous catechol siderophore of *E. coli* (O'Brien and Gibson, 1970) and *Salmonella typhimurium* (Pollock and Neilands,

1970). This siderophore is also produced by *Klebsiella pneumoniae* and *Shigella* species (Payne *et al.*, 1983; Perry and San-Clemente, 1979). The enterochelin receptor protein in *E. coli* is an 81 kDa outer membrane protein which is expressed only in cells grown in iron restricted medium (Uemura and Mizushima, 1975; McIntosh and Earhart, 1976).

The enterochelin 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.*, 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 (Nahlik *et al.*, 1989) and EntA is 2,3-dihydro 2,3-dihydroxybenzoate dehydrogenase (Liu *et al.*, 1989). EntD, E, F and G are subunits of a synthetase that forms enterochelin from dihydroxybenzoic acid and L-serine (Figure 1.1) (Nahlik *et al.*, 1987; Greenwood and Luke, 1980).

The protein products of genes *fepABCDEG* are specifically required for transport of the ferrienterochelin complex. FepA is the 81 kDa outer membrane protein receptor for ferrienterochelin, while enterochelin 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 (Ozenberg, 1987; Pierce and Earhart, 1986; Pierce *et al.*, 1983).

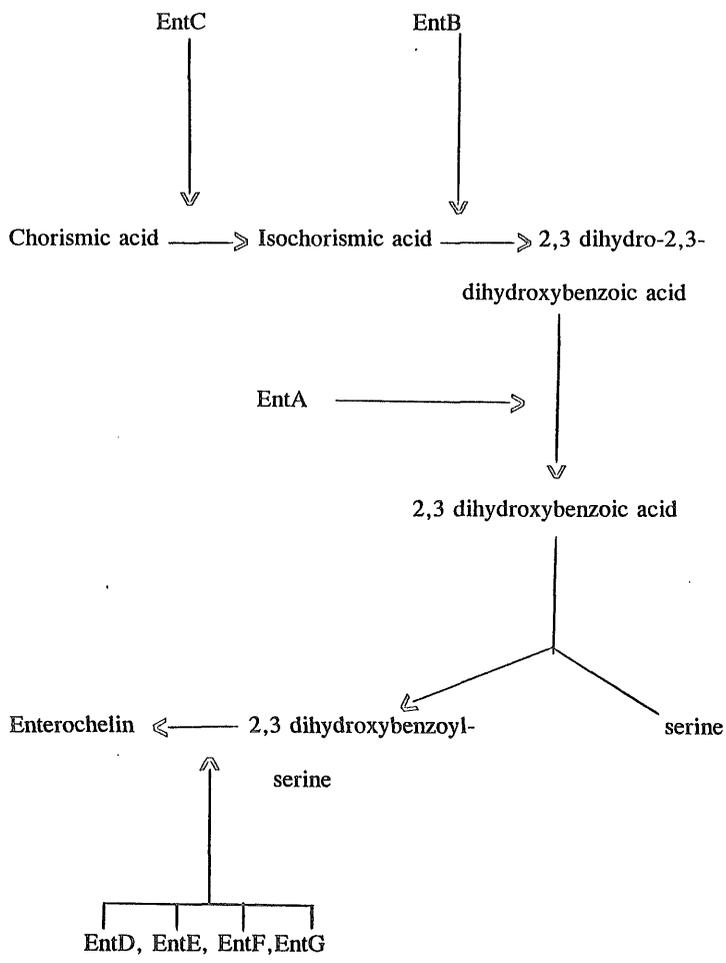


Figure 1.1: Synthesis of enterochelin (Silver and Walderhaug, 1992).

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 enterochelin (Holifield and Neilands, 1978).

1.3.2. The aerobactin system.

Aerobactin 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).

The aerobactin genes were first identified associated with plasmid ColVK30 (Williams 1979; Williams and Warner, 1980; Warner *et al.*, 1981), 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; Roberts *et al.*, 1986; - Fernandez Beros *et al.*, 1988; Gonzalo *et al.*, 1988). Furthermore, aerobactin genes have been found on the chromosomes of *E. coli* K1 isolates (Valvano and Crosa, 1984; Valvano *et al.*, 1986), *Shigella* species (Lawlor and Payne, 1984) and *Salmonella* species (McDougall and Neilands, 1984).

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

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

The aerobactin system of plasmid ColVK30 has been cloned (Bindereif and Neilands, 1983) 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 (de Lorenzo and Neilands, 1986; de

Lorenzo *et al.*, 1986). The fifth gene, *iutA*, encodes the 74 kDa outer membrane aerobactin receptor protein (Figure 1.2) (Carbonetti and Williams, 1984).

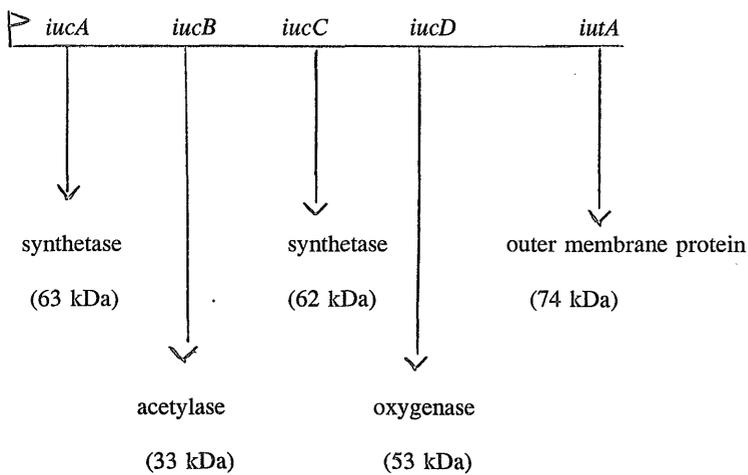


Figure 1.2: The five-gene cluster of the aerobactin system of plasmid ColV-K30 and the polypeptide products it encodes. ▴ represents the promoter and the direction of transcription.

A number of experiments have shown that aerobactin is more effective as a siderophore *in vivo* than enterochelin. 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 enterochelin which tends to promote its adsorption to proteins; enterochelin molecules attached to serum proteins are presumably less effective as siderophores. Also, serum-bound enterochelin molecules may function as haptens, since the presence of antibodies against enterochelin has been detected in normal human serum (Moore *et al.*, 1980), and such antibodies inhibit enterochelin uptake (Moore and Earhart, 1981).

1.3.3. 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 Rosenberg, 1990).

1.3.4. 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 an 80.5 kDa outer membrane protein. It was reported that citrate was an efficient iron carrier for *E. coli* (Frost and Rosenberg, 1973) and for *P. aeruginosa* (Cox, 1980; Harding and Royt, 1990) and appeared to function as a true siderophore in *Bradyrhizobium*, where it was specifically produced during iron starvation (Guerinot *et al.*, 1990; Lesueur *et al.*, 1993). Ferricitrate transport is induced by the presence of citrate in the external medium (Frost and Rosenberg, 1973). 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).

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	ferrienterochelin, colicins B and D
Fec	80.5 kDa	ferricitrate
FhuA	78 kDa	ferrichrome, ferricrysin, ferricrocin, colicin M, phage T1, T5, Φ 80
FhuE	76 kDa	coprogen, rhodotorulic acid
IutA	74 kDa	ferriaerobactin, cloacin DF13
Cir	74 kDa	monomeric catechols, colicins Ia and Ib

1.4. 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 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 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 *et 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 (Gudmundsdottir *et al.*, 1989; Bell *et al.*, 1990). TonB-dependent uptake systems also require the involvement of 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 *E. 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 1.3) (Kampfenkel and Braun, 1993).

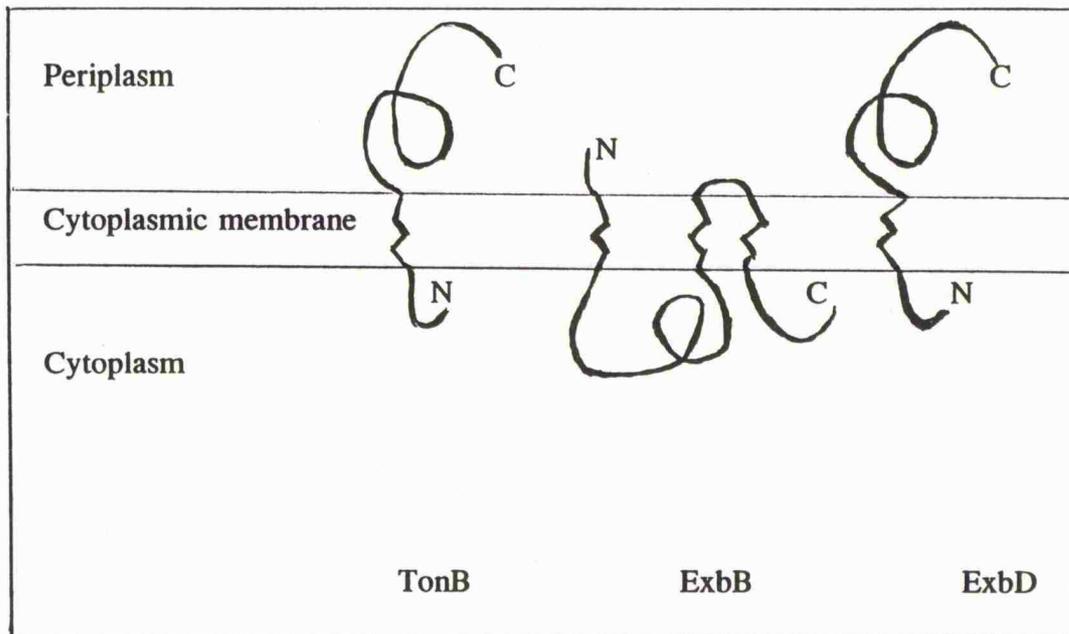


Figure 1.3: Location of TonB, ExbB and ExbD in the bacterial membrane (Roof *et al.*, 1991; Kampfenkel and Braun, 1992 and 1993).

1.5. Siderophore systems in other bacteria.

Siderophore-mediated iron uptake systems of other enteric bacteria have not been studied as extensively as those of *E. coli*, but they are generally assumed to be similar. The enterochelin system has been found in *Salmonella*, *Klebsiella* and *Shigella* species, but even though they produce the same siderophore, the receptor proteins are apparently not identical. As with *E. coli* the enterochelin receptor proteins in *Salmonella* and *Shigella* species appear as a 81 kDa protein on SDS-PAGE, but in *Klebsiella* it appears to be 83 kDa. The enterochelin genes are located on the chromosome in all these species (Payne, 1980; 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 *Enterobacter*, *Salmonella*, *Klebsiella*, *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 the *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 pyoverdine (Cox *et al.*, 1981; Cox and Adams, 1985), pyoverdine being more effective than pyochelin (Sriyosachati and Cox, 1986). In addition, it is capable of utilizing a number of heterologous siderophores, including pyoverdines produced by other pseudomonads, ferrioxamine B, aerobactin and

enterochelin (Pool *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 protein by Meyer *et al.*, 1990, or a 90 protein kDa by Poole *et al.*, 1991. The ferric enterochelin 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). It has recently been reported that the growth of *P. fragi* strains in iron-depleted medium were stimulated by siderophores of foreign origin including desferriferrioxamine B, enterobactin and some pyoverdines and all strains were capable of using transferrin, lactoferrin and haemoglobin. Although iron starvation led to the specific production of outer membrane proteins of apparent molecular mass ranging from 80 to 88 kDa, however, no siderophores were detectable in the growth supernatants of iron-starved cells (Champomier-Verges *et al.*, 1996).

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* produce 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).

Staphyloferrin A, a carboxylate-type of siderophore has been isolated and chemically characterized from both *Staphylococcus aureus* and the coagulase-negative

staphylococci (Konetschny-Rapp *et al.*, 1991). More recently, staphyloferrin B, which is produced by a large variety of staphylococci strains, has been isolated and characterized from *S. hyicus* DSM 20459 (Hoog *et al.*, 1994).

Exochelin and mycobactin are iron-binding compounds synthesized by mycobacteria (Ratledge *et al.*, 1982; Barclay and Ratledge, 1983; Hall and Ratledge, 1987). Exochelin is a water soluble compound that is capable of solubilising iron and transporting it into the cell (Hall and Ratledge, 1987) and mycobactin acts as an iron storage compound (Ratledge *et al.*, 1982). A 29 kDa iron-repressible envelope protein has been observed as the receptor of exochelin (Hall *et al.*, 1987).

1.6. High affinity iron transport system and virulence.

Since iron is essential for microbial growth but levels of free iron *in vivo* are below microbial requirements, pathogens attempting to establish infections must be able to adapt effectively to iron-limited environments. The role of iron transport systems in virulence is well illustrated in the case of *E. coli* carrying ColV plasmids. Smith and Huggins (1976) showed that loss of the plasmid rendered strains less pathogenic for chickens, mice or colostrum-deprived calves than the corresponding ColV⁺ strain, while transfer of a ColV plasmid to a ColV⁻ strain enhanced its virulence. Colicin V itself is not required for virulence (Quackenbush and Falkow, 1979), but an iron uptake system specified by ColV plasmids was identified as an important component in the virulence of invasive strains of *E. coli* (Williams, 1979). This system was shown to be mediated by a hydroxamate siderophore (Stuart *et al.*, 1980) subsequently identified as aerobactin (Warner *et al.*, 1981). Furthermore, the aerobactin-mediated iron uptake system is far more prevalent in *E. coli* isolated from

extraintestinal infections than from the faeces of healthy individuals whether of human or veterinary origin (Montgomerie *et al.*, 1984; Carbonetti *et al.*, 1986; Linggood *et al.*, 1987, Jacobson *et al.*, 1988). A high incidence of clinical isolates of *Enterobacter*, *Escherichia* and *Shigella* were found to be aerobactin producers (Martinez *et al.*, 1987; Bindereif and Neilands, 1985).

A correlation between virulence and aerobactin synthesis has also been observed in *Klebsiella pneumoniae*. Highly virulent strains all produced aerobactin and grew well in the presence of transferrin. Aerobactin non-producers, on the other hand, were relatively avirulent and were unable to grow in transferrin containing medium, despite the fact that they produced enterochelin (Nassif and Sansonetti, 1986).

By contrast, a *Shigella* mutant that was unable to make aerobactin and failed to grow in low iron media was not significantly less virulent than the parent. The mutant retained invasiveness for HeLa cells and caused keratoconjunctivitis in the guinea pig, although a low inoculum of the mutant had reduced ability to evoke keratoconjunctivitis or produce lesions in rabbit ligated ileal loops (Nassif *et al.*, 1987). A slight reduction in lethality of the mutant for chicken embryos was observed (Lawlor *et al.*, 1987). Furthermore, it was observed that *Shigella* did not utilize ferritin, but both the wild type and the mutant were found to utilize haem *in vitro* for growth in low iron media. The ability to obtain iron from transferrin and lactoferrin was also determined; the wild type aerobactin producing strain, but not the aerobactin deficient mutant, grew in low iron medium in the presence of these iron-binding proteins (Lawlor *et al.*, 1987). These data suggest that production of aerobactin may be important during extracellular multiplication of *Shigella*, but is not required for invasion and intracellular growth.

Recently, a method for determining the relative availability of transferrin-bound iron and cell-derived iron to microbial iron-scavenging mechanisms has been described. The results showed that *E. coli* strains that produced enterochelin but not aerobactin acquired predominantly transferrin-bound iron, whereas *E. coli* strains that produced aerobactin but not enterochelin showed a preference for cell-derived iron (Brock *et al.*, 1991). These results may help to explain why production of aerobactin, despite its relatively low affinity for iron, is more directly associated with invasiveness in *E. coli*.

Yancey *et al.* (1979) reported that an enterochelin defective mutant of *Salmonella typhimurium*, that failed to grow in human serum in the absence of added iron or enterobactin, was less virulent for mice. However, a more recent study using higher inoculating doses showed that such *ent*⁻ mutants were fully virulent in several mouse strains (Benjamin *et al.*, 1985). *S. typhimurium* is primarily an intracellular pathogen and so, as in the case of *Shigella*, enterochelin may be important for extracellular multiplication, but once the bacteria are within host cells, siderophores are no longer required for intracellular survival or multiplication (Payne, 1988).

In addition, Fernandez-Beros *et al.* (1989) reported that the serum of patients with typhoid fever exhibited an immunoglobulin G response to iron-deprivation-induced proteins (83kDa, 78 kDa and 69kDa); bioassay and DNA hybridization tests showed that pathogenic strains of *S. typhi* produced enterochelin but not aerobactin. The assumption that the enterochelin system plays a role in the pathogenesis of typhoid fever is supported by the data of Furman *et al.* (1994), who found that the growth of *S. typhi* mutants defective in enterochelin synthesis or enterochelin transport was inhibited in the presence of human sera and unsaturated transferrin, and restored by

fully saturated transferrin. The mutants exhibited decreased ability to grow in HeLa cell monolayers and their LD₅₀ values were increased in a mouse mucin model.

It has been reported that vibriobactin is not an important virulence factor for *Vibrio cholerae* (Sigel *et al.*, 1985; Griffiths, 1987b), while another group reported that a mutant showing reduced virulence in a new-born mouse model was defective in the expression of a 77 kDa iron regulated outer membrane protein homologous with the *E. coli* enterochelin receptor (Goldberg *et al.*, 1990).

Yersinia pestis is a highly virulent pathogen, but it apparently does not produce any siderophores. In contrast, other *Yersinia* species have been shown to produce siderophores, and in at least one case, a mouse lethal strain of *Y. enterocolitica*, the siderophore plays a role in virulence; mutants which do not produce the siderophore were not lethal for mice (Heesemann, 1987).

1.7. 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 (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 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, 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* were affected by iron availability and most (34) of these were under the control of Fur. Although many of

the Fur-dependent proteins were under negative control, a significant proportion (15 of 34) appeared to be under a form of positive control. Surprisingly, not all iron-regulated proteins were controlled by Fur and not all Fur-dependent proteins were 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 (Neiederhoffer *et al.*, 1990; Tardat and Touati, 1991; Compan and Touati, 1993).

1.8. 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 Φ 80, and for colicin M and the antibiotic albomycin. 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). 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; Van de 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), (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.* (1978) 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; Oudega *et al.*, 1977; Konisky, 1982).

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 the product of genes *tolQ*, *tolR* and *tolA* were, as expected for this group, 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), whose products are required for the uptake of group A colicins, including cloacin DF13 (Levenggod *et al.*, 1991; 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 1.3).

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 result 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 (Levengood *et al.*, 1993). TolA is strictly required for the action of all group A colicins (Levengood *et al.*, 1991).

1.9. Scope of this research.

The investigation of the pathogenesis of infectious diseases at a molecular level is an important field of research; it is hoped that a clearer understanding of the molecular basis of pathogenicity will suggest improved methods for the diagnosis, treatment and prevention of infections. The ability of pathogenic bacteria to proliferate *in vivo* and to resist host defences is essential for any infection. Siderophore systems are frequently associated with bacterial virulence because of the capability of siderophores to scavenge iron in severely iron-limited environments. Aerobactin is a siderophore produced by bacterial strains of the family *Enterobacteriaceae*, which has been shown to be important in the extracellular phases of pathogenicity. However, within the family, there is considerable variation in the structure of the receptor protein, such that its mobility in SDS-PAGE varies from an apparent molecular mass of 74 kDa in *E. coli* ColV-K30 strains (Bindereif *et al.*, 1982) to 85 kDa in *Enterobacter cloacae* (Crosa *et al.*, 1988; Krone *et al.*, 1985). The receptor protein in enteroinvasive *E. coli* and *Shigella* species appears to be 76 kDa (Derbyshire *et al.*, 1989; Griffiths *et al.*, 1985; Marolda *et al.*, 1987).

The aim of this research was to characterize aerobactin receptor function and to compare aerobactin receptor gene structure in *E. coli* ColV-K30 and *Shigella* species. The receptors in these two species have different molecular weights and show different sensitivity to cloacin DF13. The aerobactin genes of *S. flexneri* serotype 2a, *S. flexneri* serotype 6 and *S. sonnei* have been cloned to generate recombinant plasmids pLG2130, pLG2131 and pLG2132 respectively. The restriction maps of these clones were identical and so plasmid pLG2131 was chosen for further experiments. The aerobactin receptor gene (*iutA_{Sh}*) was subcloned from pLG2131 and sequenced. Hybrid *iutA_{Sh}::iutA* genes were constructed by replacing the 5'-terminus, the middle or the 3'-terminus fragments of *iutA_{Sh}* with the homologous fragment of *iutA*, and the cloacin sensitivity and the aerobactin uptake associated with these constructs were analyzed.

CHAPTER 2

CLONING THE AEROBACTIN GENES OF *SHIGELLA* spp.

2.1. INTRODUCTION

Shigella spp. are the causative agents of bacillary dysentery. The organism was first isolated in 1896 by the Japanese microbiologist, Kiyoski Shiga (Keusch, 1982). There are four species, *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. *Shigella* are very closely related to *E. coli* and cannot be distinguished on the basis of DNA homology (Brenner *et al.*, 1973). For humans, the infecting dose of *Shigella* varies from 10 to 100 organisms, in contrast to the relatively high infecting dose (10^5 cells) required for *Salmonella* food-poisoning (Dupont and Hornick, 1973). *Shigella* species are strictly enteroinvasive; they enter the human host by the faecal-oral route and proceed through the stomach to the ileum and colon, where they interact with the intestinal mucosa; they spread to basal surface of epithelial cells of the colon and cause extensive tissue damage (ulceration), fluid secretion and inflammation, producing the characteristic clinical manifestation of dysentery, diarrhoea with blood and mucus (Finlay and Falkow, 1989). There are three stages in the process of invasion, colonization, penetration and post-invasion proliferation (Williams *et al.*, 1988). Invasive bacteria usually colonize the animal mucosa before crossing mucosal barriers to enter into host cells, so that colonization factors are important for the process of invasion.

Pathogenic bacteria which follow a faecal-oral route must be able to survive in

variable conditions in the host (such as the extremely low pH of stomach acid, high concentrations of bile salts, digestive enzymes, etc.) and to establish themselves in the intestine, despite the presence of competitive bacterial populations. In this phase, bacteriocins may play a part in inhibiting resident bacteria, but they are not absolutely required.

The first major interaction between a pathogenic microorganism and its host entails attachment to a eukaryotic cell surface. Some organisms such as *V. cholerae* multiply at and remain on the surface, but other organisms use attachment as the first essential step before proceeding to deeper tissues or other locations. In its simplest form, microbial adherence requires the participation of two factors, a receptor and an adhesin. The receptors so far defined are usually specific carbohydrate residues on the eukaryotic cell surface. The bacterial adhesin is typically a protein structure on the bacterial cell surface which interacts with the host cell receptor (Finlay and Falkow, 1989).

When invasive bacteria bind to the host cell surface, condensation of filamentous actin (F-actin) and myosin into microfilaments occurs beneath the site of adherence. Microfilament rearrangements result in formation of pseudopods that engulf the bacteria and bring them inside the host cell within a phagocytic vesicle (Vosselon *et al.*, 1991). Many genes involved in invasion of cultured cells by *Shigella flexneri* have been identified; they are termed *ipaABCD* (invasion plasmid antigens) (Buysse *et al.*, 1987). Transposon insertions in *ipaA* have no effect on invasiveness (Watanabe *et al.*, 1990), *ipaD* mutants are less adherent to tissue culture cells than wild-type shigellae and *ipaB* and *ipaC* are essential for expression of the invasive phenotype (Sasakawa *et al.*, 1989). Proteins IpaB (62 kDa) and IpaC (43 kDa) are exported to

the bacterial surface, and release into the extracellular fluid is triggered by contact with epithelial cells (Watasai *et al.*, 1995). The export process and/or assembly of IpaB and IpaC at the bacterial surface require the *mxi* (membrane expression of Ipa) gene products (Hromockyj and Maurelli, 1989; Andrews *et al.*, 1991; Allaoui *et al.*, 1993). After uptake by the host cell, the bacteria rupture the wall of the vesicle and release themselves into the cytoplasm. IpaB may be responsible for this process, because when a mutant lacking IpaB was incubated with a macrophage-like cell line, the mutant could not escape the phagocytic vesicle. Once in the cytoplasm, bacteria multiply rapidly and spread to adjacent cells. This process requires an outer membrane protein, IcsA (intracellular spread; also called VirG) which promotes actin condensation at one end of bacterium. Bacteria are propelled through the cytoplasm by actin tails, and pushed into the adjacent host cells, forming protrusions from which they eventually escape (Bernardini *et al.*, 1989; Makino *et al.*, 1986; Goldberg *et al.*, 1993). A protein called IcsB appears to be involved in lysis of the protrusions (Allaoui *et al.*, 1992).

There are many specific tests for invasiveness, including the guinea-pig keratoconjunctivitis assay devised by Sereny (1957), infection of chick embryos, and the rabbit ligated ileal loop model. When invasive bacteria survive the host immune system, they proliferate at the site of infection and then spread throughout the body to other sites.

A generalised host response to bacterial invasion is reduction of the amount of iron in the blood plasma (Weinberg, 1978). In order to survive in this environment, many microorganisms synthesize siderophores capable of competing with host iron-binding proteins for the iron they require for growth (Hider, 1984). Aerobactin,

which is an hydroxamate siderophore, is considered to be a virulence determinant of *Shigella* (Hale, 1991). The aerobactin genes were found in all *Shigella* species except *S. dysenteriae* type 1; the genes are located on the *Shigella* chromosomal DNA, and share considerable homology with the aerobactin genes of the *E. coli* plasmid ColV (Lawlor and Payne, 1984). The presence of lactoferrin in the mucosal secretions limits the availability of iron at the mucosal surface, and so it is assumed that the role of aerobactin in the pathogenesis of *Shigella* is in the initial stage during adherence, rather than in the intracellular phase of infection (Williams *et al.*, 1988). It has been demonstrated that the ability of aerobactin-negative mutants to invade and multiply within HeLa cells is similar to that of wild-type strains. These data indicate that an adequate pool of iron is available under the reducing conditions prevalent in the mammalian cell cytoplasm or that haem-iron is sufficient to satisfy the requirements for intracellular bacterial growth in the absence of siderophore expression (Payne, 1989). Whatever the explanation, it is generally assumed that siderophores are not required in the intracellular phase.

As has been mentioned in chapter I, there are two distinct differences between IutA of ColV-K30 and the aerobactin receptor of *Shigella*. First, IutA of ColV-K30 exists as 74 kDa protein on SDS-PAGE while the IutA homologue of *Shigella* appears as 76 kDa. The second difference concerns the function of IutA as the receptor of cloacin DF13; it has been observed that *Shigella* expressing IutA was less sensitive than *iutA*⁺ *E. coli* ColV-K30. The aim of this project was to look at the molecular nature of these differences. This chapter describes the molecular cloning and analysis of the aerobactin genes in *Shigella*.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains and plasmids.

Strain AN1937 is a FepA⁺ strain of *E. coli*, while strain LG1522 is a FepA⁻ derivative of AN1937 carrying an *iuc* derivative of plasmid ColV-K30 (Carbonetti and Williams, 1984). DH5 α is an *E. coli* strain used for transformations. F205 is an *E. coli* strain carrying plasmid pJPN73 which produces cloacin DF13. *Shigella flexneri* serotype 2a is a wild type strain which produces aerobactin, does not produce enterochelin but gives a positive result in the hybridization test with enterochelin genes. *Shigella flexneri* serotype 6 and *Shigella sonnei* are wild type strains; both produce aerobactin and enterochelin.

Recombinant plasmid pABN1 contains a 16.3 kb *Hind*III fragment of plasmid ColV-K30 cloned into the vector pPlac, which expresses the complete aerobactin system (Bindereif and Neilands, 1983). Plasmid pUC19 is a 2.69 kb vector which contains, multi-cloning site in a fragment of the *lacZ* gene capable of complementary defective *lacZ* genes in certain bacterial strains (Yanisch-Perron *et al.*, 1985) (Table 2.1).

2.2.2. Culture media.

Bacteria were grown in either nutrient broth (Oxoid no. 2) or M9 minimal salts medium (Roberts *et al.*, 1963) containing 0.2% w/v glucose, 0.5% w/v casamino acids and 20 μ g/ml tryptophan. Ampicillin (100 μ g/ml) or chloramphenicol (10 μ g/ml) were included in media to select for the presence of pABN1 or pLG141 respectively. α, α' -dipyridyl (200 μ M) was added as required to limit availability of

Table 2.1: Bacterial strains and plasmids used.

Bacterial strains & plasmids	Relevant properties	Reference
<i>E. coli</i> K-12		
W3110	Wild type	Bachmann, 1972.
BZB1022	W3110, <i>cir</i> , <i>gyrA</i> (Nal ^R)	Pugsley, 1985.
AN1937	<i>ara entA lac leu mtl proc</i> <i>rpsL supE thi fhuA xyl</i>	Williams, 1979.
LG1522	An1937, <i>fepA</i> (pColV-K30, <i>iuc</i>)	Carbonetti and Williams, 1984.
DH5 α	<i>supE44 lacU169</i> (80 <i>lacZ</i> M15 <i>hsdR17 recA1 gyrA96</i> <i>thi-1 relA1</i>	Hanahan, 1983.
F205		Graaf <i>et al.</i> , 1969
<i>Shigella flexneri</i> serotype 2a	Wild type	Struelens <i>et al.</i> , 1990.
<i>Shigella flexneri</i> serotype 6	Wild type	Struelens <i>et al.</i> , 1990.
<i>Shigella sonnei</i>	Wild type	Struelens <i>et al.</i> , 1990.
Plasmids:		
ColV-K30	<i>iutA</i> ⁺ <i>iuc</i> ⁺ <i>cva</i> ⁺	Williams, 1979.
pABN1	<i>iutA</i> ⁺ <i>iucA</i> ⁺ <i>bla</i> ⁺ (Ap ^R)	Bindereif and Neilands, 1983.
pLG141	<i>iutA</i> ⁺ <i>cat</i> ⁺ (Cm ^R)	Carbonetti and Williams, 1984.

pUC19	Ap ^R	Yanisch-Peron <i>et al.</i> , 1985.
pLG2130	Cloned aerobactin genes of <i>S. flexneri</i> ser.2a in pUC19	This study.
pLG2131	Cloned aerobactin genes of <i>S. flexneri</i> ser.6 in pUC19	This study.
pLG2132	Cloned aerobactin genes of <i>S. sonnei</i> in pUC19.	This study.
pLG2133	<i>KpnI</i> fragment subcloned of pLG2131 in pUC19	This study.
pLG2135	<i>HincII</i> fragment subcloned of pLG2131 in pUC19	This study.
pJPN73	Cloacin DF13 producer	Graaf <i>et al.</i> , 1969.

iron. Solid media contained 1.5% w/v agar (Difco).

2.2.3. Bioassay for siderophore production.

Siderophores were detected by the bioassay described by Carbonetti and Williams (1984). Indicator strains *E. coli* AN1937 and LG1522, for the detection of enterochelin and aerobactin respectively, were grown overnight in nutrient broth, diluted 1:100 in PBS (0.8% w/v NaCl, 0.2% w/v KCl, 0.02% w/v KH₂PO₄, 0.12% w/v Na₂HPO₄) and 200µl were spread over the surface of an M9 agar plate containing dipyriddy. Strains to be tested were stab inoculated onto the plate and incubated overnight at 37°C. Production of a siderophore was indicated by the presence of a halo of growth of the indicator strain around the point of inoculation.

2.2.4. Preparation of cloacin DF13.

Cloacin DF13 was prepared essentially as described by de Graaf *et al.* (1969). A 10 ml culture of *E. coli* strain F205 (pJPN73) grown overnight at 37°C in nutrient broth containing ampicillin was used to inoculate 500 ml of prewarmed (37°C) nutrient broth containing ampicillin. After 20 min incubation at 37°C with vigorous agitation, mitomycin-C (Sigma) was added to a final concentration of 25 ng/ml and incubation was continued for a further 2 h. Cells were pelleted by centrifugation at 10,000 x g, and 121.5 g of ammonium sulphate were dissolved in the supernatant to produce a 40% saturated solution. After stirring overnight at 4°C the solution was subjected to centrifugation at 15,000 x g for 20 min at 4°C to remove precipitated proteins. A further 66 g of ammonium sulphate were added to increase the saturation to 60% and the solution was again stirred overnight at 4°C. The solution was

centrifuged as described above and the supernatant fraction was discarded. The pellet was redissolved in 10 ml of 50mM Tris-HCl (pH 8.0), 20 mM EDTA and dialysed overnight at 4°C against 4 l of the same buffer. The solution was sterilized by filtration through a 0.22 μ m diameter pore size filter (Millipore).

2.2.5. Cloacin sensitivity assay.

The strain to be tested was grown overnight in nutrient broth, diluted 1:100 in PBS and poured over the surface of an M9-dipyridyl agar plate. Surplus fluid was poured off and the plate dried inverted at 37°C. Samples (15 μ l) of serial dilutions of cloacin were spotted onto the plate, allowed to dry and incubated at 37°C overnight. Zones of clearing in the region at the spots indicated sensitivity to cloacin, for example due to the expression of the aerobactin receptor protein IutA.

2.2.6. Plasmid DNA preparation.

DNA was prepared by a method based on that of Morelle (1990). 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 μ l of 50 mM glucose, 25 mM Tris.HCl (pH8.0), 10mM EDTA and incubated at room temperature for 5 min. 400 μ l of 0.2 M NaOH, 1% SDS were added, and the mixture was incubated on ice for 5 min. 300 μ l of 3M sodium acetate (pH5.0) 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 μ l of propan-2-ol were 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 μ l of water to which 200 μ l of 4.4M LiCl were 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 3M sodium acetate (pH 5.0) and 2 volumes of absolute ethanol, and incubation on ice for 15 min. The precipitate was harvested by centrifugation at 10,000 x g for 10 min. The final pellet was resuspended in 50 μ l of sterile distilled water containing 0.1 mg/ml RNase A (Sigma). 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 μ l of 10 mM Tris HCl (pH8.0), 1mM EDTA (TE).

2.2.7. Preparation of DNA Probes.

Plasmid DNA was cleaved with an appropriate restriction enzyme and the DNA fragments separated in a 0.8% low gelling temperature agarose (FMC Sea Plaque LGT) gel. The gel was examined using a u.v. transilluminator, and the required bands were excised with a scalpel; gel slices were placed in a preweighed 1.5 ml Eppendorf tube, and water was added in the ratio 1.5 ml per gram of agarose.

The mixture was boiled for 7 min and stored at 20°C. Prior to labelling, aliquots were boiled again for 3 min and placed at 37°C. The following solutions were added in order to a 1.5 ml Eppendorf tube: 5.5 μ l H₂O, 5 μ l OLB (Oligolabelling buffer with Pharmacia hexadeoxyribonucleotides), 1 μ l bovine serum albumin (BSA, Pharmacia enzyme grade; 10 mg/ml stock), 10 μ l DNA fragment, 2.5 μ l [³²P]dCTP

(Amersham, 0.55 MBq at 110 TBq/mMol), 1 μ l Klenow fragment (Pharmacia, 1 Unit/ μ l). The contents were mixed by gently tapping the tube and the polymerization reaction was allowed to proceed at 37°C for 30 min or at room temperature overnight.

2.2.8. Colony DNA Blot.

Nylon hybridization transfer membranes (82 mm diameter disc; Hybond N, Amersham) were placed on the surface of nutrient agar plates and the strains to be tested were spot inoculated onto the surface. The plates were incubated at 37°C overnight to allow colonies to grow. The filters were removed and placed, colony side up, on a piece of Whatman 3MM paper previously soaked in 0.5M NaOH and drained. After 7 min the filter was removed and the process was repeated with 0.1M NaOH, 1.5M NaCl for 10 min, with 1M Tris for 2 min and with 1.5M NaCl, 0.5M Tris-HCl pH7.4 for 4 min. The filters were washed vigorously in 2x SSC (1x SSC is 0.15M NaCl, 0.015M trisodium citrate) and dried on Whatman 3MM paper at room temperature. The filters were wrapped in a single layer of plastic film and placed colony side down on an ultraviolet transilluminator for 5 min to allow the chromosomal DNA to stick onto the filter.

2.2.9. Chromosomal DNA preparation.

Bacterial strains were grown overnight in nutrient broth containing 0.5% 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 STE (15% sucrose, 50 mM Tris-HCl pH 7.5, 50 mM EDTA) containing 10 mg of solid lysozyme and incubated for 5 min at room temperature. 2 ml of TE (10 mM

Tris-HCl pH 8.0 and 1 mM EDTA) containing 10% w/v SDS were added, mixed gently and incubated for 15 min at room temperature. An equal volume of phenol/chloroform mixture 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 3M Na 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, extracted with phenol/chloroform followed by ethanol precipitation as described above, and redissolved in 300 μ l of TE.

2.2.10. DNA-DNA Hybridizations.

Filters were placed in Perspex hybridization chambers. All washings were performed in prewarmed (65°C) solutions in a shaking water bath at 65°C. Filters were washed in hybridization solution (0.5M Na₂HPO₄H₃PO₄ pH 7.2, 1mM EDTA, 7% w/v SDS) for 30 min, and placed in a minimal volume of fresh hybridization solution with the appropriate DNA probe, and incubated at 65°C overnight. The filters were washed three times for 15 min each in 0.4M Na₂HPO₄H₃PO₄, pH 7.2, air dried, and autoradiographed on X-Ray film.

2.2.11. Immunoblotting.

Outer membrane protein preparations were mixed with equal volumes of SDS-PAGE

sample buffer, boiled for 5 min, loaded onto an 11% SDS polyacrylamide gel and electrophoresed at 35 mA for 4 hours. The proteins were transferred to nitrocellulose paper (Hybond C, Amersham) by the method of Towbin *et al.* (1979). The gel and its attached nitrocellulose filter were sandwiched between pieces of Whatman 3MM paper. This sandwich was then placed between electrodes, with the nitrocellulose filter on the anodic side, in the transfer apparatus which contained transfer buffer (39 mM glycine, 48 mM Tris base, 0.027% SDS, 20% methanol). Transfer of the protein was carried out at room temperature for 1.5 - 2 h with a current of 0.65 mA/cm². To check for efficient transfer of proteins the filter was stained with Ponceau solution {Ponceau S [3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2, 7-naphthalenedisulfonic acid] (Sigma) 0.2%, trichloroacetic acid 3% and sulfosalicylic acid 3%} for 5 to 10 min with gentle agitation. When the bands of protein were visible, the nitrocellulose filter was washed in several changes of deionized water at room temperature, and the positions of proteins were marked. The filter was incubated at 4°C overnight in PBS-Tween (0.05% v/v Tween 20 in PBS), reacted with primary antiserum raised against Triton/urea-soluble outer membranes of *E. coli* strain B2B1022 (pABN1) (Roberts *et al.*, 1989), appropriately diluted in PBS-Tween for 30 min at room temperature and washed four times over 40 min with PBS-Tween. The filter was reacted with secondary antiserum (DAKO swine anti-Rabbit) diluted 1:800 in PBS-Tween for 30 min at room temperature, and washed as above. The filters were then incubated with rabbit antihorseradish peroxidase (PAP; DAKO) diluted 1:800 in PBS-Tween and washed as above. The filter was stained for 2 min in 30 mg of 4-chloro 1-naphthol in 20 ml of methanol to which 50 ml 50 mM Tris.HCl, pH 7.6, and 100 μ l H₂O₂ (100 vol) had been added immediately before use.

The filter was rinsed in water and air dried.

2.2.12. Cloning strategy.

Vector plasmid DNA was digested with appropriate restriction enzymes. When digestion was complete, the plasmid DNA was extracted with phenol-chloroform, precipitated with 2 volumes of absolute ethanol for 15 min at 0°C, recovered by centrifugation at 12,000 x g for 10 min at 4°C, and redissolved in 90 μ l of 10mM Tris.HCl (pH8.3). 10 μ l of 10x CIP (Calf intestinal alkaline phosphatase) dephosphorylation buffer {10 mM ZnCl₂, 10 mM MgCl₂, 100 mM Tris.HCl (pH8.3)} and the appropriate amount of CIP were added to the linear plasmid DNA and incubated for 30 min at 37°C. At the end of the incubation, SDS and EDTA (pH8.0) were added to final concentrations of 0.5% and 5 mM respectively and mixed well. Proteinase K was added to a final concentration of 100 μ g/ml and incubated for 30 min at 56°C. The mixture was cooled to room temperature and extracted once with phenol and once with phenol-chloroform. 0.1 volume of 3M sodium acetate (pH7.0) and 2 volumes of ethanol were added, mixed well and stored at 0°C for 15 min. The DNA was recovered by centrifugation at 12,000 x g for 10 min at 4°C. The pellet was washed with 70% ethanol at 4°C, centrifuged and redissolved in TE (pH7.6).

A DNA mixture containing the same amount of dephosphorylated plasmid DNA and *Shigella* chromosomal DNA fragments was added to 1 μ l of 10x ligation buffer (0.5 Tris.HCl (pH7.6), 100 mM MgCl₂, 100 mM dithiothreitol and 500 μ g/ml bovine serum albumin), 1 μ l of 10 mM ATP and H₂O to 10 μ l. Finally, T4 DNA ligase was added to this mixture and incubated for 4-8 h at 16°C.

2.2.13. Transformation.

Plasmid DNA was introduced into recipient cells by the method of Hanahan (1983). Recipient cells were grown in nutrient broth 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) (pH7.0), 10 mM RbCl and harvested by centrifugation as above. Then cells were resuspended in 1 ml of 100 mM MOPS (pH6.5), 50 mM CaCl₂, 10 mM RbCl and incubated on ice for 15 min. Cells were harvested as above and resuspended in 200 µl of 100 mM MOPS (pH6.5), 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 nutrient broth 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 purified for further analysis.

2.2.14. Southern blot analysis.

This was carried out by the method of Southern (1975). DNA was digested with restriction enzymes according to the manufacturers' conditions, and loaded in an 0.8% agarose gel in electrophoresis buffer (40 mM Tris acetate, pH 7.7, 1mM EDTA, 0.5 µg/ml ethidium bromide). Electrophoresis was for about 3 h at constant voltage (70 V). Before blotting, the gel was photographed with a ruler beside it. The gel was washed in 0.25 M HCl for 7 min with gentle shaking, rinsed briefly in distilled water, washed for 30 min in 250 ml of denaturing solution (0.5M NaOH,

1.5M NaCl), rinsed again in distilled water and washed for 30 min in 250ml of neutralizing solution (3M NaCl, 0.5M Tris-HCl, pH7.4).

The blotting apparatus was prepared by placing a glass plate across a large plastic tray, with a sheet of Whatman 3MM paper over it with the ends hanging into the tray; 20X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) was poured into the tray. The neutralized gel was placed onto the blotting apparatus, and the Whatman paper surrounding the gel was covered with clingfilm. Nylon blotting membrane and a sheet of Whatman No:1 filter paper were soaked in 20X SSC. The nylon blotting membrane was placed directly on the gel, followed by the Whatman paper. Paper towels were placed on top followed by a glass plate and a weight. Blotting was allowed to proceed for 3-20 h, after which the membrane was removed from the gel and rinsed briefly in 3X SSC, dried with filter paper at room temperature and wrapped in clingfilm. The wrapped membrane was placed on a u.v. transilluminator, DNA side down, for 15 sec to 4 min in order to allow the DNA to stick to the filter.

2.2.15. Double stranded DNA sequencing.

Double stranded DNA sequencing was carried out by the chain termination method of Sanger *et al.* (1977) using a Sequenase™ version 2.0 kit (United States Biochemical Corporation) and protocols recommended by the manufacturer for reading sequences close to the primer. 5 µg of plasmid DNA were denatured in 0.2 M NaOH, 0.2mM EDTA for 30 min at 37°C. The mixture was neutralized by adding 0.1 volume of 3 M sodium acetate (pH 5.0) and the DNA was precipitated by addition of 3 volumes of ethanol at -70°C for 15 min. DNA was pelleted by centrifugation at 10,000 x g for 10 min, washed with 70% ethanol and redissolved

in 7 μ l of distilled water, 2 μ l of 200 mM Tris.HCl (pH7.5), 100 mM $MgCl_2$, 250 mM NaCl (5 x Sequenase TM reaction buffer) and 1 μ l of primer. The mixture was incubated at 65°C for 2 minutes and allowed to cool below 35°C over a period of about 30 min. The tube was placed on ice and the following were added: 1 μ l of 0.1 M DTT (dithiothreitol), 1 μ l of labelling mix (7.5 μ M each of dGTP, dCTP and dTTP) diluted 1:14 in water, 0.5 μ l of [$\alpha^{35}S$]dATP (approximately 10 μ Ci/ μ l), 1 μ l of Sequenase TM Version 2.0 (diluted 1:8 in 10mM Tris.HCl, pH7.5, 5 mM DTT, 0.5 mg/ml BSA). After mixing, the solution was incubated for 3 min at room temperature. 2.5 μ l of termination mixes (80 μ M each of dATP, dCTP, dGTP or dTTP, 50 mM NaCl and 8 μ M of an appropriate ddNTP) were added into tubes labelled A,C,G,T and prewarmed to 37°C. 3.5 μ l of the labelling mix were added to each termination mix and incubated at 37°C for 5 min before addition of 4 μ l of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The mixtures were heated to 75°C for 3 min before analysing by denaturing gel electrophoresis using wedge gels composed of 6% acrylamide (19:1 acrylamide: N-methylbisacrylamide) and 8.3 M urea in 45 mM Tris.HCl, 45 mM boric acid (pH8.3), 125 mM EDTA (0.5 x TBE). Running buffer was TBE and electrophoresis was at 60 V for about 3 h. Gels were fixed twice for 15 min each in 10% acetic acid, 12% methanol, dried and exposed to Fuji RX100 X-ray film overnight and developed.

2.3. RESULTS

2.3.1. Identification of the aerobactin genes in *Shigella* spp.

In order to confirm that the *Shigella* strains used in these experiments contained the aerobactin genes, bioassays were carried out on minimum medium, using the indicator strains LG1522 for aerobactin and AN1937 for enterochelin. The results showed growth of strain LG1522 surrounding the inocula of *S. flexneri* serotype 2a, *S. flexneri* serotype 6 and *S. sonnei*, confirming that they all express the aerobactin genes. On the other hand, the growth of strain AN1937 only appeared surrounding the inocula of *S. flexneri* serotype 6 and *S. sonnei*, confirming that only both *Shigella* strains express enterochelin although all of them contain enterochelin genes (Struelens *et al.*, 1990).

It has been mentioned previously that the aerobactin receptor is also the receptor for cloacin DF13, so that the presence of the aerobactin receptor in bacterial strains is indicated by their sensitivity to cloacin DF13. The *Shigella* strains used in these experiments (*S. flexneri* serotype 2a, *S. flexneri* serotype 6 and *S. sonnei*) were all sensitive to cloacin but their sensitivity was significantly lower (turbid zones) than the sensitivity of *E. coli* strain W3110(CoIV-K30) and W3110(pABN1) which showed a clear zone.

In order to identify the location of the aerobactin gene in *Shigella* spp., Southern blot hybridization of chromosomal DNA was carried out using *iutA* gene sequences as probes. The chromosomal DNA was digested with *HindIII* and run on an agarose gel, transferred to Hybond N nylon membrane, and then hybridized with a 2 kb *BglIII* fragment carrying sequences of the *iutA* gene. Autoradiography of filters indicated

that the probe hybridized to 11 kb fragments of *Shigella* chromosomal DNA (Figure 2.1).

Furthermore, the presence of the aerobactin receptor protein was identified in each strain by immunoblotting. The 76 kDa outer membrane proteins of *Shigella* species and the 74 kDa proteins of *E. coli* strains containing the aerobactin gene clones gave a positive reaction with antiserum raised against Triton/urea-soluble outer membranes of *E. coli* strain BZB1022(pABN1) (Roberts *et al.*, 1989) (Figure 2.2).

2.3.2. Cloning of the aerobactin genes from *Shigella* sp.

Chromosomal DNA was digested with *Hind*III, and separated on a 0.8% agarose gel. Fragments of about 11 kb were isolated and ligated at 16°C overnight with pUC19 which had been digested with *Hind*III and treated with calf intestinal alkaline phosphatase, followed by transformation into competent cells of *E. coli* strain DH5 α .

White transformant colonies were tested for their ability to hybridize with the *iutA* gene in colony blots, for aerobactin production in a bioassay, and for sensitivity to cloacin DF13. Colonies that were positive in all three tests were isolated among transformants from all three *Shigella* strains from which chromosomal DNA was isolated. Recombinant plasmids were purified from these transformant strains, digested with *Hind*III and analysed by agarose gel electrophoresis. The results indicated that the recombinant plasmids each contained an 11 kb *Hind*III insert. Restriction maps of these clones were built up by digesting with restriction enzymes (singly or in combination), separation on agarose gels, and analysis by Southern blot hybridization using the *iutA* gene as a probe. The results indicated that clones derived from *S. flexneri* serotype 2a, *S. flexneri* serotype 6 and *S. sonnei* DNA designated



Figure 2.1: Southern blot analysis of *Hind*III digested *Shigella* chromosomal DNA using *E. coli* ColV-K30 *intA* gene as the probe

1. *Hind*III digested cloned of aerobactin gene from *S. flexneri* ser.6.
2. *Pvu*II digested pABN1.
3. *Hind*III digested chromosomal DNA of *Shigella flexneri* ser.2a.
4. *Hind*III digested chromosomal DNA of *Shigella flexneri* ser.6.
5. *Hind*III digested chromosomal DNA of *Shigella sonnei*.

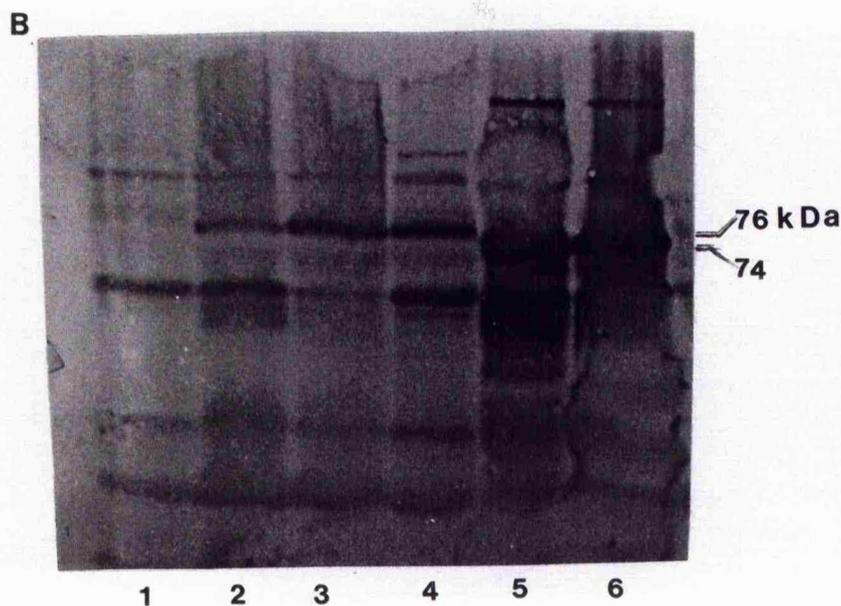
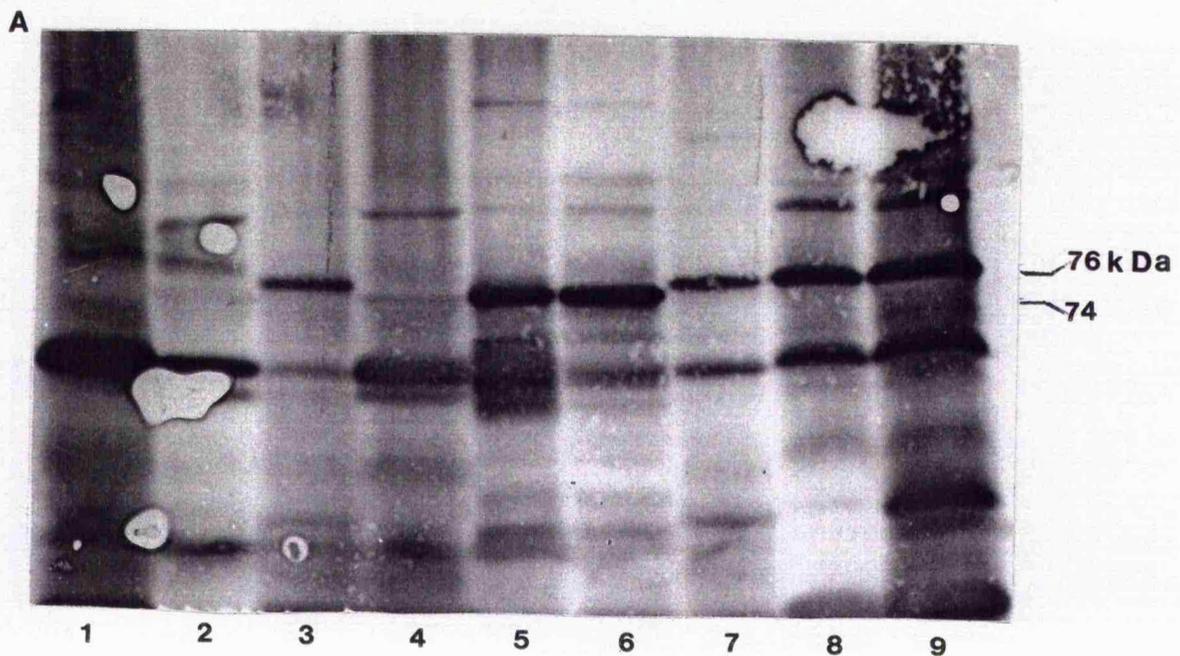


Figure 2.2: Western blot analysis using antibodies raised against triton/urea-soluble outer membrane of *E. coli* expressing IutA of outer membrane proteins of *Shigella* and *E. coli* strains expressing IutA.

- A: 1. DH5 α grown in nutrient broth, 2. DH5 α , 3. DH5 α (pLG2131), 4. W3110(pLG141), 5. BZB1022 (pABN1), 6. W3110(CoIV K30), 7. *S. sonnei*, 8. *S. flexneri* ser. 6., 9. *S. flexneri* ser.2a (2-9 grown in M9-dipyridyl).
- B: 1. DH5 α , 2. DH5 α (pLG2132), 3. DH5 α (pLG2131), 4. DH5 α (pLG2130), 5. BZB1022(pABN1), 6. DH5 α (pABN1), all grown in M9-dipyridyl.

pLG2130, pLG2131 and pLG2132 respectively, had identical restriction maps.

Compared to the aerobactin genes of the ColV-K30 plasmid cloned as plasmid pABN1 (Bindereif and Neilands, 1983), the restriction maps of pLG2130/1/2 were similar over about 7 kb (figure 2.3). The main difference was the existence of a *Bam*HI site downstream of the central *Eco*RI site in the *Shigella* DNA but not in ColV-K30. This *Bam*HI site is located within the aerobactin receptor genes. A 5 kb *Kpn*I fragment of plasmid pLG2131 was subcloned into pUC19 vector and introduced into *E. coli* strain DH5 α ; this subclone is termed pLG2133. *E. coli* strain DH5 α carrying this subclone did not produce aerobactin, but was sensitive to cloacin DF13, indicating that the aerobactin receptor gene of plasmid pLG2131 is located within the subcloned fragment. However, the *iutA* gene of ColV-K30 is located in the central 6.5 kb *Bam*HI fragment of pABN1; this has been subcloned into vector plasmid pACY184 to form a recombinant plasmid designated pLG141 (Carbonetti and Williams, 1984).

2.3.3. DNA sequence of the aerobactin receptor gene from *Shigella flexneri* ser.6.

Small subclones and nested deletions from the *Kpn*I fragment of plasmid pLG2131 were prepared for sequencing. An open reading frame was identified from an ATG triplet at position 211-213 to a TGA triplet at position 2407-2409 (indicated by * in figure 2.4). Upstream of the translation initiation codon was observed a possible ribosomal binding site at position 201-206 (figure 2.4). Two palindromes were observed at positions 2420-2443 and 2444-2469 adjacent to the translation terminating codon (figure 2.5). The nucleotide sequences of *Shigella iutA* (*iutA_{Sh}*) showed a similarity of about 90% to those of *iutA*. The *iutA_{Sh}* gene is located entirely



Figure 2.3: The cloned aerobactin genes of ColV-K30 (A) and of *Shigella* spp. (B). B: *Bam*HI; E: *Eco*RI; H: *Hind*III; G: *Bg*II; Hc: *Hinc*II; K: *Kpn*I; P: *Pst*I; S: *Sma*I; C: *Cl*aI.

— *Bg*II fragment of *iutA* used as a probe.

- - - - - *Kpn*I fragment of *iutA_{SH}* subcloned into pUC19 to form plasmid pIG2133.

- · - · - · *Hinc*II fragment of *iutA_{SH}* which has been sequenced.

```

      10              30              50
GTCAACGCCAGCATGCAAACTCATGGCATCGCGAACCCAGCTCAGCCTGATGGCCTGGC
-----+-----+-----+-----+-----+-----+-----+-----+
CAGTTGCGGTTCGTACGTTTGTAGTACCGTAGCGCTTGGGGTCGAGTCGGACTACCGGACCG
      70              90              110
GATCTGCACGTATTCTTAATCGCGTACTGGGGCGTGATTTATTCGATCTCAGTATGCCGC
-----+-----+-----+-----+-----+-----+-----+-----+
CTAGACGTGCATAAGAATTAGCGCATGACCCCGCACTAAATAAGCTAGAGTCATACGGCG
      130             150             170
CCGCACTGATTCAGTGGCGCACGGCAGCCGGAAAAAACCGCAGCCGGACGTTTCTTTAAC
-----+-----+-----+-----+-----+-----+-----+-----+
GGCGTGAATAAGTCACCGCGTCCCGTCCGCCCTTTTGGCGTCGGCCTGCAAAGAAATTG
      190             210             230
TCACTATACAGCAAATATTAGGAATAACGATGATGCGCAAAAAGTATATGCCCCGGGCT
-----+-----+-----+-----+-----+-----+-----+-----+
AGTGATATGTCGTTTATAAGTCCTTATTGCTACTACGCGTTTTTCATATACGGGGCCGA
      250             270             290
                                M M R K K Y M P R A
CTTGGTCCGCTGCTTCTTGTGCTGCTGCCAGCTGTCGCCAGCAAAAACGATGATAAT
-----+-----+-----+-----+-----+-----+-----+-----+
GAACAGGCGACGAAGAACAGCAGCAGAGTGGTCGACAGCGGGTCGTTTGTACTATTA
L G P L L L V V L S P A V A Q Q N D D N
      310             330             350
GAGATCATAGTGTCTGCCAGCCGAGCAATCGAACTGTAGCGGAGATGGCGCAAACCACT
-----+-----+-----+-----+-----+-----+-----+-----+
CTCTAGTATCACAGACGGTCGGCGTCGTTAGCTTGACATCGCCTCTACCGCGTTTGGTGA
E I I V S A S R S N R T V A E M A Q T T
      370             390             410
TGGATTATCGAAAAATGCCGAACTGGAGCAGCAGATTCAGGGCGGTAAGAGCTGAAAGAC
-----+-----+-----+-----+-----+-----+-----+-----+
ACCTAATAGCTTTTACGGCTTGACCTCGTCTAAGTCCCGCCATTTCTCGACTTCTG
W I I E N A E L E Q Q I Q G G K E L K D
      430             450             470
GCACTGGCTCAGTTAATCCCGGCCCTTGATGTCAGCAGCCAGAGTCGAACCAACTACGGT
-----+-----+-----+-----+-----+-----+-----+-----+
CGTGACCGAGTCAATTAGGGGCCGGAACACTACAGTCGTCGGTCTCAGCTTGGTTGATGCCA
A L A Q L I P G L D V S S Q S R T N Y G
      490             510             530
ATGAACATGCGTGGCCGCCCGCTGGTTGTCCTGATTGACGGTGTGGCCTCAACTCTTCA
-----+-----+-----+-----+-----+-----+-----+-----+
TACTTGTACGCACCGGGGGGACCAACAGGACTAACTGCCACACGCGGAGTTGAGAAGT
M N M R G R P L V V L I D G V R L N S S
      550             570             590
CGTTCCGACAGCCGACAACTGGACTCTGTGATCCTTTTAAATATCGACCATATTGAAGTG
-----+-----+-----+-----+-----+-----+-----+-----+
GCAAGGCTGTCCGGCTGTTGACCTGAGACAGCTAGGAAAATTATAGCTGGTATAACTTCAC
R S D S R Q L D S V D P F N I D H I E V
      610             630             650

```

ATCTCCGGCGCGACGGCCCTGTACGGTGGCGGGAGTACCGGATGGTTGATCAACATCGTG
-----+-----+-----+-----+-----+
TAGAGGCCGCGCTGCCGGGACATGCCACCGCCCTCATGGCCTACCAACTAGTTGTAGCAC
I S G A T A L Y G G G S T G W L I N I V
670 690 710
ACCAAAAAAGGTGAGCCGGAAACCATGATGGAGTTTGAGGCTGGCACAAAAAGTGGCTTT
-----+-----+-----+-----+-----+
TGGTTTTTTCCAGTCGGCCTTTGGTACTACCTCAAACCTCGACCGTGT'TTTCACCGAAA
T K K G Q P E T M M E F E A G T K S G F
730 750 770
AACAGCAGTAAAAGATCACGATGAGCGCATTGCCGGTGTCTCTCCGGCGAAAATGACCAT
-----+-----+-----+-----+-----+
TTGTCGTCA'TTCTAGTGTACTACTCGCGTAACGGCCACGACAGAGGCCCGCCTTTACTGGTA
N S S K D H D E R I A G A V S G G N D H
790 810 830
ATCTCCGGACGCTCTTTCCGTGGCATATCAGAAATTTGGCGGCTGGTTTGACGGTAACGGC
-----+-----+-----+-----+-----+
TAGAGCCCTGCAGAAAGGCACCGTATAGTCTTTAAACCGCCGACCAAACCTGCCATTGCCG
I S G R L S V A Y Q K F G G W F D G N G
850 870 890
GATGCCACCCTGCTTGATAACACCCAGACCGGCTGCAGCACTCCAATCGGCTGGACATC
-----+-----+-----+-----+-----+
CTACGGTGGGACGAACATTTGTTGGGTCTGGCCGGACGTCGTGAGGTTAGCCGACCTGTAG
D A T L L D N T Q T G L Q H S N R L D I
910 930 950
ATGGGAACCGGTACGCTGAACATCGATGAATCCCGGCAGCTTCAACTGATAACGCAGTAC
-----+-----+-----+-----+-----+
TACCCTTGCCATGCGACTTGTAGTACTTAGGGCCGTCGAAGTTGACTATTGCGTCATG
M G T G T L N I D E S R Q L Q L I T Q Y
970 990 1010
TATAAAAGTCAGGGAGACGACAATTACGGGCTTAATCTCGGAAAGGCTTTTCCGCCATC
-----+-----+-----+-----+-----+
ATATTTTCAGTCCCTCTGCTGTTAATGCCCGAATTAGAGCCCTTTCCGAAAAGGCGGTAG
Y K S Q G D D N Y G L N L G K G F S A I
1030 1050 1070
AGCGGGAGCAGCACACCATAACGTCAGTAAGGGGCTGAATTCGACCGCAT'TCCGGCACT
-----+-----+-----+-----+-----+
TCGCCCTCGTTCGTGTGGTATGCAGTCATTCCCGACTTAAGACTGGCGTAAGGGCCGTGA
S G S S T P Y V S K G L N S D R I P G T
1090 1110 1130
GAGCGGCATTTGATCAGCCTGCAGTACTCTGACAGTGATTTCTTGGGACAGGAACTGGTC
-----+-----+-----+-----+-----+
CTCGCCGTAAACTAGTCGGACGTCATGAGACTGTCACTAAAGGACCCTGTCTT'GACCAG
E R H L I S L Q Y S D S D F L G Q E L V
1150 1170 1190
GGTCAGGTTTACTACCGCGATGAGTCGTTGCGGTACTACCCGTTCCCGACGGTAAATGCG
-----+-----+-----+-----+-----+
CCAGTCCAAATGATGGCGTACTCAGCAACCCATGATGGGCAAGGGCTGCCATTTACCG
G Q V Y Y R D E S L R Y Y P F P T V N A
1210 1230 1250

AATAAACAGGCGACGGCTTTCTCCTCGTCACAGCAGGATACCGACCAGTACGGCATGAAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
TTATTTGTCCGCTGCCGAAAGAGGAGCAGTGTCTCCTATGGCTGGTCATGCCGTA
N K Q A T A F S S S Q Q D T D Q Y G M K
1270 1290 1310

CTGACTCTGAACAGCCAACCTGATGGACGGCTGGCAAATCACCTGGGGGCTGGATGCTGAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
GACTGAGACTTGTCCGTTGACTACCTGCCGACCGTTTAGTGGACCCCGACCTACGACTC
L T L N S Q L M D G W Q I T W G L D A E
1330 1350 1370

CATGAGCGCTTTACCTCCAACCAGATGTTCTTCGATCTGGCTCAGGCAAGTGCTTCCGGÀ
-----+-----+-----+-----+-----+-----+-----+-----+-----+
GTACTCGCGAAATGGAGGTTGGTCTACAAGAAGCTAGACCGAGTCCGTTACGAAGGCCT
H E R F T S N Q M F F D L A Q A S A S G
1390 1410 1430

GGGCTGAACAACCATAAGATTTACACCACCGGGCGCTATCCGTCAATGACATCACCAAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
CCCGACTTGTGGTATCTAAATGTGGTGGCCCGGATAGGCAGTATACTGTAGTGGTTA
G L N N H K I Y T T G R Y P S Y D I T N
1450 1470 1490

CTGGCGCCTTCCTGCAATCCAGCTATGACATTAATGATATTTTTACCGTTAGCGGTGGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
GACCGCCGGAAGGACGTTAGGTCGATACTGTAATTAATAAAAATGGCAATCGCCACCG
L A A F L Q S S Y D I N D I F T V S G G
1510 1530 1550

GTACGCTATCAGTATACTGAGAACAGGGTAGATGATTPCATCGACTACACGCAGCAACAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
CATGCGATAGTCATATGACTCTTGTCCCATCTACTAAAGTAGCTGATGTGCGTTCGTTGT
V R Y Q Y T E N R V D D F I D Y T Q Q Q
1570 1590 1610

AAGATTGCTGCCGGGAAGGCGATATCTGCCGACGCCARRCCTGGTGGTTCCGGTAGATTAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
TTCTAACGACGGCCCTCCGCTATAGACGGCTGCGGTYGGACCACCAAGCCATCTAATG
K I A A G K A I S A D A X P G G S V D Y
1630 1650 1670

GATAACTTTCTGTCAATGCTGGTCTGCTGATGCACATCACCGAACGTCAGCAGGCATGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
CTATTGAAAGACAAGTTACGACCAGACGACTACGTGTAGTGGCTTGCAGTCGTCGGTACC
D N F L F N A G L L M H I T E R Q Q A W
1690 1710 1730

TTCAATTTTTCCAGGGGGTGGCATTGCCGGATCCGGGGAAATATATGTTGTCGGGCATC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
AAGTTAAAAAGGGTCCCCACCGTAACGGCCTAGGCCCTTTATAATACCAGCGCGTAG
F N F S Q G V A L P D P G K Y Y G R G I
1750 1770 1790

TATGGTGCAGCAGTGAACGGCCATCTTTCCCTGACAAAGAGCGTGAACGTCAGCGACAGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
ATACCAGTTCGTCACCTGCCGGTAGAAAGGGACTGTTTCTCGCACTTGCAGTCGCTGTCA
Y G A A V N G H L S L T K S V N V S D S
1810 1830 1850

AAGCTGGAAGGCGTGAAAGTCGATTCTTATGAACTGGGCTGGCGCTTTACCGGTGACAAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
TTCGACCTFCCGCACCTTTCAGCTAAGAATACTTGACCCGACCGGAAATGGCCACTGTTG
K L E G V K V D S Y E L G W R F T G D N
1870 1890 1910
CTGCGGACTCAAATCGCGGCATATTACTCGCTTTCCAATAAGAGCGTGAAAGGAATAAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GACGCCGTGAGTTTAGCGCCGTATAATGAGCGAAAGGTTATTCTCGCACCTTTCCTTATTT
L R T Q I A A Y Y S L S N K S V E R N K
1930 1950 1970
GATCTGACCATCAGTGTGAAGGACGACAGGCGCCGTATTTACGGCGTGAAAGGTGCGGTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CTAGACTGGTAGTCACACTTTCCTGCTGTCCGCGGCATAAATGCCGCACCTTCCACGCCAC
D L T I S V K D D R R R I Y G V E G A V
1990 2010 2030
GACTACCTGATCCCGGATACTGACTGGAGTACCGGTGTGAACTTCAATGTGCTGAAAACC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CTGATGGACTAGGGCCTATGACTGACCTCATGGCCACACTTGAAGTTACACGACTTTTGG
D Y L I P D T D W S T G V N F N V L K T
2050 2070 2090
GAGTCGAAAAGTGAACGGTGAATGGCAAAAATATGACGTGAAGGAATCAAGTCCATCGAAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CTCAGCTTTCACTTGCCACTTACCGTTTTTATACTGCACTTCCTTAGTTTACAGGTAGCTTT
E S K V N G E W Q K Y D V K E S S P S K
2110 2130 2150
GGCAGCGTTACATTAACCTGGGCGCCGGAACCGTGGAGTGTGCGGTGACAGACCACT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CGCGTGCGAATGTAATTGACCCGCGCCTTGGCACCTCACACGCACATGTCTCGTGGTGA
A H A Y I N W A P E P W S V R V Q S T T
2170 2190 2210
TCTTTCGACGTAAGCGATGCAGAGGGTAACGATATTAATGGTTACTACTACCGTCGATTTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AGAAAGCTGCATTCGCTACGTTCCCATTTGCTATAATTACCAATGTGATGGCAGCTAAAA
S F D V S D A E G N D I N G Y T T V D F
2230 2250 2270
ATCAGTAGTTGGACGCTTCCGGTGGGAACACTCAGCTTCAGCGTTGAGAACCCTTTCGAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
TAGTCATCAACCTGCGAAGGCCACCTTGTGAGTTCGAAGTCGCAACTCTTGGAGAAGCTG
I S S W T L P V G T L S F S V E N L F D
2290 2310 2330
CGTACTATACCCTGTCTGGGGACAGCGTGCACCTCTGTACTACAGCCCGGGTTACGGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GCACTGATATGGTGACAGACCCCTGTGCGACGTGGAGACATGATGTCGGGCCCAATGCCG
R D Y T T V W G Q R A P L Y Y S P G Y G
2350 2370 2390
CCTGCTTCACTGTACGACTACAAAAGGCCGGGGCCGAACCTTTGGTCTGAACTACTCAGTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GGACGAAGTGACATGCTGATGTTTCCGGCCCCGGCTTGGAAACCAGACTTGATGAGTCAC
P A S L Y D Y K G R G R T F G L N Y S V
2410 2430 2450

```

CTGTTCTGACGGGGATGCTTTTGCACAAGGGTATGCTGTAAACAACATGGTCCTTGGTG
-----+-----+-----+-----+-----+-----+-----+
GACAAGACTGCCCCCTACGAAAACGTTGTTCCCATACGACATTTGTTGTACCAGGAACCAC
L  F  *
      2470                2490                2510
GCCATGTTGTTTACAGCCAGTATAAATATACAGCCTTAACAAGATATTCCGTCFCCGGAAT
-----+-----+-----+-----+-----+-----+-----+
CGGTACAACAAATGTCGGTCATATTATATGTCGGAATTGTTCTATAAGGCAGAGGCCTTA
      2530                2550                2570
GGACTGTTCACTGAATAACATTATTTCTGTTTATGGATCTTTGACTGAAATTTATC
-----+-----+-----+-----+-----+-----+-----+
CCTGACAAGTGGTGACTTATGTAATAAAGACAAAATACCTAGAAACTGACTTTAAATAG
      2590                2610                2630
GCATATCCTCATTTCATTTGTCGGTCTGAAAACCTAACCTTACAAGTAAGCGTCGTCAGC
-----+-----+-----+-----+-----+-----+-----+
CGTATAGGAGTAAAGTAACAGGCAGACTTTTGATTGGAATGTTTCATTTCGCAGCAGAGTCG
      2650                2670                2690
ACCGTCTGGCAGATCCTGAAATTCCTGAGAGGATGATCTGCGCGTAAGCGTCGAC
-----+-----+-----+-----+-----+-----+-----+
TGGCAGACCGTCTAGGACTTTAAGGACTCTCCTACTAGACGCGCATTTCGCAGCTG

```

Figure 2.4 A: DNA sequence of the aerobactin receptor gene of *Shigella flexneri* serotype 6 and the amino acid translation of the open reading frame. (*) start/stop codon.

761. CTGTCTCCGG CGGAAATGAC CATATCTCCG GACGTCTTTC CGTGGCATAT
||||| ||||||| ||||||| ||||||| |||||||
719. CTGTCTCCGG CGGAAATGAC CATATCTCCG GACGTCTTTC CGTGGCATAT

811. CAGAAATTTG GCGGCTGGTT TGACGGTAAC GGCATGCCA CCCTGCTTGA
||||| ||||||| ||||||| ||||||| || |||||||
769. CAGAAATTTG GCGGCTGGTT TGACGGTAAC GGCATGCCA CCTTGCTTGA

861. TAACACCCAG ACCGGCCTGC AGCACTCCAA TCGGCTGGAC ATCATGGGAA
||||| ||||||| || ||||| | ||||||| |||||||
819. TAACACCCAG ACCGGCCTGC AGTACTCCGA TCGGCTGGAC ATCATGGGAA

911. CCGGTACGCT GAACATCGAT GAATCCCGGC AGCTTCAACT GATAACGCAG
| ||||||| ||||||| ||||||| ||||| | || || |||
869. CTGGTACGCT GAACATCGAT GAATCCCGGC AGCTTCAACT GATCACACAG

961. TACTATAAAA GTCAGGGAGA CGACAATTAC GGGCTTAATC TCGGGAAAGG
||||| | ||||| || ||||| ||||| ||||||| |||||||
919. TACTATAAAA GCCAGGGCGA CGACGATTAC GGGCTTAATC TCGGGAAAGG

1011. CTTTTCGCC ATCAGCGGGA GCAGCACACC ATACGTCAGT AAGGGGCTGA
||| | ||| ||||| ||||| ||||| || ||||||| || |||||||
969. CTTTATGCC ATCAGAGGGA CCAGCACGAA ATTGTCAGT AACGGGCTGA

1061. ATCTGACCG CATTCCCGGC ACTGACGGGC ATTTGATCAG CCTGCAGTAC
||||| ||||||| ||||||| ||||||| ||||||| |||||||
1019. ATCCGACCG TATTCCCGGC ACTGACGGGC ATTTGATCAG CCTGCAGTAC

1111. TCTGACAGTG ATTTCTGGG ACAGGAACTG GTCGGTCAGG TTTACTACCG
||||| | ||| ||||| ||||| ||| ||||||| |||||||
1069. TCTGACAGTG CTTTCTGGG ACAGGAGCTG GTCGGTCAGG TTTACTACCG

1161. CGATGAGTCG TTGCGGTACT ACCCGTTCCC GACGGTAAAT GCGAATAAAC
||||| ||||| | || ||||||| ||||||| ||||| |||||
1119. CGATGAGTCG TTGCGATTCT ACCCGTTCCC GACGGTAAAT GCGAATAAAC

1211. AGGCGACGGC TTTCTCCTCG TCACAGCAGG ATACCGACCA GTACGGCATG
||| ||||| || ||||||| | ||||||| |||||||
1169. AGGTGACGGC TTTCTCCTCG TCACAGCAGG ATACCGACCA GTACGGCATG

1261. AAACGACTC TGAACAGCCA ACTGATGGAC GGCTGGCAA TCACCTGGGG
||||| ||||||| || || ||||||| ||||||| |||||||
1219. AAACGACTC TGAACAGCAA ACCGATGGAC GGCTGGCAA TCACCTGGGG

1311. GCTGGATGCT GATCATGAGC GCTTTACCTC CAACCAGATG TTCTTCGATC
||||| ||||| ||||| ||||| ||||| |
1269. GCTGGATGCT GATCATGAGC GCTTTACCTC CAACCAGATG TTCTTCGACC

1361. TGGCTCAGGC AAGTGCTTCC GGAGGGCTGA ACAACCATAA GATTTACACC
||||| ||||| ||||| ||||| ||||| |||||
1319. TGGCTCAGGC AAGCGCTTCC GGAGGGCTGA ACAACAAGAA GATTTACACC

1411. ACCGGGCGCT ATCCGTCATA TGACATCACC AATCTGGCGG CCTTCCTGCA
||||| ||||| ||||| ||||| ||||| |||||
1369. ACCGGGCGCT ATCCGTCGTA TGACATCACC AACCTGGCGG CCTTCCTGCA

1461. ATCCAGCTAT GACATTAATG ATATTTTAC CGTTAGCGGT GCGGTACGCT
||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
1419. ATCAGGCTAT GACATCAATA ATCTCTTTAC CCTCAACGGT GCGGTACGCT

1511. ATCAGTATAC TGAGAACAGG GTTGATGATT TCATCGACTA CACGCAGCAA
||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
1469. ATCAGTACAC TGAAAACAAG ATTGATGATT TCATCGGCTA CGGCAGCAA

1561. CAGAAGATG CCGCCGGGAA GGCGATATCT GCCGACGCCA TTCCTGGTGG
| | ||||| ||||| ||||| ||||| ||||| |||||
1519. CGGCAGATG GCGCCGGGAA GGCTACATCC GCCGACGC.. ATTCTGGCGG

1611. TTCGGTAGAT TACGATAACT TTCTGTTCAA TGCCGGTCTG CTGATGCACA
|| || ||| ||||| ||||| ||||| ||||| ||||| |||||
1567. CTCAGTCGAT TACGA.CACT TCCTGTTCAA CGCCGGTCTG CTGATGCACA

1661. TCACCGAACG TCAGCAGGCA TGGTCAATT TTTCCAGGG GGTGGCATTG
||||| ||||| ||||| ||||| ||||| ||||| |||||
1616. TCACCGAACC GCAGCAGGCA TGGCTCAACT TCTCCAGGG CCTGGAGCTG

1711. CCGGATCCGG GGAATATTA TGGTCGCGGC ATCTATGGTG CAGCAGTGAA
||||| ||||| ||||| ||||| ||||| ||||| |||||
1666. CCGGACCCGG GTAAATACTA TGGTCGCGGC ATCTATGGTG CTGCAGTGAA

1761. CGGCCATCTT TCCCTGACAA AGAGTGTGAA CGTCAGCGAC AGTAAGCTGG
||||| ||||| ||||| ||||| ||||| ||||| |||||
1716. CGGCCATCTT CCTCTAACAA AGAGTGTGAA CGTCAGCGAC AGCAAGCTGG


```

2361. CTACAAAGGC CGGGGCCGAA CCTTTGGTCT GAACTACTCT GTGCTGTTCT
      |||          |||          |||          |||          |||
2316. CTACAAAGGC AGGGGCCGAA CCTTTGGTCT GAACTACTCT GTGCTGTTCT

```

2411. GA

||

2366. GA

Figure 2.4 B: The comparison of nucleic acid sequences of *iutA_{Sh}* and *iutA* (bold) genes.

(|): shows identical nucleic acids in both genes. The frame-shift region referred to in the text is shown in underlines.

```

                A G
                G A
2420 T T T   C A   C A           G
                G
2443 A A A   G T   G T           G
                T   C
                A   T
                T C
                C
2444 C A A C A T G G           T
                T
2469 G T T G T A C C           T
                G   G

```

Figure 2.5: Two palindromes observed adjacent to the translation terminating codon:

T2420-A2443 and C2444-G2469.

within a 2.7 kb *HincII* fragment of plasmid pLG2131, and indeed an *E. coli* strain carrying the *HincII* fragment subcloned in the pUC19 vector (pLG2135) was sensitive to cloacin DF13.

Comparison of the predicted amino acid sequences of the *Shigella* and ColV-K30 IutA proteins showed a similarity of 93%. The DNA sequences predict that both primary gene products consist of 732 amino acid residues, with calculated molecular weight of 81550 and 81855 respectively. The major difference is due to frame shifts between the *EcoRI* and *BamHI* sites of the *Shigella* gene located at C₁₅₉₅ and A₁₆₂₄; there are three additional bases: C₁₅₉₆, A₁₅₉₇ and T₁₆₂₃. Consequently, the amino acid sequences of the IutA proteins within this region are very different (figure 2.6) and the differences in the hydrophilicity, surface profile and flexibility based on the prediction of Hopp and Woods (1981), Emimi *et al.*(1985) and Karplus and Schulz (1985) respectively are shown on figure 2.7. The difference was also identified in the central region between *ClaI* and *EcoRI* sites (figure 2.6 and figure 2.7).

It has been mentioned before that siderophores, vitamin B12, many colicin groups and bacteriophages require the TonB protein for transport across the outer membrane of *E. coli*. The respective outer membrane receptor proteins of these agents contain a conserved peptide sequence called the "TonB box". Comparing the sequence data for the IutA_{Sh} protein to other TonB-dependent outer membrane proteins (Baumler and Hantke, 1992), it can be seen that the invariant asparagine and aspartate residues of region II, and the two invariant glycine residues and the single invariant asparagine residue of region III were conserved in the IutA_{Sh} protein. However, in region I only the invariant valine residue was conserved in the IutA_{Sh} protein, but not the threonine residue (fig. 2.8). Compared to the ColV-K30 IutA protein, the *Shigella* molecule has

Figure 2.7.A: The hydrophilicity, surface probabilities and the chain

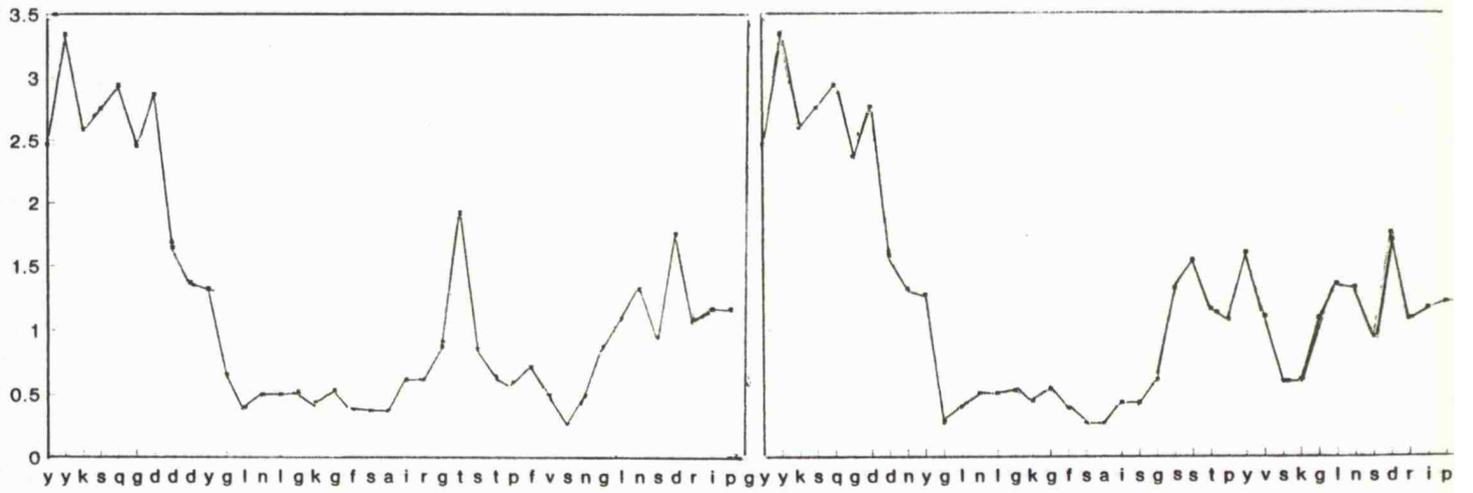
flexibility predictions of IutA and IutA_{Sh} in the *Clal-EcoRI* region.

- (a) The hydrophilicity of this region of the IutA (left) and IutA_{Sh} (right) proteins as predicted by the algorithm of Hopp and Woods (1981). Positive values indicate hydrophilic regions.
- (b) The surface probabilities of IutA (left) and IutA_{Sh} (right) proteins in this region according to the method of Emini *et al.* (1985).
- (c) The chain flexibility as predicted by Karplus and Schulz (1985) of IutA (left) and IutA_{Sh} (right) proteins in this region.

a



b



c

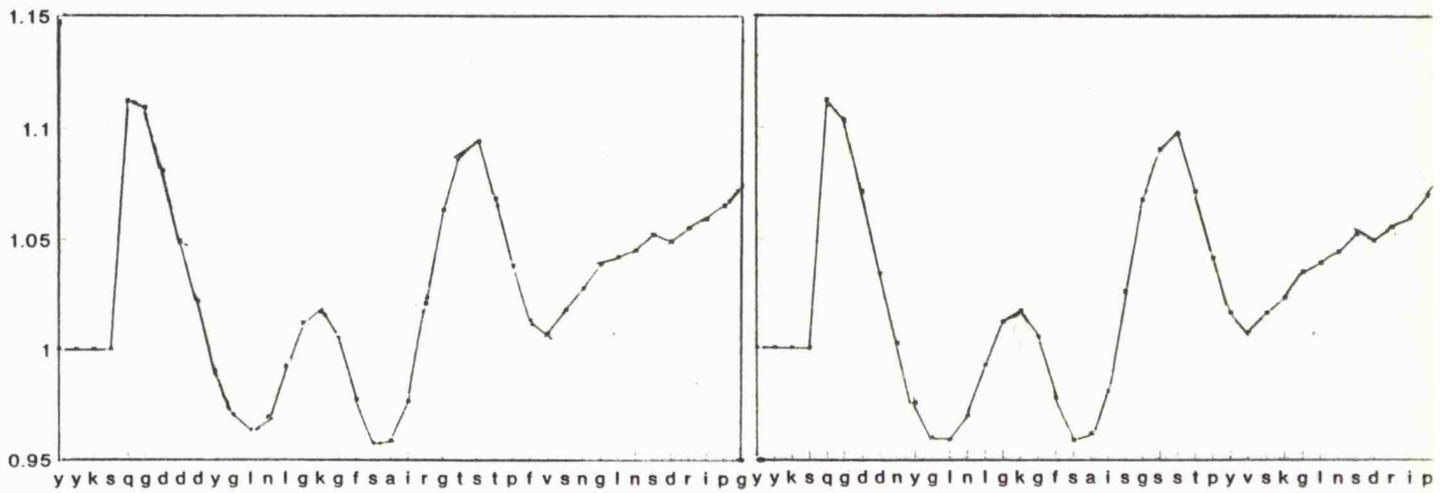
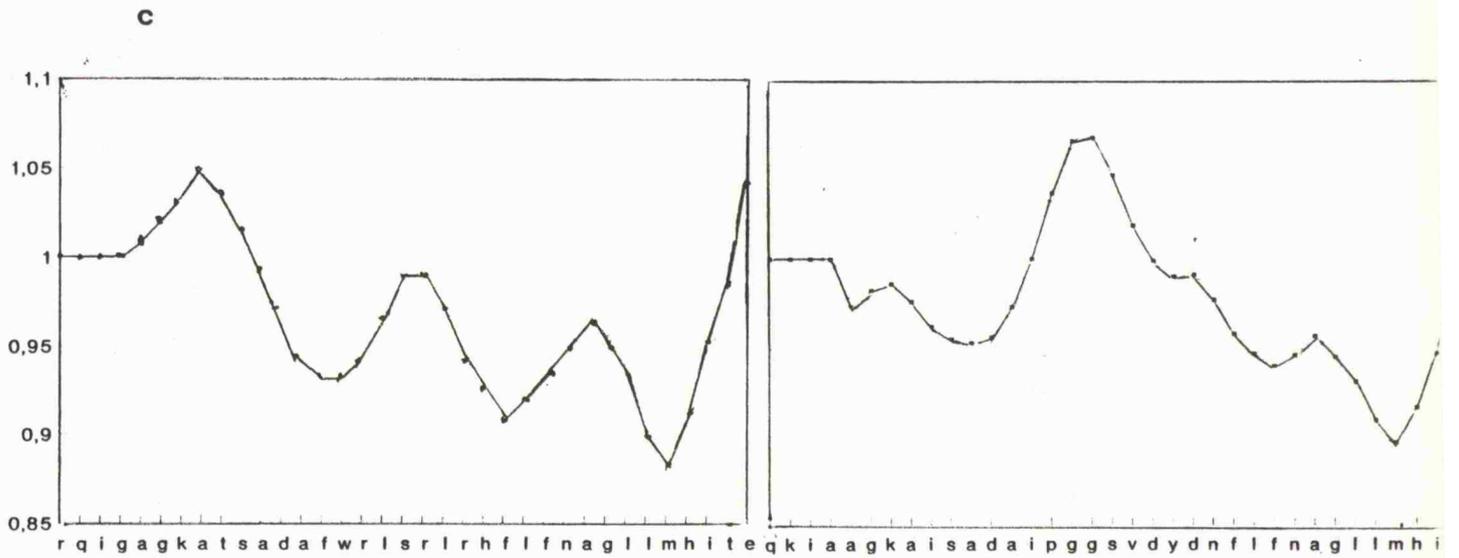
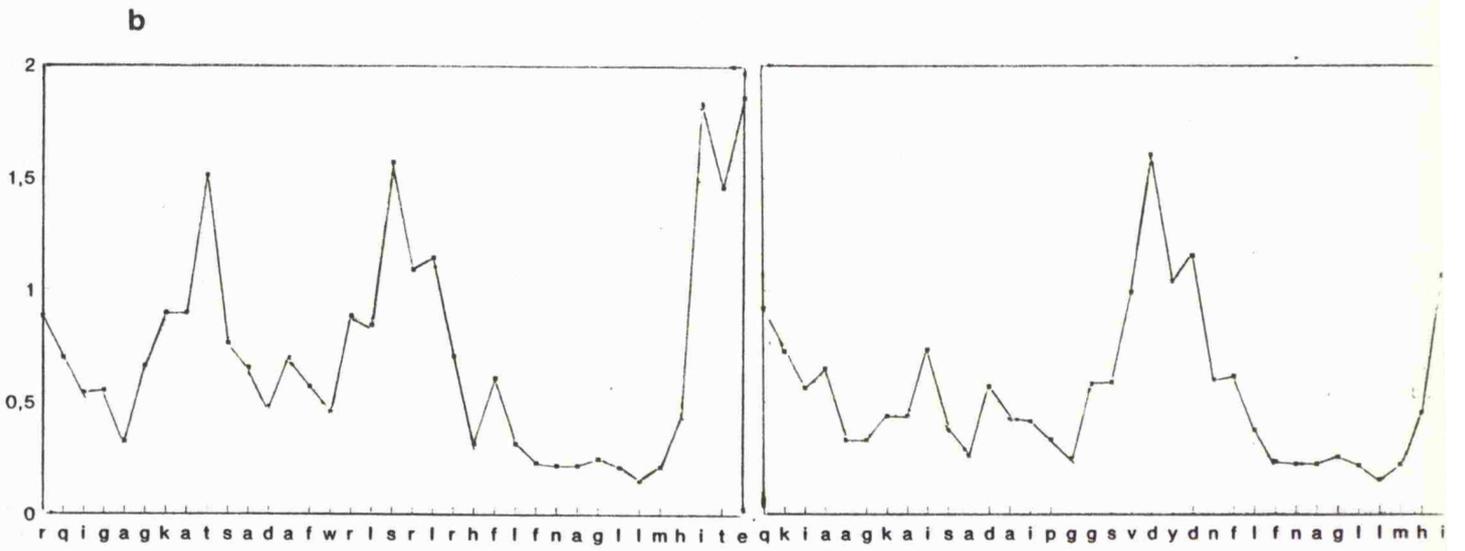
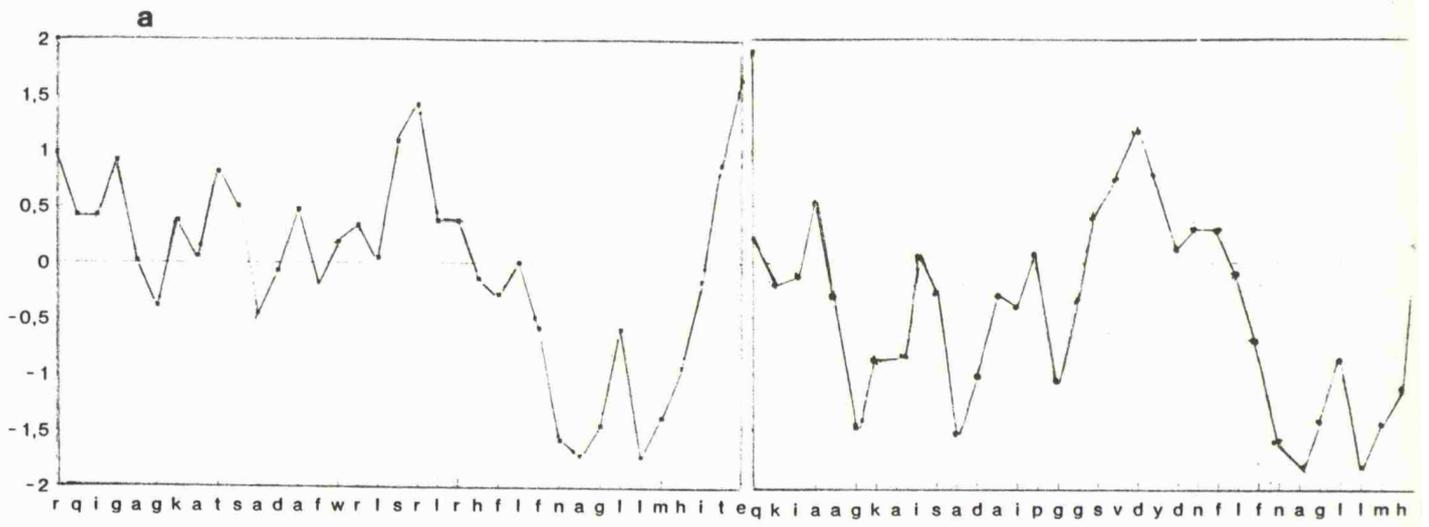


Figure 2.7.B : The hydrophilicity, surface probabilities and the chain flexibility of IutA and IutA_{Sh} in the frame shift region.

- (a) The hydrophilicity of this region of the IutA (left) and IutA_{Sh} (right) proteins as predicted by the algorithm of Hopp and Woods (1981). Positive values indicate hydrophilic regions.
- (b) The surface probabilities of IutA (left) and IutA_{Sh} (right) proteins in this region according to the method of Emini *et al.* (1985).
- (c) The chain flexibility as predicted by Karplus and Schulz (1985) of IutA (left) and IutA_{Sh} (right) proteins in this region.



		I			II
		* *			* *
BtuB	4	SPDTLVVTAN	BtuB	565	IANLFDKRY
Cir	4	DGETMVVTAS	Cir	608	VLNLFDKDL
FecA	21	SGFTLSVDAS	FecA	705	VKNIFDQDY
FepA	10	HDDTIVVTAA	FepA	674	VDNLFDKRL
FhuA	5	DEDTIVVTAA	FhuA	680	VNNLFDREY
FhuE	4	TEETVIVEGS	FhuE	661	VNNLFDKTY
IutA	4	DDETFVVSAN	IutA	660	IENLFDRDY
IutA _{Sh}	3	DDNEIIVSAS	IutA _{Sh}	660	VENLFDRDY

		III
		* * *
BtuB	105	QRVEYIRGPRSAVYGS DAIGGVVNIITTR
Cir	106	ERIEVVRGPMSSLYGSDALGGVVNIITKK
FecA	190	DAIDVVRGGGAVRYGPQSVGGVVNEVTRA
FepA	120	ERIEVLRGPARARYNGA-GGVVNIITKK
FhuA	127	ERAEIMRGPVSVLYGKSSGPGLLNMVSKR
FhuE	120	ERVEVVRGATGLMTGTGNPSAAINMVRKH
IutA	103	HHIEVIFGAT-SLYGGGSTGGLINIVTKK
IutA _{Sh}	102	DHIEVISGAT-ALYGGGSTGWLINIVTKK

Figure 2.8: Conserved sequences in TonB-dependent receptors (Baumler and Hantke, 1992) and IutA_{Sh}. The numbers at the left represent the position of the first amino acid in each homology region in the mature sequence of the protein. Invariant amino acids are indicated by asterisks.

5 different residues out of 10 in region I, 1 out of 9 in region II and 4 out of 28 in region III of the TonB box.

To confirm that the other two clones (from *S. flexneri* serotype 2a and *S. sonnei*) also have the same DNA sequence, particularly within the region of the frame-shift mutations, clones pLG2130 and pLG2132 were sequenced using primers located between *Cla*I and *Bam*HI sites (Figure 2.3). The DNA sequences within this particular region were found to be identical.

2.4. DISCUSSION.

The *iutA* genes of different members of the family *Enterobacteriaceae* display considerable polymorphism in gene structure (Marolda *et al.*, 1987; Loper *et al.*, 1993). The migration rate of IutA protein in SDS-PAGE is also variable, indicating apparent molecular weights ranging from 74 and 76 kDa in *E. coli* and *Shigella* strains (Bindereif *et al.*, 1982; Marolda *et al.*, 1987) up to 80 and 85 kDa in *Erwina carotovora* (Ishimaru and Loper, 1992) and *Enterobacter cloacae* (Crosa *et al.*, 1988; Krone *et al.*, 1985) respectively.

The restriction maps of the aerobactin genes of *E. coli* plasmid ColV-K30, *Shigella flexneri* serotype 2a and enteroinvasive *E. coli* are very similar (Marolda *et al.*, 1987; Johnson, 1991). This observation is not surprising, since *Shigella* are very closely related to *E. coli*. The main difference in the restriction maps of the aerobactin receptor genes was the presence of a *Bam*HI site 655 bp downstream of the *Eco*RI site in both the *Shigella* and the enteroinvasive *E. coli* genes, but not in that of ColV-K30

(Marolda *et al.*, 1987). In *Shigella*, the existence of *Bam*HI site did not change the amino acid sequence in this region.

All secretory proteins are synthesized with an amino terminal extension known as the signal peptide, whose primary function is to help direct them to the cytoplasmic membrane. Although there is no consensus sequence of signal peptides, they usually consist of three distinct regions: a positively charge amino-terminal region (n-region), a central hydrophobic region (h-region) and a polar C-terminal region (c-region). The carboxy-terminal end of a signal peptide is usually an alanine or a glycine residue (Duffaud *et al.*, 1985).

In IutA, there were 25 amino acid residues in the signal sequence and the cleavage site is at the alanine₂₅ residue (Krone *et al.*, 1985). Based on this observation, it is likely that the cleavage site of the signal peptide of IutA_{Sh} is at the alanine₂₄ residue, thus the mature protein of IutA_{Sh} begins with glutamine and consists of 708 amino acid residues. Thus the calculated molecular weights of the mature IutA and IutA_{Sh} proteins are 78614 and 78654 respectively; however, these proteins appear as the 74 and 76 kDa proteins respectively on SDS-PAGE. The different apparent molecular weights of these proteins on SDS-PAGE may be due to different conformational changes in the protein molecules upon heating in SDS solution (Nakamura and Mizushima, 1976).

The signal sequences of IutA and IutA_{Sh} are 44% different; however, the different appearance of these proteins on SDS-PAGE is not likely to be due to signal sequence differences. It has been reported previously (Klose *et al.*, 1988) that several *ompA* mutants which had substitutions in the region encoding the signal sequence were not defective in assembly of the protein into the membrane. Comparison of the signal

peptides of the same protein from different species reveal that they are not always identical. The signal peptides of the prolipoproteins from *Serratia marcescens* and *Erwinia amylovora* are identical and differ by only two residues in the amino-terminal region from the signal peptide of the *E. coli* prolipoprotein, and more changes are observed in *Morganella morganii* and *Proteus mirabilis*. However, all these prolipoproteins can be processed in *E. coli* to form the fully modified mature lipoprotein assembled in the outer membrane and they are conserved in many places. In addition, signal peptides of the OmpA from *E. coli* and *S. dysenteriae* are highly conserved and differ only at one position, while for signal peptides of the *E. coli* and *V. cholerae* enterotoxins, the only significant similarities are observed at the cleavage site and at the positively charge amino termini (Duffaud *et al.*, 1985). On the other hand, an OmpA protein which had substitutions leucine₁₆₄ to proline and valine₁₆₆ to aspartate was translocated across the plasma membrane but could not be incorporated into the outer membrane (Klose *et al.*, 1988). In addition, substitution of glycine₁₄₄ with leucine also affected the efficiency of outer membrane incorporation, and after in vitro synthesis this mutant protein was less efficiently precipitated with monoclonal antibodies that recognize conformational epitopes than the wild type PhoE (de Cock *et al.*, 1991). These observations show that the correct assembly of proteins into the outer membrane was less dependent on the signal sequence than on certain amino acid sequences within the mature polypeptides.

The frame shift mutations in the region between *EcoRI* and *BamHI* sites in IutA_{Sh} resulted in very similar predictions for hydrophilicity, surface profile and flexibility compared with IutA. However, there were differences in the region upstream of the frame shift; the hydrophilicity prediction of IutA_{Sh} showed that it was more

hydrophobic in this region. The surface profile showed that the IutA protein in this region extended further from the membrane than the IutA_{Sh} protein. Therefore, there is a possibility that the difference in the cloacin sensitivity function in IutA is located in this region. The central regions between *Clal* and *EcoRI* sites of IutA or IutA_{Sh} which have many different amino acid residues also showed differences in protein predictions. The hydrophilicity predictions for these regions showed a hydrophobic region in IutA but a hydrophilic region in IutA_{Sh}. The protein surface profile prediction of this region in IutA showed the existence of a peak in the middle of a low surface region, and two low peaks in the same region in IutA_{Sh}. Possibly this central region of IutA also influences the cloacin susceptibility function. Because of these differences, hybrid *iutA::iutA_{Sh}* genes were constructed in order to identify locations of the aerobactin and cloacin DF13 receptor functions in these genes.

IutA is one of the TonB-dependent outer membrane transport proteins, which consequently contain a consensus sequence called TonB box. This sequence plays an important role in active transport; in the BtuB protein, for example, substitutions of leucine₈ with proline and valine₁₀ with glycine or proline completely inactivated the protein. In contrast, the invariant threonine₇ could be changed to leucine, proline, serine and threonine₁₁ could be replaced by isoleucine without effect. In addition, alanine₁₂ could be changed to threonine, glycine, aspartate or valine without impairing transport activity (Gudmundsdottir *et al.*, 1989). It was also reported that the majority of amino acid substitutions within the TonB box of FhuA had little or no effect on transport function (Schoffler and Braun, 1989).

Invariant amino acids threonine and valine in region I (figure 2.8) were conserved in 11 TonB-dependent proteins (Gudmundsdottir *et al.*, 1989; Poole *et al.*, 1993).

However, the threonine is not conserved in IutA_{Sh}, but is replaced by glutamine. Similarly the ferripyoverdine receptor protein, FpvA, contains alanine and isoleucine instead of the invariant threonine and valine residues respectively (Poole *et al.*, 1993). As observed by Gudmundsdottir *et al.*, (1989), it is probable that the TonB protein tolerates some variations in the TonB box of receptor proteins.

CHAPTER 3

CONSTRUCTION OF HYBRID COLV-K30::*SHIGELLA FLEXNERI*

AEROBACTIN RECEPTOR GENES

3.1. INTRODUCTION

Several of the iron-repressible outer membrane proteins (IROMPs) of *Escherichia coli* are receptors of bacteriocins in addition to any function in iron uptake. For example, the ferrichrome receptor FhuA (78 kDa) is also the receptor of colicin M, while the 81 kDa FepA protein is the receptor of both enterochelin and colicins B and D (Braun *et al.*, 1976). Similarly the aerobactin receptor protein IutA also serves as the receptor for cloacin DF13, a bacteriocin produced by strains of *Enterobacter cloacae* (Van Tiel-Menkveld *et al.*, 1982). Despite a common target for binding, however, the uptake mechanisms are clearly different. Aerobactin receptor function is TonB dependent, also requiring the involvement of the products of the *exbB* and *D* genes (Frost and Rosenberg, 1973; Hantke and Zimmermann, 1981); on the other hand, the uptake of cloacin is TonB independent and involves the products of the *tolQ*, *tolR* and *tolA* genes (Thomas and Valvano, 1992; Thomas and Valvano, 1993). There is significant variation among the aerobactin receptor proteins of different species of the Enterobacteriaceae, at least as indicated by their apparent molecular weights in SDS-PAGE. For example, the aerobactin receptor protein of *E. coli* ColV-K30 is 74 kDa (Bindereif *et al.*, 1982; Grewal *et al.*, 1982), of enteroinvasive *E. coli* and *Shigella* species are 76kDa (Griffiths *et al.*, 1985), of *Enterobacter cloacae* is 85 kDa (Krone *et al.*, 1985; Crosa *et al.*, 1988) and of *Klebsiella pneumoniae* is 76 kDa

(Miles and Khinji, 1975).

With respect to the function of IutA as the receptor of cloacin DF13, bacterial strains expressing this protein are not equally sensitive to cloacin. It has been reported that *Shigella* were less sensitive to cloacin DF13 than *E. coli* strains carrying plasmid ColV-K30 (Payne *et al.*, 1983). An *E. coli* strain carrying a recombinant plasmid containing the cloned aerobactin receptor gene from an enteroinvasive strain of *E. coli* showed a 16-fold decrease in susceptibility to cloacin compared with cells containing the *iutA* gene cloned from ColV-K30, although aerobactin uptake was similar (Marolda *et al.*, 1991). Presumably these differences reflect differences either in the ability of cloacin to bind to the receptor, or in other steps in the killing pathway (Griffiths *et al.*, 1985).

Hybrid genes are usually constructed to study the topology of proteins and to localize several function in a particular protein. For example, ExbB-/ExbD- β -lactamase fusion proteins were constructed for analyzing the topology of ExbB and ExbD proteins (Kampfenkel and Braun, 1993; Kampfenkel and Braun, 1992), and various *ompF-ompC*, *ompC-ompF* and *ompF-ompC-ompF* chimeric genes were used to locate the domains of the OmpF protein involved in cellular sensitivity to colicins (Mizuno *et al.*, 1987; Fourel *et al.*, 1990).

In order to determine regions in the aerobactin receptor protein required for aerobactin and cloacin binding, hybrid *iutA* genes have been constructed by swapping homologous fragments of the *iutA* genes of *E. coli* ColV-K30 and *Shigella flexneri* serotype 6. The fragments used represent three distinct regions of the genes, (a) the 5' terminal fragment upstream of the *ClaI* site, (b) the central region between the *ClaI* and *EcoRI* sites, and (c) the 3' terminal fragment downstream of the *EcoRI* site.

3.2. MATERIALS AND METHODS

3.2.1. Outer membrane protein preparation.

Cells from 50 ml overnight cultures were harvested by centrifugation at 12,000 x 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, and then centrifuged at 12,000 x g for 5 min to remove any remaining whole cell debris. The inner membrane was selectively solubilised by incubation at room temperature for 1 h with 2% (w/v) sodium lauroyl sarcosinate (Sigma Chemicals Co.); insoluble outer membrane proteins were pelleted by centrifugation at 20,000 x g for 60 min.

3.2.2. Cloacin adsorption by outer membrane proteins.

Outer membrane proteins prepared from *E. coli* strains containing cloned native or hybrid *iutA* genes were added to a serial doubling dilution of cloacin DF13 in 2 ml aliquots of LUB. These mixtures were left for 30 min at room temperature to allow outer membrane proteins to adsorb cloacin; the cloacin remaining in the medium was assayed by adding 0.5 ml of *Klebsiella pneumoniae* strain M51a at an optical density at 620 nm of 0.5 and incubating at 37°C for 4 h. The optical densities of these cultures were then determined at 620 nm.

3.2.3. Preparation of [¹⁴C]aerobactin.

¹⁴C-labelled aerobactin was purified from culture supernatants of *Aerobacter aerogenes* strain 62-1 (Gibson and Magrath, 1969). Bacteria were grown overnight

Table 3.1: Bacterial strains and plasmids used.

Bacterial strains/ plasmids	Relevant properties	Reference
<i>E. coli</i> DH5 α	<i>supE44 lacU169</i> (80 <i>lacZ</i> M15) <i>hsdR17 recA1 endA1</i> <i>gyrA96 thi-1 relA1</i>	Hanahan, 1983.
<i>Aerobacter</i> <i>aerogenes</i> 62-1	Aerobactin producer	Gibson & Magrath, 1969.
<i>Klebsiella</i> <i>pneumoniae</i> M51a	Cloacin DF13 sensitive	Cooper & James, 1985.
pLG2131	Cloned aerobactin genes of <i>S. flexneri</i> in pUC19	This study.
pLG2133	<i>KpnI</i> fragment subcloned of pLG2131 in pUC19	This study.
pLG2134	<i>BamHI</i> fragment subcloned of pABN1 in pUC19 (<i>iutA</i> ⁺)	This study.
pLGC34	Construct 1 in pUC19	This study.
pLGC43	Construct 2 in pUC19	This study.
pLGE34	Construct 3 in pUC19	This study.
pLGE43	Construct 4 in pUC19	This study.
pLGV33	Construct 5 in pUC19	This study.
pLGS34	Construct 6 in pUC19	This study.

in 10 ml of M9 salts medium containing dipyriddy harvested by centrifugation, and resuspended in the same volume of medium containing 0.3 mM L-lysine. After incubation at 37°C with aeration for 30 min, the bacteria were again harvested and resuspended in 10 ml of fresh medium containing 185 kBq [¹⁴C]lysine (specific activity 11.99 GBqm/mol, Amersham). The suspension was incubated at 37°C for a further 4 h before the bacteria were again pelleted by centrifugation, and the supernatant fluid lyophilized. The resulting powder was dissolved in 1 ml of distilled water, 5 ml of cold ethanol were added and the mixture was incubated on ice for 5 min before centrifugation to remove cellular debris. The supernatant fraction was lyophilized, redissolved in 0.5 ml distilled water and separated by ascending chromatography on Whatman 3MM paper with a butanol:acetic acid:water (12:3:5) solvent system. Labelled products were located by autoradiography, and material of $r_f=0.53$ was eluted in 1 ml distilled water.

3.2.4. Uptake of labelled aerobactin.

Cells from 1.5 ml of overnight Luria broth cultures were harvested by centrifugation at 13,000 x g for 4 min, washed in PBS (140 mM NaCl, 27 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄) and resuspended in 1 ml of PBS containing 0.4 % glucose. [¹⁴C]aerobactin was added to each sample and incubated for 30 min at room temperature with continuous agitation. The cells were harvested by centrifugation at 13,000 x g for 4 min, washed in PBS and resuspended in 200 µl of PBS. After addition of 2 ml of Packard Emulsifier Safe scintillation fluid and vortexing, the radioactivity associated with the cell pellets was determined in a Packard TriStar liquid scintillation spectrometer.

3.2.5. Preparation and characterisation of hybrid *iutA* constructs.

Recombinant plasmids containing the *Bam*HI fragment encoding ColV-K30 *iutA* or the *Kpn*I fragment carrying the *Shigella iutA* were digested with the appropriate restriction enzymes and the DNA fragments were separated and isolated as described in 2.2.7. Appropriate DNA fragments were ligated and transformed into *E. coli* DH5 α as described in 2.3.2. Constructs were selected by isolating recombinant plasmid from transformants using the method described in 2.2.6. *E. coli* strains carrying each construct were examined for their sensitivity to cloacin (as described in 2.2.5), cloacin adsorption, ¹⁴C-aerobactin uptake and the DNA sequence of the fragment between *Cla*I and *Eco*RI sites using double stranded DNA sequencing method (as described in 2.2.14).

3.3. RESULTS

3.3.1. Hybrid gene constructions.

In order to determine which domains of the IutA or IutA_{Sh} proteins are responsible for aerobactin uptake or sensitivity to cloacin DF13, hybrid genes were constructed and introduced into *E. coli* DH5 α .

Constructs were prepared by combining restriction fragments of plasmid pLG2133 (the *Kpn*I fragment of *iutA*_{Sh} in pUC19) and pLG2134 (the *Bam*HI fragment of *iutA* from pLG141 in pUC19) as shown in figure 3.1. Construct 1 was a hybrid of the *iutA*_{Sh} gene from the 5'-terminus to the *Cla*I site joined to a fragment of *iutA* downstream of the *Cla*I site. Construct 2 was the reverse of this. Constructs 3 and

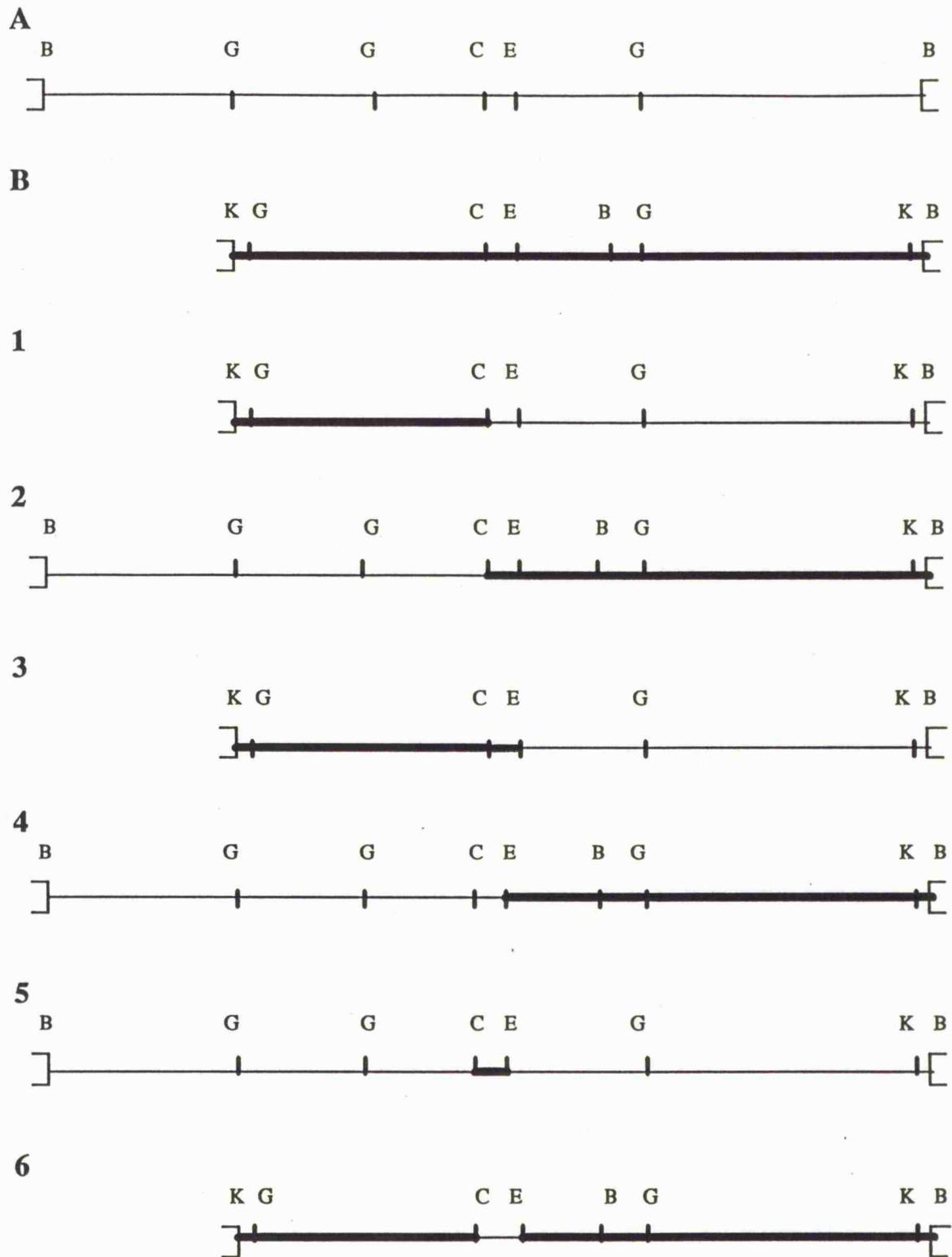


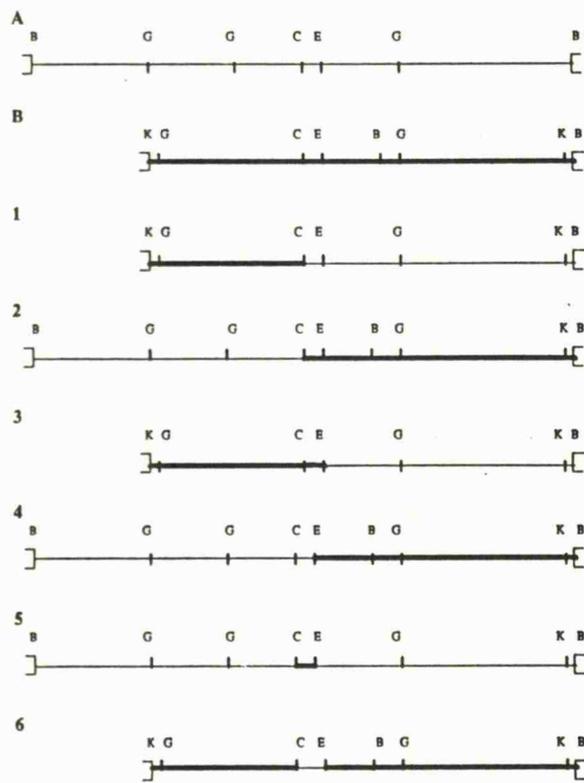
Figure 3.1: Restriction maps of aerobactin receptor genes from ColV-K30 (A), *S. flexneri* ser. 6 (B) and hybrid aerobactin gene constructs 1-6 (1-6).
 B: *Bam*HI, Bg: *Bgl*III, C: *Cl*aI, E: *Eco*RI, H: *Hind*III, K: *Kpn*I.

4 were similar to constructs 1 and 2 respectively, except that the junctions were at the *EcoRI* site within the genes. Constructs 5 and 6 were derived from constructs 1 and 2 by digesting the latter with *EcoRI*, and swapping the 5'-terminal fragments.

The structures of these constructs were proved by analysis of restriction patterns, especially with reference to digestion with *BamHI* and *BglII*. Thus, plasmid pLG2133 produced two fragments of 2.4 and 5.2 kb with *BamHI* and two fragments of 2.8 and 4.8 kb with *BglII* digestions. Plasmid pLG2134, on the other hand, produced two fragments of 2.6 and 6.5 kb with *BamHI* and three fragments of 1, 2 and 6 kb with *BglII* digestions. Constructs 1 and 3 produced one fragment of 8 kb with *BamHI* and two fragments of 2.8 and 5.2 kb with *BglII* digestions. Constructs 2 and 4 produced three fragments of 2.4, 2.6 and 3.8 kb with *BamHI* and three fragments of 1, 2 and 5.8 with *BglII* digestions. With regard to *BamHI* and *BglII* digestions, constructs 5 and 6 gave the same restriction fragments as pLG2134 and pLG2133 respectively (Figure 3.2). In order to distinguish between pairs of plasmids with identical restriction enzyme digestion patterns, DNA sequencing of all constructs was performed using oligonucleotide sequences located between the *ClaI* and *EcoRI* sites as the primer (figure 3.3). In this way the structures illustrated in figure 3.1 were confirmed.

3.3.2. Outer membrane proteins.

Although the aerobactin receptor proteins of ColV-K30 and *S. flexneri* ser.6 have the same functions they do not behave identically upon analysis by SDS-PAGE. The IutA protein appeared as a 74 kDa protein, while the IutA_{Sh} protein was 76 kDa. It was important therefore to analyze the outer membrane protein profiles of strains



Reference restriction maps of aerobactin receptor genes.

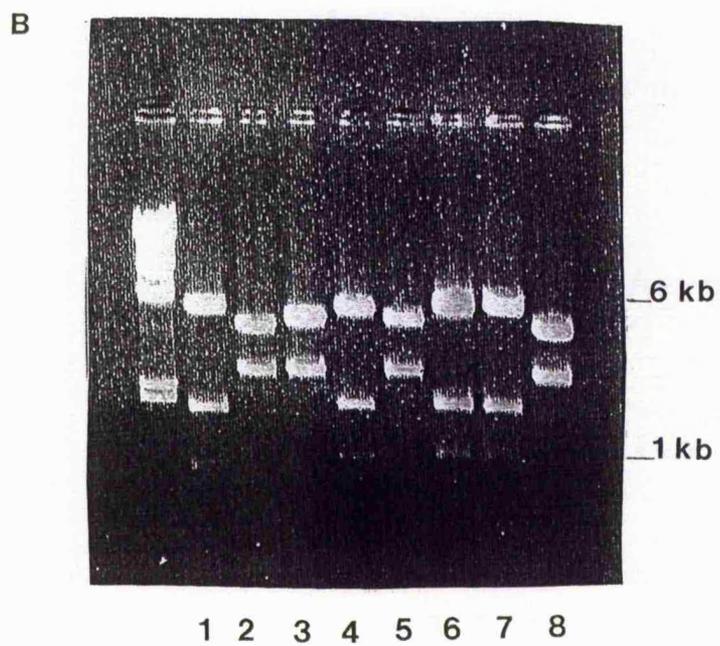
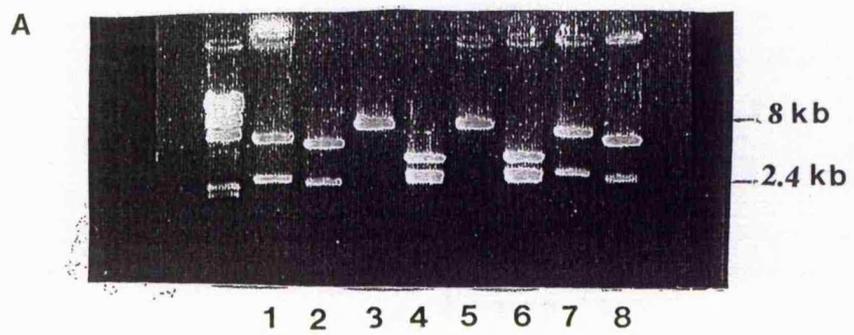


Figure 3.2: Digestion of 1:*iutA* (pLG2134), 2:*iutA_{Sh}* (pLG2133), 3-8:constructs 1-6 with *Bam*HI (A) and *Bgl*III (B).

TCGATGAATC CCGGCAGCTT CAACTGATAA CGCAGTACTA TAAAAGTCAG
GGAGACGACA ATTACGGGCT TAATCTCGGG AAAGGCTTTT CCGCCATCAG
CGGGACGAGC ACACCATACG TCAGTAAGGG GCTGAATTCT

Figure 3.3: DNA sequences between *ClaI* and *EcoRI* sites of *iutA_{Sh}* and the primer sequences (bold) used to confirm the DNA sequences of all constructs.

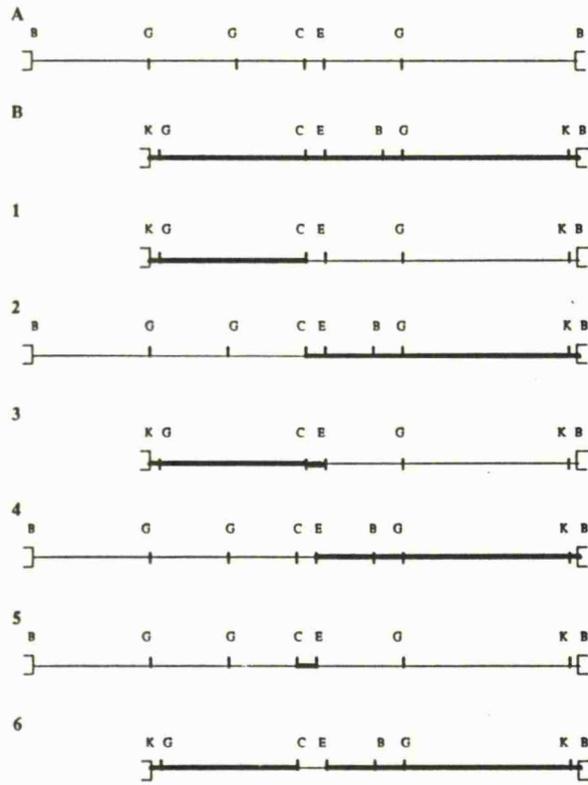
harbouring the cloned hybrid *iutA-iutA_{Sh}* genes in comparison with those expressing normal IutA and IutA_{Sh} proteins (Figure 3.4).

In all cases the apparent molecular size of the hybrid protein was dependent on the origin of the 3'-terminus of the gene (i.e. downstream of the *EcoRI* site). All structures with the 3'-terminus from *iutA* (i.e. pLG2134 and constructs 1, 3 and 5) expressed a 74 kDa protein, while structures with the 3'-terminus from *iutA_{Sh}* (i.e. pLG2133 and constructs 2, 4 and 6) expressed a 76 kDa species. Strains carrying construct 5 showed an additional 70 kDa band in addition to the 74 kDa protein. It may be that the central *ClaI-EcoRI* fragment is important for the stability of the IutA protein product; replacing this fragment may change the conformation of the protein.

3.3.3. Cloacin sensitivity

Strains carrying constructs 1-5 were significantly more sensitive to cloacin than was a strain harbouring a recombinant plasmid containing *iutA_{Sh}*. A strain carrying construct 6, on the other hand, was only marginally more sensitive. Comparing the phenotype of constructs 1-4 associated with that of pLG2133 (*iutA_{Sh}*), it is clear that fragments both upstream of *ClaI* and downstream of *EcoRI* sites influence cloacin sensitivity (table 3.2).

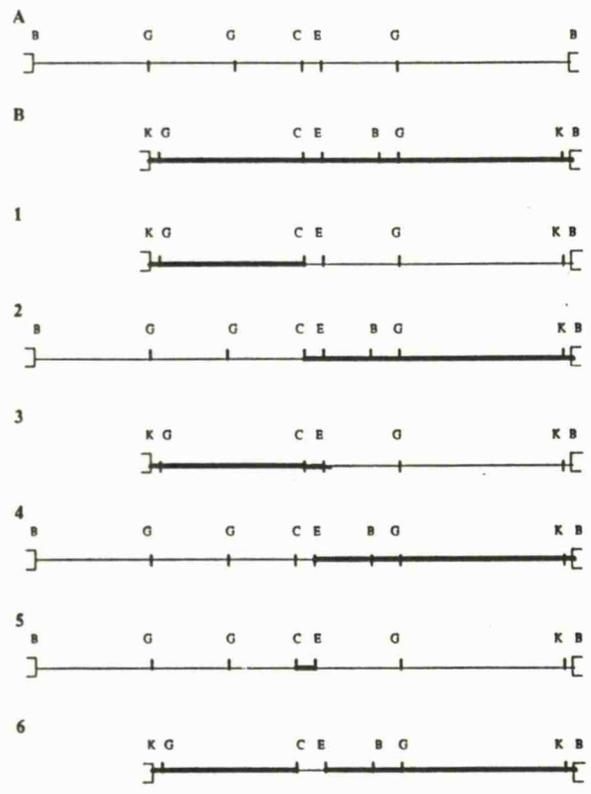
Strains carrying constructs with the 3'- terminus of *iutA* from both the *ClaI* or *EcoRI* sites (constructs 1 and 3) were more sensitive to cloacin DF13 than strains carrying constructs with the 5'-terminus of *iutA* (constructs 2, 4). Comparing constructs 1 with 3 and 2 with 4 indicates that the origin of the small fragment between *ClaI* and *EcoRI* did not influence cloacin sensitivity in this genetic



Reference restriction maps of aerobactin receptor genes.

Table 3.2: Cloacin sensitivity of *E. coli* strains containing cloned *iutA*, or *iutA_{Sh}* or constructs 1-6. Zones of inhibition: clear: + + +, slightly turbid: + +, turbid: +, very turbid: (+) and negative: -.

<i>E. coli</i> +	Sensitivity to cloacin DF13	
	undiluted	diluted 1:10
pLG2134 (<i>iutA</i>)	+++	+++
pLG2133 (<i>iutA_{Sh}</i>)	+	—
Construct 1	+++	++
Construct 2	++	++
Construct 3	+++	++
Construct 4	++	++
Construct 5	++	+
Construct 6	+	(+)



Reference restriction maps of aerobactin receptor genes.

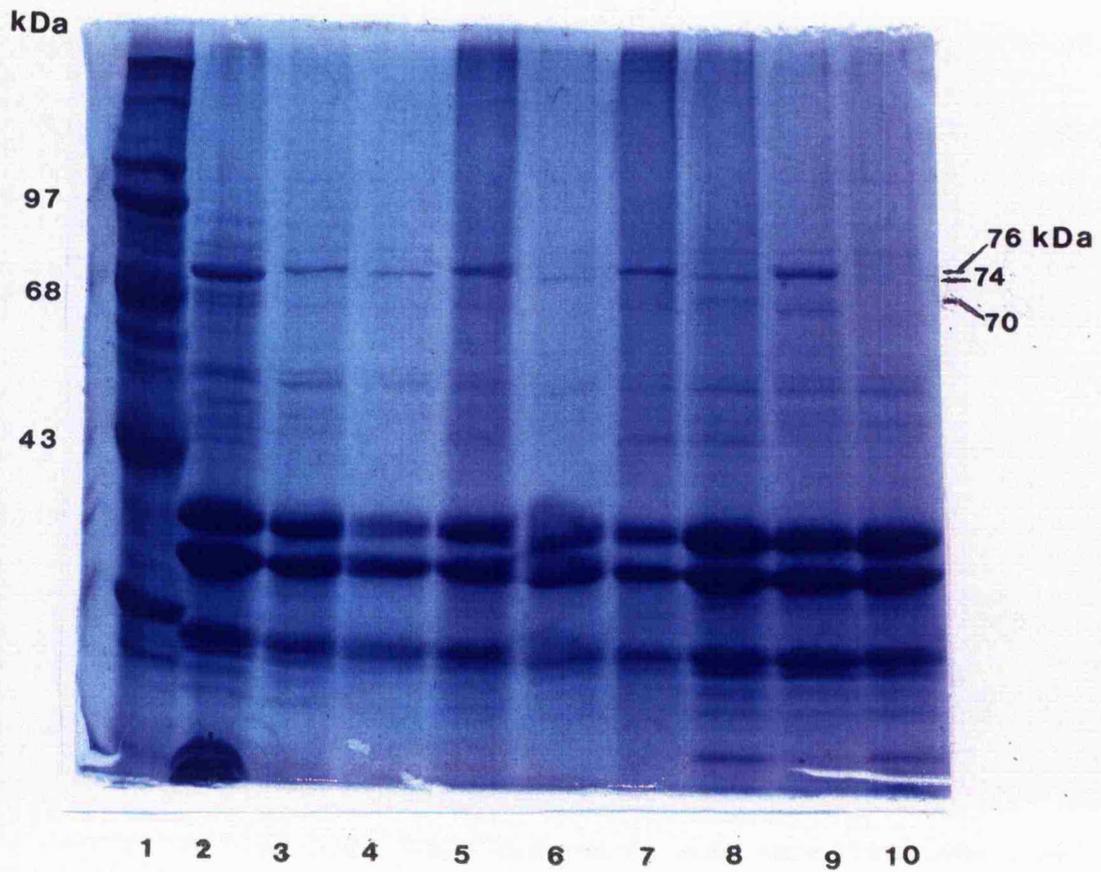


Figure 3.4: Apparent molecular weight of IutA proteins on SDS-PAGE of *E. coli* containing: 2. *iutA* (pLG2134), 3. *iutA_{sh}* (pLG2133), 4-9. constructs (1-6), 10. DH5 α (pUC19). Lane 1 contains protein marker.

background. Surprisingly, however, if the *ClaI-EcoRI* fragments of *iutA* and *iutA_{Sh}* were swapped with each other (to generate constructs 5 and 6 respectively), the cloacin sensitivities conferred by gene products were significantly altered. Thus, replacement of the *ClaI-EcoRI* fragment of *iutA* with the homologous fragment of *iutA_{Sh}* (construct 5) reduced cloacin sensitivity of a strain carrying this construct compared with that of a strain expressing normal IutA. On the other hand, replacement of the *ClaI-EcoRI* fragment of *iutA_{Sh}* with the equivalent fragment from *iutA* (construct 6) increased cloacin sensitivity compared with wild type IutA_{Sh}. These results indicate that the *ClaI-EcoRI* fragment of *iutA* may also influence cloacin sensitivity.

3.3.4. Adsorption of cloacin DF13 by outer membrane proteins.

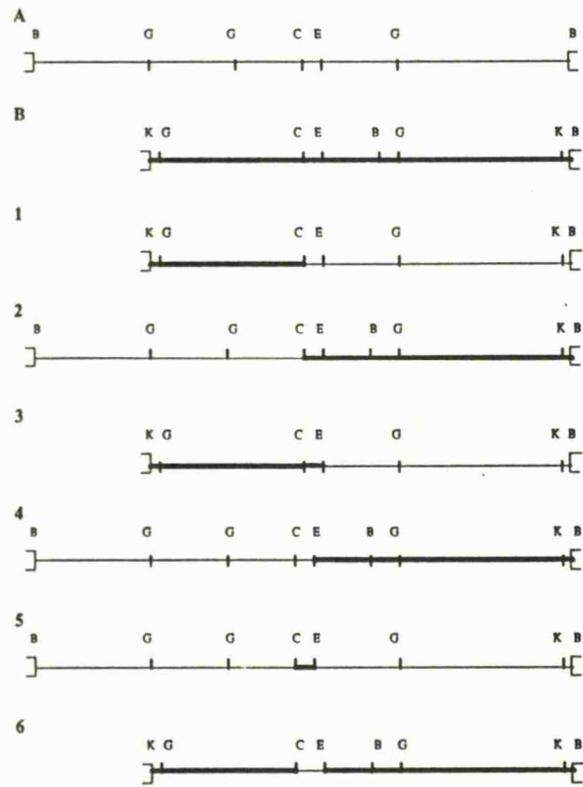
In order to study the sensitivity to cloacin DF13 of *E. coli* strains harbouring *iutA* or the hybrid genes, outer membrane protein preparations of various strains were used in a quantitative cloacin binding assay. After an incubation period of 30 min to allow the outer membrane proteins to adsorb cloacin, the cloacin remaining in the medium was quantified by adding an indicator strain for cloacin DF13, *Klebsiella aerogenes* M51a, and measuring its growth after incubating for 4 h. High levels of growth of *K. pneumoniae* M51a indicated relative absence of cloacin due to efficient binding by outer membrane protein preparations being tested. Low levels of growth indicated poor binding of cloacin by test samples.

Crude sensitivity tests (Table 3.2) had shown that *E. coli* harbouring the cloned *iutA_{Sh}* gene (pLG2133) was relatively insensitive to cloacin. This result was reflected by the cloacin adsorption experiment, which revealed that there was little or no

detectable adsorption of cloacin by membrane preparations of a strain carrying plasmid pLG2133 (Figure 3.5). The graph is in fact very similar to that of both controls, the reaction mixture lacking outer membrane protein and the reaction mixture with outer membranes of *iutA* DH5 α .

The capability of an outer membrane preparation of a strain carrying construct 1 to adsorb cloacin was higher than those of a strain expressing *IutA_{Sh}* and similar to that of a strain expressing *IutA*. This suggests that the *iutA* fragment downstream of *ClaI* site is more influential for efficient cloacin adsorption than the equivalent fragment of *iutA_{Sh}*. This result is similar to the results with the outer membrane of a strain carrying construct 3, which had significantly higher cloacin adsorption than those of a strain harbouring construct 4. In this case the data suggest that the cloacin DF13 receptor function of *iutA* is located in the fragment downstream of *EcoRI* site.

However, the cloacin adsorption by an outer membrane preparation of *E. coli* strain carrying construct 3 was significantly higher than that of a strain harbouring construct 1. The difference between these constructs lies in the origin of the fragment between the *ClaI* and *EcoRI* restriction sites, *iutA_{Sh}* in construct 3, *iutA* in construct 1. This result suggests that the central *ClaI-EcoRI* fragment of *iutA_{Sh}* may be able to enhance the capacity of the *iutA* fragment downstream of *EcoRI* to influence cloacin adsorption. This phenomenon was also shown by the cloacin adsorption of outer membrane preparations of strains carrying constructs 2 and 4, although they only showed a slight difference. The difference in degree of cloacin adsorption between outer membrane preparations of strains carrying constructs 1 and 3 or 2 and 4 may be due to the greater influence on cloacin adsorption of the 3'-terminus than the 5'-terminus of *iutA*.



Reference restriction maps of aerobactin receptor genes.

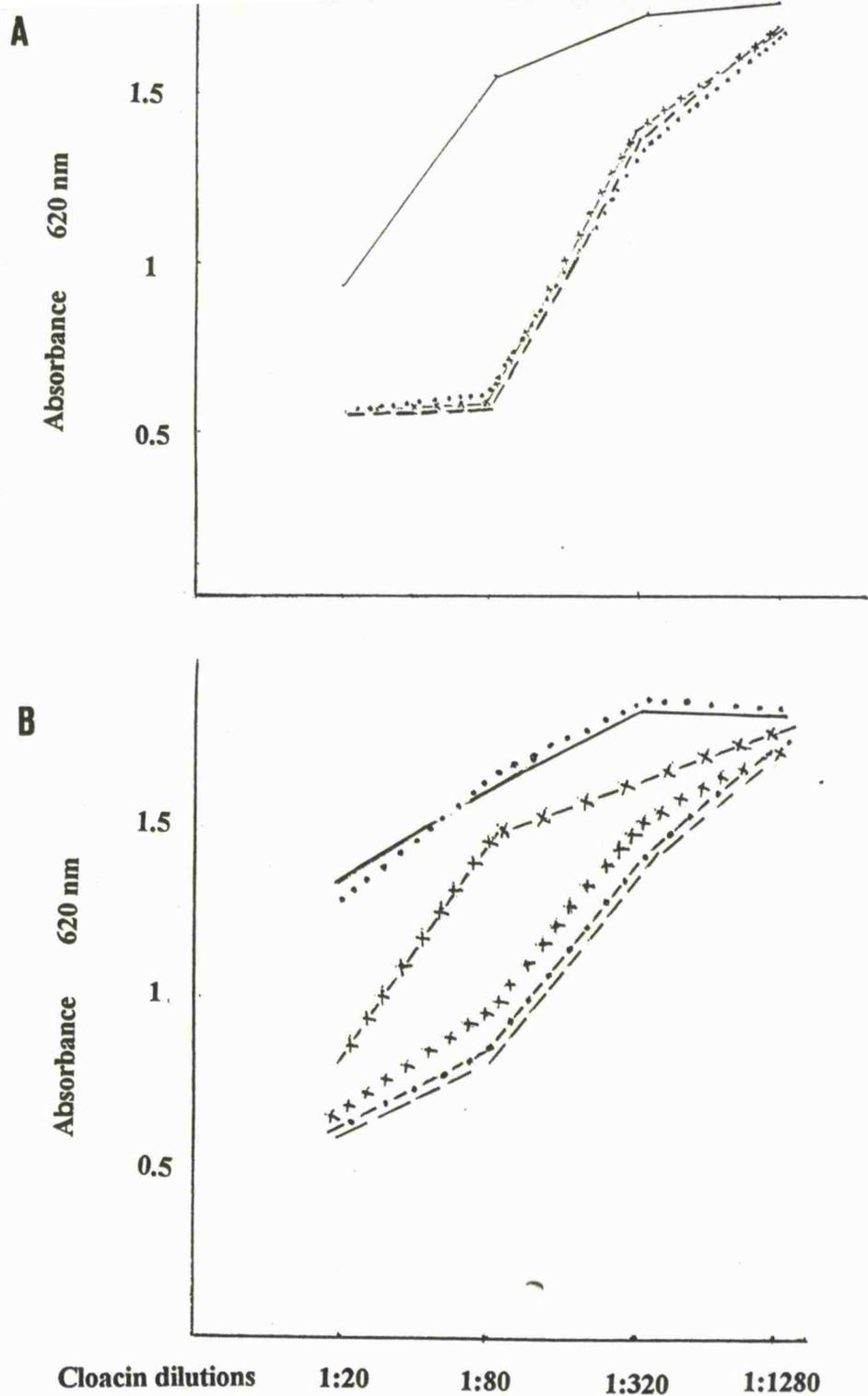


Figure 3.5: Growth (absorbance at 620 nm) of the indicator strain *Klebsiella pneumoniae* M51a in LUB containing residual cloacin DF13 in serial dilutions after adsorption by outer membrane protein preparation of *E. coli* DH5 α expressing: A: *iutA* (—), *iutA_{Sh}* (---), no receptor (-X -X); (.....) represents a control without adsorption by outer membrane proteins. B: construct number 1 (-X - X), 2 (X X X X), 3 (.....), 4 (-.-.-.-), 5 (—), 6 (---).

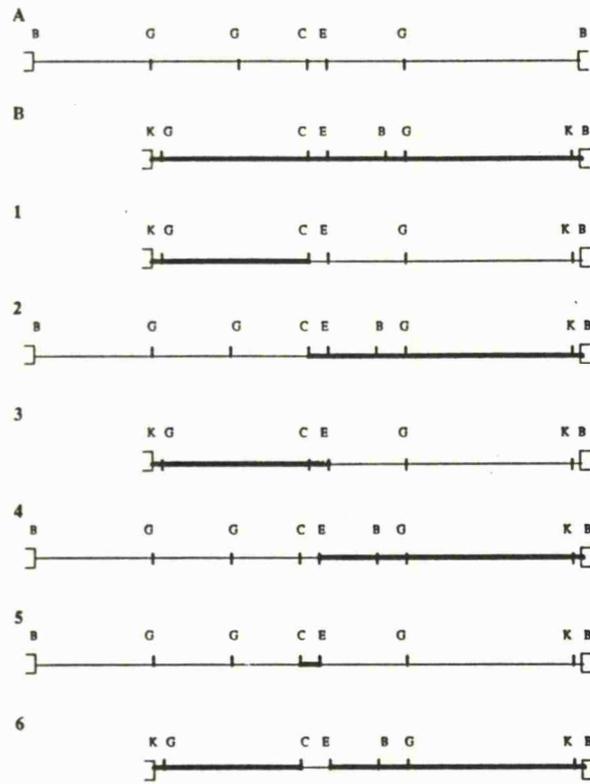
The cloacin adsorption by outer membranes of a strain carrying construct 5, which is the result of a replacement of the central fragment between *ClaI* and *EcoRI* sites of *iutA* with the equivalent fragment of *iutA_{Sh}*, was higher than that of a strain expressing the original IutA. Again, this observation suggested that the *iutA_{Sh}* fragment between the *ClaI* and *EcoRI* sites may be able to increase the cloacin adsorption function of the *iutA* fragment downstream of *EcoRI* site.

Construct 6, which has the opposite structure to construct 5, only slightly increased cloacin adsorption by outer membrane preparations compared with those of a strain expressing normal IutA_{Sh}. In this case, there is a possibility that the central fragment of *iutA* between *ClaI* and *EcoRI* was able to influence cloacin adsorption although to a lesser extent than the other fragments of *iutA* (upstream and downstream of the *ClaI* and *EcoRI* sites respectively).

3.3.5. Aerobactin uptake

The uptake of [¹⁴C] aerobactin (measured as described in section 3.2.4) of *E. coli* carrying the cloned *iutA_{Sh}* and *iutA* genes was similar (figure 3.6), suggesting that, despite difference in cloacin binding, the two receptors were equally efficient in siderophore binding. Aerobactin uptake by strains carrying constructs 1 and 5 was significantly less than the wild type, constructs 3 and 6 were similar and constructs 2 and 4 were significantly higher than that of a strain expressing the *E. coli* ColV-K30 or *Shigella* IutA proteins (figure 3.6).

Comparing aerobactin uptake by strains carrying constructs 1-4, the data showed that aerobactin uptake by strains carrying constructs 2 and 3 was higher than that of strains carrying constructs 4 and 1 respectively. The difference between these



Reference restriction maps of aerobactin receptor genes.

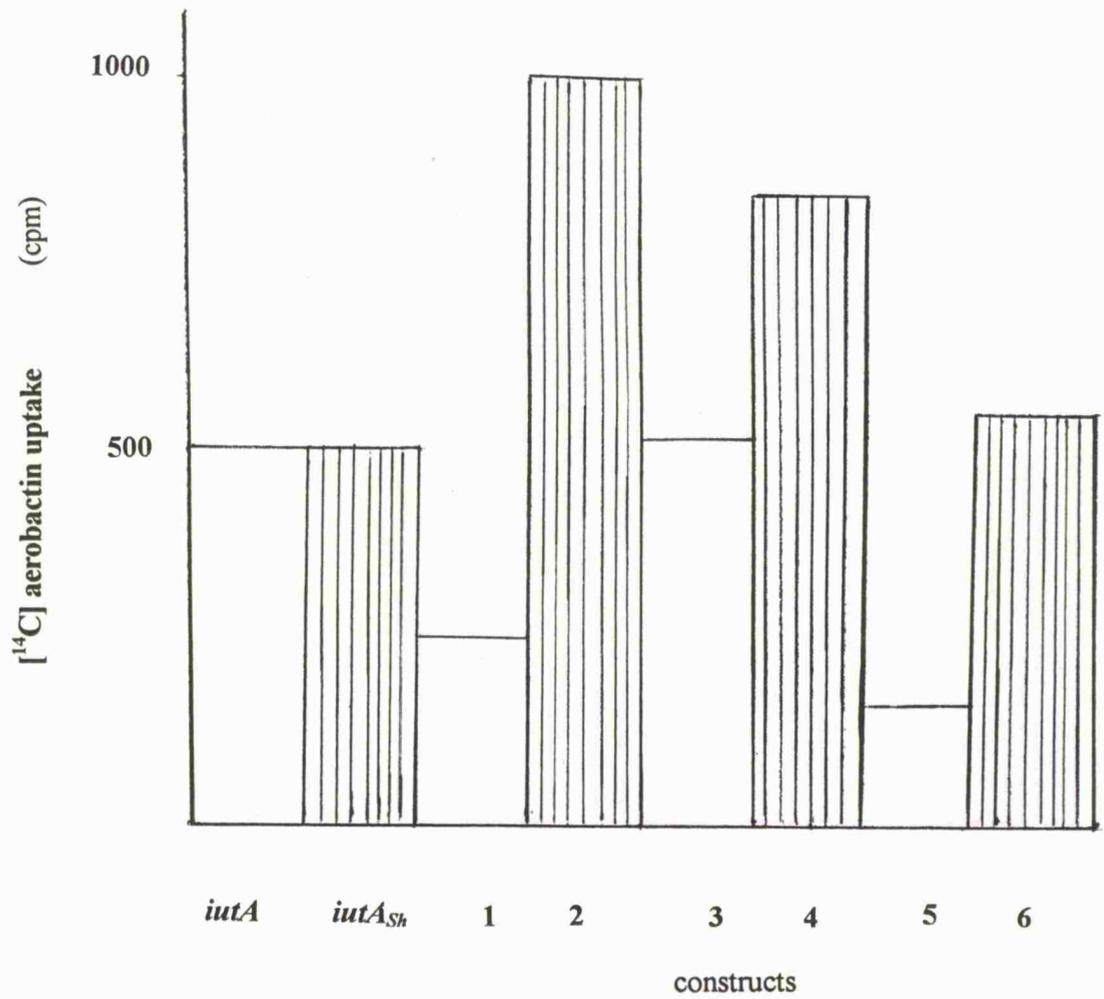


Figure 3.6: [¹⁴C] aerobactin uptake of *E. coli* expressing *iutA* and constructs with the 3'-terminus of *iutA* (empty blocks) or *iutA_{Sh}* and constructs with the 3'-terminus of *iutA_{Sh}* (striped blocks). Numbers 1-6 represent constructs 1-6.

constructs was the presence of the *ClaI-EcoRI* fragment of *iutA_{Sh}* in constructs 2 and 3, while constructs 1 and 4 contained the equivalent fragment of *iutA*. Thus, it seems that the *ClaI-EcoRI* fragment of *iutA_{Sh}* is more influential in the aerobactin uptake function than the homologous fragment of *iutA*.

On the other hand, replacement of the central *iutA* fragment between the *ClaI* and *EcoRI* sites with the equivalent fragment of *iutA_{Sh}* (construct 5) decreased aerobactin uptake compared with a strain expressing *IutA*. In addition, a strain carrying construct 6, which is the reciprocal construct of construct 5, showed a similar level of aerobactin uptake to that of a strain expressing the original *iutA_{Sh}*.

Comparison of aerobactin uptake by strains carrying construct 1 with 2 and 3 with 4 indicates that the 3'-terminus of *iutA_{Sh}* and the 5'-terminus of *iutA* (as in constructs 2 and 4) cooperate more efficiently than in the natural genes, suggesting that functions involved with aerobactin translocation are dispersed within the receptor genes.

3.4. DISCUSSION

The result of cloacin sensitivity and cloacin adsorption experiments revealed that replacement of each fragment of *iutA* with the equivalent fragment from *iutA_{Sh}* decreased cloacin sensitivity, regardless of the migration of the outer membrane proteins on SDS-PAGE. For example, although the outer membrane protein expressed by construct 2 is 76 kDa, the same as *IutA_{Sh}*, cloacin adsorption and cloacin sensitivity conferred by construct 2 were significantly higher than those conferred by

iutA_{Sh}. All constructs with the 3'-terminus from *iutA* expressed a 74 kDa protein, while constructs with the 3'-terminus from *iutA_{Sh}* expressed a 76 kDa protein on SDS-PAGE. These results also revealed that these protein conformations do not depend on the signal peptide sequences, but depend on certain amino acid sequences within the polypeptides, as mentioned in section 2.4.

Marolda *et al.* (1991) also observed that an enteroinvasive *E. coli* 978-77::pColV-K30 chimeric *iutA* gene encoded a protein of 74 kDa determining a level of cloacin susceptibility identical to that mediated by ColV-K30, while the pColV-K30::*E. coli* 978-77 type encoded a 76 kDa which mediated a low level of cloacin susceptibility. The second hybrid genes was similar to construct 4 (3'-terminus of *iutA_{Sh}*) with a junction at the *EcoRI* site. This construct (4) also encoded a 76 kDa outer membrane protein, but it mediated cloacin sensitivity higher than that of the original *IutA_{Sh}*, although less than that of the *IutA*.

Although the restriction maps of the aerobactin receptor genes of enteroinvasive *E. coli* and *Shigella* were very similar (Marolda *et al.*, 1987), the similarity of their nucleotide sequences has not been determined. In addition, the cloacin sensitivity conferred by aerobactin receptor proteins does not depend on the apparent molecular weight determined on SDS-PAGE; for example, the 76 kDa protein from *K. edwardsii* confers a high susceptibility to cloacin (Krone *et al.*, 1985), while the cloacin sensitivity of *Shigella* are very low, although they express the aerobactin receptor as a 76 kDa protein.

Two factors are involved in cloacin sensitivity, cloacin adsorption to the surface and translocation to the cytoplasmic site of action (Tiel-Menkveld *et al.*, 1982; Krone *et al.*, 1986). If a bacterial strain is less sensitive to cloacin but cloacin adsorption is

the same as or higher than that of a cloacin sensitive strain, it is possible that there is a defect in the translocation system.

The results of cloacin sensitivity and cloacin adsorption experiments revealed that each fragment of *iutA* (a: the fragment upstream of the *ClaI* site, b: the fragment between the *ClaI* and *EcoRI* sites, and c: the fragment downstream of the *EcoRI* site) is probably involved in cloacin adsorption and translocation, since replacement of each fragment of *iutA_{Sh}* with the homologous fragment of *iutA* give higher levels of cloacin adsorption and sensitivity compared to the *iutA_{Sh}*. Alternatively, mixing and matching fragments may alter the conformation of the resulting proteins, thus indirectly influencing cloacin sensitivity.

Analysis of constructs 1 and 3 showed that strains carrying these constructs had the same level of cloacin sensitivity despite the fact that cloacin adsorption by a strain harbouring construct 3 is higher than that of a strain carrying construct 1 or the cloned *iutA* gene. These constructs have the 3'-terminus fragment of *iutA* downstream of *ClaI* site (construct 1) and downstream of *EcoRI* site (construct 3), so the difference between these constructs is the presence of the *ClaI-EcoRI* fragment of *iutA* in construct 1. This phenomenon was also observed with construct 2 and 4, and so there is a possibility that this fragment has a role in cloacin translocation and aerobactin transport (constructs 2 and 4 conferred significantly higher aerobactin uptake than the original *iutA* and *iutA_{Sh}*). If the similarity in cloacin susceptibility was due to the insensitivity of the semi-quantitative assay method, there is a possibility that constructs 3 and 2 conferred higher cloacin sensitivity than constructs 1 and 4 respectively; in this case, it is difficult to distinguish whether the *ClaI-EcoRI* fragment of *iutA* influences cloacin binding or cloacin translocation or both. In addition,

construct 6, which is *iutA_{Sh}* with its *ClaI-EcoRI* fragment replaced by the homologous fragment of *iutA*, conferred a slightly higher cloacin sensitivity and cloacin adsorption than those conferred by *iutA_{Sh}*. This suggests an influence of the *ClaI-EcoRI* fragment of *iutA* in cloacin sensitivity and cloacin adsorption.

Construct 5 (*iutA* in which the *ClaI-EcoRI* fragment is replaced by the equivalent fragment of *iutA_{Sh}*) encoded a 74 kDa and an additional 70 kDa outer membrane protein and mediated significantly higher cloacin adsorption than that conferred by *iutA*, although cloacin sensitivity and aerobactin uptake conferred by this structure were less than those conferred by *iutA*. These results showed the influence of the *ClaI-EcoRI* fragment of *iutA* in cloacin sensitivity and aerobactin uptake. Another possibility is that this fragment may also function in maintaining the stability of *iutA*; the outer membrane protein profile of a strain harbouring this construct showed an additional smaller protein species which may be a degradation product of the 74 kDa protein.

Although the *ClaI-EcoRI* regions of *iutA* and *iutA_{Sh}* are small, consisting of 45 amino acids with only 5 residues different between them, this fragment has an important influence on cloacin sensitivity and in aerobactin uptake. In construct 5, it appears that the presence of five altered residues in this particular fragment may be important for the stability of IutA protein, because replacement of this fragment with the homologous fragment from *iutA_{Sh}* affects the outer membrane profiles. Perhaps the smaller protein is also able to adsorb cloacin, but does not have the ability to translocate cloacin or to take up aerobactin. On the other hand, the presence of this small fragment of *iutA* in *iutA_{Sh}* slightly increased cloacin sensitivity and aerobactin uptake, as shown by construct 6.

CHAPTER 4

THE ISOLATION OF BACTERIOPHAGE B74K

4.1. INTRODUCTION

The cell envelope of Gram-negative bacteria is composed of two distinct membranes, the inner cytoplasmic membrane and the outer membrane. The space between the two membranes is termed the periplasmic region (Osborn *et al.* 1972). The outer membrane constitutes a physical and functional barrier between the cell and its surroundings which effectively controls access of solutes and external agents to the cytoplasmic membrane. The major function of the outer membrane is to act as a barrier to antibiotics, detergent, and other toxic chemicals, but this function is accompanied by permeability properties that allow entry of nutrients from the medium. Another important function of the outer membrane is evasion of phagocytosis and avoidance of specific immune attack by altering the surface antigen constitution. The outer membrane of Gram-negative bacteria contains two types of lipids, lipopolysaccharides (LPS) confined to the outer leaflet and phospholipids confined to the inner leaflet in the *Enterobacteriaceae*, as well proteins (Nikaido and Vaara, 1985). Some of these outer membrane components function as receptors for bacteriophages and bacteriocins (Osborn and Wu, 1980).

The outer membrane proteins of *Escherichia coli* can be classed as major or minor proteins reflecting their levels of expression. The major outer membrane proteins consist of murein lipoprotein, OmpA and the porins. Murein lipoprotein is

a small protein (7200 daltons), present in a large number of copies (Nikaido and Vaara, 1985), which appears not to be essential for growth under laboratory conditions (Hirota *et al.*, 1977). Lipoprotein defective mutants are also normal in the diffusion of small, hydrophilic solutes (Nikaido *et al.*, 1977), but the cell wall structure in these mutants appear to unstable, resulting in the release of outer membrane vesicles and periplasmic enzymes (Hirota *et al.*, 1977; Suzuki *et al.*, 1978). The OmpA protein has a molecular weight of approximately 40 kDa, similar to that of the porins. Mutants lacking the OmpA protein show reduced overall transport rates for amino acids (Manning *et al.*, 1977), produce unstable outer membranes and are defective in conjugation functions (Manning and Achtman, 1979). In addition, it has been reported that OmpA serves as a receptor for phage TuII* and K3 (Schwartz, 1980; Lugtenberg and Alphen, 1983).

Porins are proteins encoded by the *ompF*, *ompC* and *phoE* genes of *E. coli* K-12; their molecular weights are in the range 30-40 kDa. They are called porins because they form relatively nonspecific pores or channels that allow the passage of small hydrophilic molecules with molecular weights less than about 600 daltons across the outer membrane (Nakae and Nikado, 1975). The physiological importance of porins in allowing diffusion of nutrients, antibiotics or inhibitors across the outer membrane has been established by the use of porin-deficient mutants (Bavoil *et al.*, 1977; Lutkenhaus, 1977). The OmpF porin of *E. coli* acts as the receptor for phage TuIa and for colicin A, while OmpC acts as the receptor for bacteriophages TuIb and Me1 (Datta *et al.*, 1977; Chai *et al.*, 1982).

Some minor proteins are also present in the outer membrane. The term "minor" may be misleading since under certain growth conditions some of these

proteins are made in quantities comparable to those of the major proteins. Many minor proteins have been identified as receptors for phages and colicins, besides their primary functions (DiRienzo *et al.*, 1978; Konisky, 1979) (table 1.2).

Generally individual phages recognize only a single receptor protein; however, there are exceptions. For example, a host-range mutant of phage TC23 is able to use protein Ia, protein Ib or protein E as its receptors (Chai and Foulds, 1978). An analogous situation was described in *Salmonella typhimurium* where one phage, PH42, could use either a 34 kDa or a 36 kDa protein as its receptors (Johansson *et al.*, 1978). These findings are not, however, a serious divergence from the specificity rule, since proteins Ia, Ib and E, and the proteins of 34 and 36 kDa had very similar amino acid sequences (Ichihara and Mizushima, 1978). Surprisingly, phage TP1, which is a host-range mutant of phage Tu1a, was able to use OmpF and LamB proteins as its receptors. Although these proteins bear some functional similarities (Boehler-Kohler *et al.*, 1979), their molecular weights and amino-terminal sequences were quite different (Endermann *et al.*, 1978).

The first step in phage infection is the interaction between a phage particle and a receptor molecule on the bacterial cell (Schwartz, 1980). Pelzcar *et al.*, (1993) described that the initial adsorption of phage to receptor is reversible when only the tail fibres are attached to the cell surface, but becomes irreversible when the tail pins attach to the cell surface. After the phage has attached to the host cell, its DNA passes through the cell wall and into the cytoplasm of the bacterial cell.

The receptor activities of iron regulated outer membrane proteins were listed in Table 1.2. In addition the vitamin B12 receptor BtuB is also a receptor of phage BF23, colicins E1, E2, and E3; the maltose uptake receptor LamB is the phage λ

receptor, and Tsx, the receptor for nucleoside uptake, is also the receptor for phage T6 and colicin K.

These data show that outer membrane proteins are often exploited by xenobiotic agents such as bacteriophage and bacteriocin in addition to their primary function as receptors. It has been mentioned above that IutA is the receptor of aerobactin and cloacin DF13, but it has not previously been determined whether IutA also serves as a receptor for any bacteriophage. The aim of this research was to isolate a bacteriophage from the environment that could utilize IutA as its receptor, as a potential tool for the analysis and identification of the IutA protein.

4.2. MATERIALS AND METHODS

4.2.1 Isolation of bacteriophage.

The method used was based on the method described by Makela (1985). Crude sewage was sterilized using 0.2-0.45 μm pore diameter membrane filters. 10-100 ml of sterile sewage was added to 100 ml of M9 medium containing 0.2 mM dipyriddy and 1 ml of an overnight broth culture of *E. coli* K-12 strain W3110(CoIV-K30). The mixture was incubated overnight at 37°C with shaking. 10 ml of the mixture was centrifuged to remove bacteria and the supernatant was filter sterilized. 1 ml of the supernatant was added to 100 ml of M9 medium containing 0.2 mM dipyriddy, 1 ml of an overnight culture of W3110(CoIV-K30) was added, followed by incubation at 37°C overnight with shaking. Again, 10 ml of the mixture was centrifuged to remove

Table 4.1: Bacterial strain and plasmids used.

Bacterial strains/ plasmids	Relevant properties	Reference
<i>E. coli</i> K-12		
W3110	Wild type	Bachmann, 1972.
BZB1022	W3110, <i>cir gyrA</i> (Nal ^R)	Pugsley, 1985.
AN1937	<i>ara entA lac leu mtl proc</i> <i>rspL supE thi fhuA xyl</i>	Williams, 1979.
LG1522	AN1937, <i>fepA</i> (pColV-K30, <i>iuc</i> ⁻)	Carbonetti & Williams, 1984
LG1703	<i>araD139 (ara, leu)</i> 7697 <i>lacX74 phoA 20 galE galK</i> <i>thi rpsE rpoB argE_{am} recA</i> <i>ompC⁺ ompF⁺</i> (pLG141) cloacin tolerant	Wooldridge, 1991
DH5 α	<i>supE44 lacU169</i> (80 <i>lacZ</i> M15) <i>hsdR17 recA1 endA1</i> <i>gyrA96 thi-1 relA1</i>	Hanahan, 1983
MH760	MC4100 { <i>araD139 lacU169</i> <i>rpsL relA thiA fibB (ompC⁺</i> <i>ompF⁺)</i> }, <i>ompR472 (ompC ompF⁺)</i>	Taylor <i>et al.</i> , 1983
MH1160	MC4100, <i>ompR101 (ompC⁻ ompF⁻)</i>	Taylor <i>et al.</i> , 1983.
MH1460	MC4100, <i>envZ11 (ompC⁺ ompF⁻)</i>	Taylor <i>et al.</i> , 1983.

MW4	W3110, phage B74K ^R	This study
B12	BZB1022, phage B74K ^R	This study
MC34	LG1703, phage B74K ^R	This study
Plasmids:		
ColV-K30	<i>iutA</i> ⁺ <i>iuc</i> ⁺ <i>cva</i> ⁺	Williams, 1979
pABN1	<i>iutA</i> ⁺ <i>iucA</i> ⁺ <i>bla</i> ⁺ (Ap ^R)	Bindereif & Neilands, 1983
pLG141	<i>iutA</i> ⁺ <i>cat</i> ⁺ (Cm ^R)	Carbonetti & Williams, 1984
pLG2134	<i>iutA</i> subcloned (<i>Bam</i> HI fragment) in pUC19	This study
pLG2136	<i>iuc</i> ⁺ <i>iutA</i> ⁺ in pUC19	This study

bacteria and the supernatant was filtered sterilized. The supernatant was serially diluted in PBS. 100 μ l of each dilution were added to tubes each containing 3 ml of soft agar and 100 μ l of an overnight culture of W3110(CoIV-K30), mixed and poured on to M9 minimal medium agar containing dipyriddy. Plates were incubated at 37°C overnight, and single plaques were tested for their ability to grow on W3110(pLG141) but not on W3110.

4.2.2. Preparation of bacteriophage lysates.

Plate lysates were prepared by mixing approximately 10^4 to 10^5 bacteriophages in 2.5 ml of soft agar (nutrient broth + 0.5% agar) containing 10^8 cells of strain W3110(pLG141), and the mixture was overlaid on fresh nutrient agar plates. After overnight incubation at 37°C, soft agar layers were collected and centrifuged to pellet bacteria. The supernatant containing bacteriophage was decanted and sterilized with chloroform. Lysates were also prepared in broth by mixing approximately 10^3 bacteriophages with 10^8 cells of strain W3110(pLG141) per ml, and incubating with vigorous aeration for 2 hours at 37°C or until visible lysis had occurred. Lysates are sterilized with chloroform and clarified by centrifugation. These methods were based on methods of Chai and Foulds (1978).

4.2.3. Phage particle preparation.

DNase and RNase were added to lysates to a final concentration of each of 1 μ g ml⁻¹ followed by incubation at room temperature for 30 min. Solid sodium chloride

was added to this mixture to a final concentration of 1M and allowed to dissolve, and the mixture was incubated on ice for one hour. Debris was removed by centrifuging the mixture at 11,000 x g for 10 min at 4°C. PEG 6000 was added to the supernatant to a final concentration of 10% (w/v), followed by slow stirring on a magnetic stirrer at room temperature, and then cooling in iced water for 1 hour to allow phage particles to form a precipitate. The mixture was centrifuged at 11,000 x g for 10 min at 4°C, and the pellet was resuspended in PBS.

4.2.4. Extraction of bacteriophage genetic material.

0.5 M EDTA solution (pH8.0) was added to bacteriophage suspension to a final concentration of 20 mM. Proteinase K and 10% w/v SDS were added to this mixture to final concentrations of 50 µg/ml and 0.5% respectively, then incubated at 56°C for 1 h. An equal volume of phenol equilibrated with 50 mM Tris (pH8.0) was added to this mixture, mixed well, and then centrifuged at 3000 x g for 5 min at room temperature to separate the phases. The aqueous phase was collected and extracted with an equal volume of phenol-chloroform. The aqueous phase was collected as before and the DNA was recovered by adding 3 M sodium acetate (pH7.0) to a final concentration of 0.3 M and 2 volumes of ethanol, mixing well and storing the solution at room temperature for 30 min. The DNA was transferred to a microfuge tube containing 1 ml of 70% ethanol, centrifuged at 12,000 x g for 2 min at 4°C and redissolved in TE (pH 7.6).

4.2.5. Bacteriophage binding assay.

Cells from 1.5 ml of overnight nutrient broth cultures of W3110 or W3110(pLG141) were harvested by centrifugation at 10000 x g for 5 min, washed in PBS, and resuspended in either PBS alone or in PBS containing appropriately diluted antibodies against Triton/urea-soluble outer membrane preparation of *E. coli* cells expressing IutA. Incubation was at 37°C for 15 min. Phage (10^5 p.f.u. in 0.1 ml) were added to each suspension and incubated at 37°C for 15 min. The cells were pelleted by centrifugation at 10000 x g for 5 min, and chloroform-sterilized supernatant fractions were titrated in standard phage assays with W3110(pLG141) as the indicator lawn, in order to determine the residual phage titre.

4.2.6. Competitive binding of aerobactin, cloacin DF13 and bacteriophage to IutA.

Samples of ferric-aerobactin (10 μ l of a 10 μ g /ml solution) were spotted at each of three positions on a lawn of W3110(pLG141) on nutrient agar and allowed to dry. 5 μ l samples of undiluted cloacin DF13 or bacteriophage preparations and of 10-fold and 100-fold dilutions of these preparations were applied in two rows, one coincident with and one away from the aerobactin spots. The plate was incubated overnight at 37°C.

Interference by cloacin DF13 of phage adsorption was determined as follows: 25 μ l of cloacin or PBS was added to 1 ml of a bacterial cell suspension (cells were collected from overnight cultures and suspended in PBS), incubated at 37°C for 30 minutes, and then centrifuged at 10000 x g for 2 min at 4°C. Cells were resuspended in 0.9 ml PBS. 100 μ l of PBS or phage suspension (10^6 p.f.u. /ml) were added to the

suspension and incubated at 37°C for 10 min, and then 100 µl of chloroform were added. The mixture was centrifuged at 1000 x g for 2 min at 4°C, diluted 100-fold, and 100 µl of the dilution were mixed with 100 µl of a culture of W3110(pLG141) in 3 ml of soft nutrient agar. This was poured onto nutrient agar plates, and incubated overnight at 37°C

4.3. RESULTS

4.3.1. Isolation and characterization of bacteriophage B74K.

Bacteriophage B74K was isolated among single plaques that were selected for the ability to grow on the *E. coli* strain expressing IutA, W3110(pLG141), but not on the IutA⁻ strain W3110. Electron microscopy showed that the phage has a hexagonal head and a tail with fibres; the size of the head is 57.6 nm by 54.5 nm, while the tail is 24.2 nm long and 9.1 nm wide (figure 4.1). The genetic material of the phage was isolated by extracting protein components with phenol and precipitating the genetic material with ethanol (section 4.2.4) Treatment with either DNase or RNase and analysis on agarose gels showed that it was DNA. The absorbance at 260 nm of diluted DNA in 0.5M NaOH was higher than in distilled water (0.067 and 0.031 respectively); in addition, the absorbance of the DNA in distilled water increased after heating at 80°C for 10 min (0.009 and 0.017 respectively). These data demonstrated that the DNA was double stranded (figure 4.2). Furthermore, electron microscopy indicated that the DNA was linear (figure 4.3).

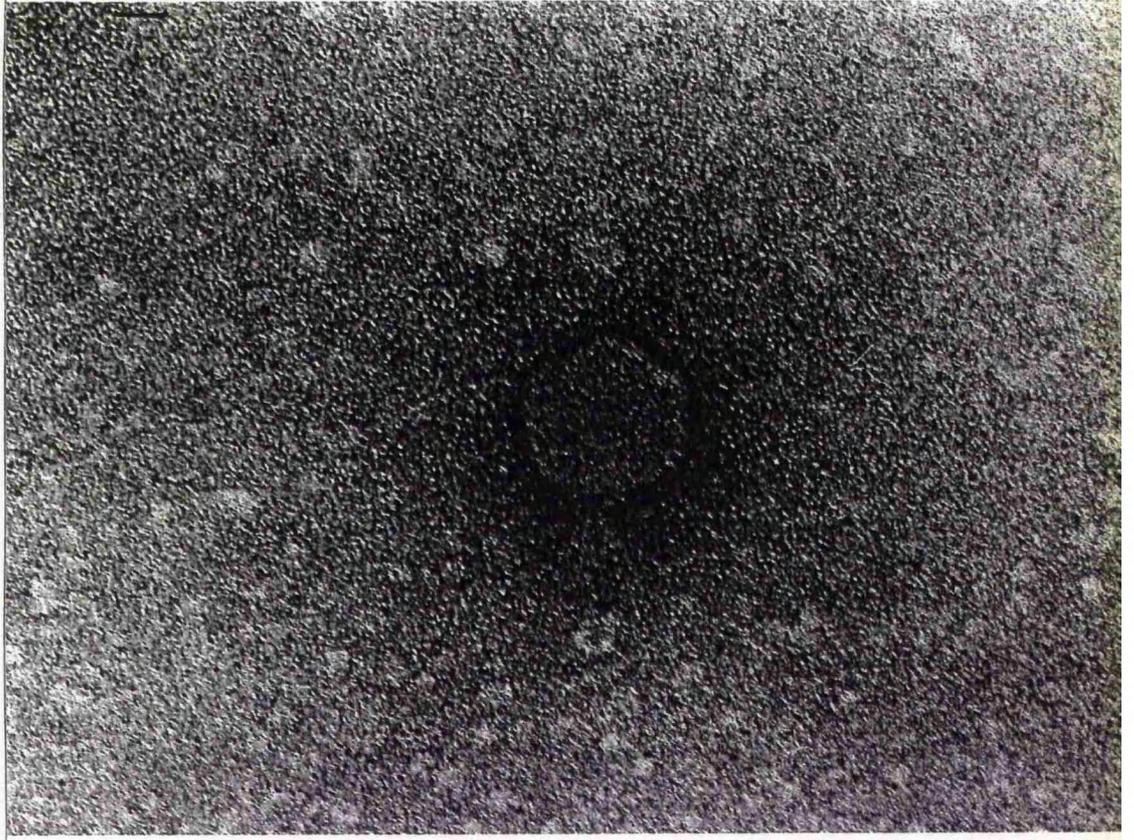


Figure 4.1: Electron micrograph of bacteriophage B74K.

(magnification x 100,000)

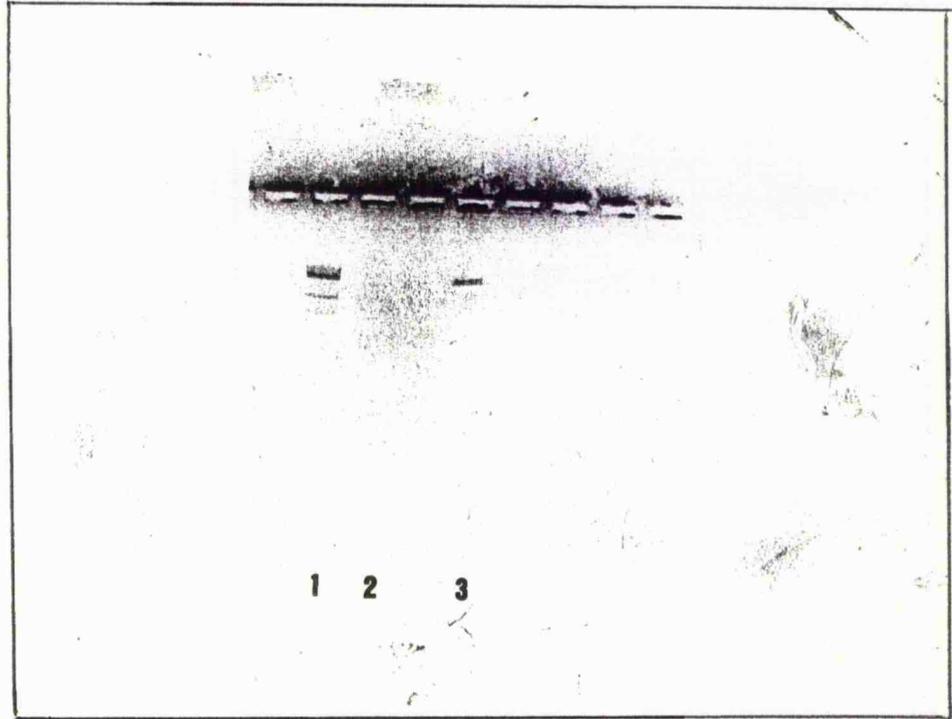


Figure 4.2: Agarose electrophoresis of the marker (1); genetic material of phage B74K after treatment with DNase (2); and RNase (3).

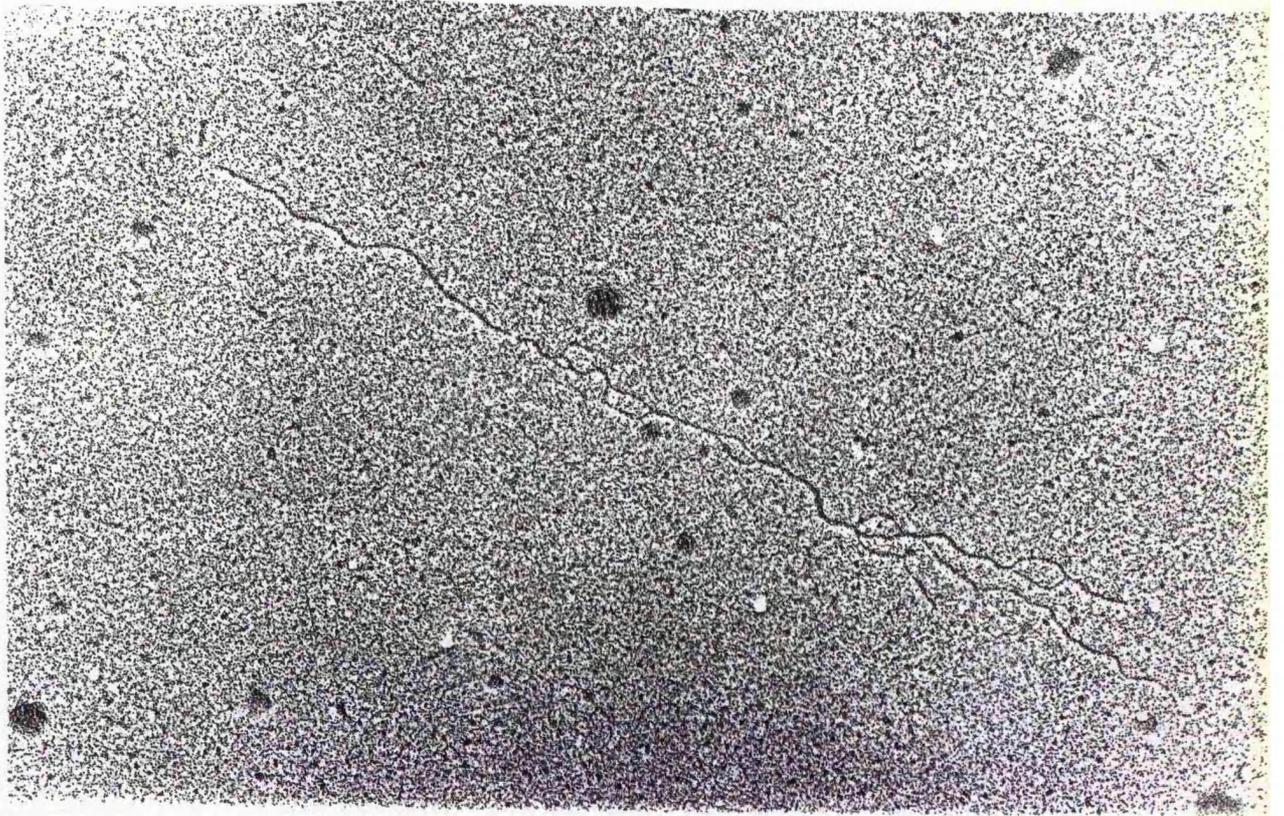


Figure 4.3: Electron micrograph of phage B74K DNA.

(magnification x 100,000)

4.3.2. Phage adsorption.

Phage adsorption to *E. coli* strains W3110 and W3110(pLG141) was determined by assaying residual phage titres after allowing for adsorption. As expected, adsorption to W3110 was less than to W3110(pLG141), and adsorption to the latter strain was inhibited by antiserum raising against Triton/urea-soluble outer membrane of *E. coli* strain BZB1022(pABN1).

The effect of phage adsorption on aerobactin binding was determined by spot tests on agar plates containing aerobactin. The spot test experiment showed that although aerobactin inhibited cloacin DF13 killing activity, it did not inhibit infection of W3110(pLG141) by B74K (Roberts *et al.*, 1989) (table 4.2). On the other hand, although plaque titres were higher on *E. coli* strains carrying *iutA* than on *iutA* strains in the absence of the complete aerobactin biosynthesis genes, they were lower if the strains were able to synthesize aerobactin (figure 4.4). These data suggest that endogenous aerobactin may interfere with adsorption of B74K.

To observe the effect of cloacin DF13 on adsorption of B74K, strains W3110 and W3110(pLG141) were incubated with cloacin, the cells were collected by centrifugation and incubated with bacteriophage to evaluate their ability to adsorb the phage. The results indicated that the ability of W3110(pLG141) to adsorb the bacteriophage was decreased by cloacin treatment, while bacteriophage adsorption of W3110 was not significantly different with or without cloacin treatment (figure 4.5).

4.3.3. The sensitivity of *E. coli* strains to B74K.

The ability of bacteriophage B74K to produce plaques on *E. coli* strains is shown in table 4.3. Because the plaques of phage B74K are big, plaque counts per plate in

Table 4.2: Inhibition of phage B74K adsorption to IutA by antiserum raised against Triton/urea-soluble outer membranes of *E. coli* strain BZB1022 (pABN1). Phage lysate were incubated with bacteria (untreated/treated with the antiserum), after removal of the bacteria, the residual phage titre was determined.

Strains	Antiserum	10 ⁻² residual phage titre (p.f.u.)
W3110	-	80
W3110	Antiserum (1:2)	94
W3110 (pLG141)	-	9.5
W3110 (pLG141)	Antiserum (1:2)	80
W3110 (pLG141)	Antiserum (1:10)	68
W3110 (pLG141)	Pre-immune serum (1:2)	12

Table 4.3: The ability of several *E. coli* strains to support phage B74K growth on nutrient agar (NA) and on minimum medium agar (MMA)+dipyridyl.

Strains	The number of plaques of B74K on	
	NA	MMA+dipyridyl.
W3110	0	25
W3110(pLG141)	22	35
BZB1022 (W3110 <i>cir</i> ⁻)	0	27
BZB (pABN1)	0	15
AN1937 (<i>entA</i> ⁻ <i>tonA</i> ⁻)	26	13
LG1466 (<i>entA</i> ⁻ <i>tonA</i> ⁻ <i>fepA</i> ⁻)	11	27
LG1522 {LG1466 (pLG141)}	19	22

Table 4.4: The sensitivity of B74K resistant mutants to several phages and colicins, s: sensitive; r: resistant; -: could not be observed.

Strains	The sensitivity to									
	phage					colicins				
	B74K	BF23	TuIa	TuIb	T6	A	D	G	N	K
W3110	s	s	s	s	s	s	s	s	s	s
MW4	r	r	r	r	r	r	r	r	r	r
MW4 (pLG141)	r	s	s	-	s	r	s	s	r	r
B12	r	r	r	r	r	r	r	s	r	r
MC34	r	s	s	s	s	r	s	s	r	r

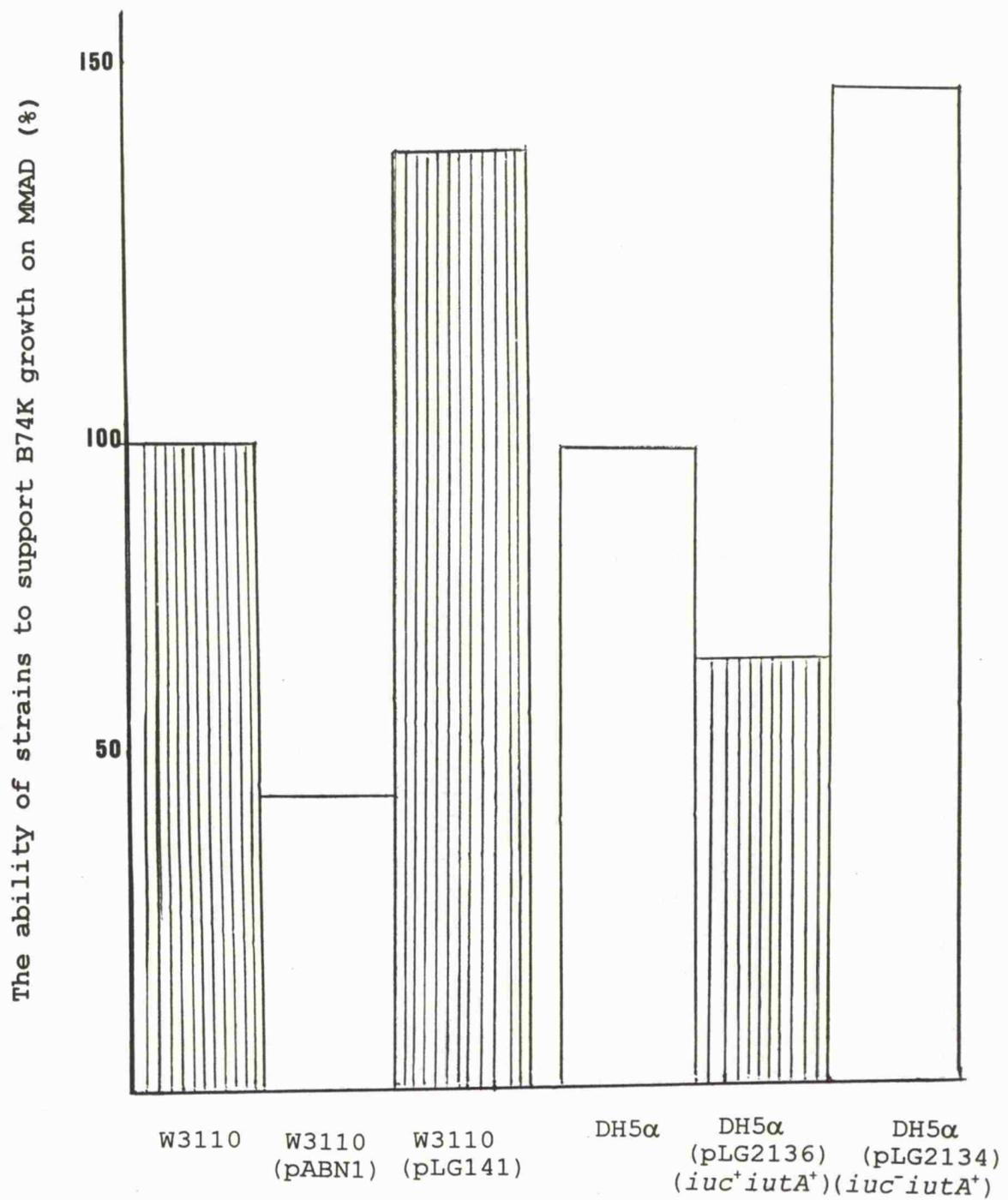


Figure 4.4: the ability of *E. coli* expressing IutA to support B74K growth on minimum medium agar containing dipyrindyl in the presence or absence the *iuc* genes.

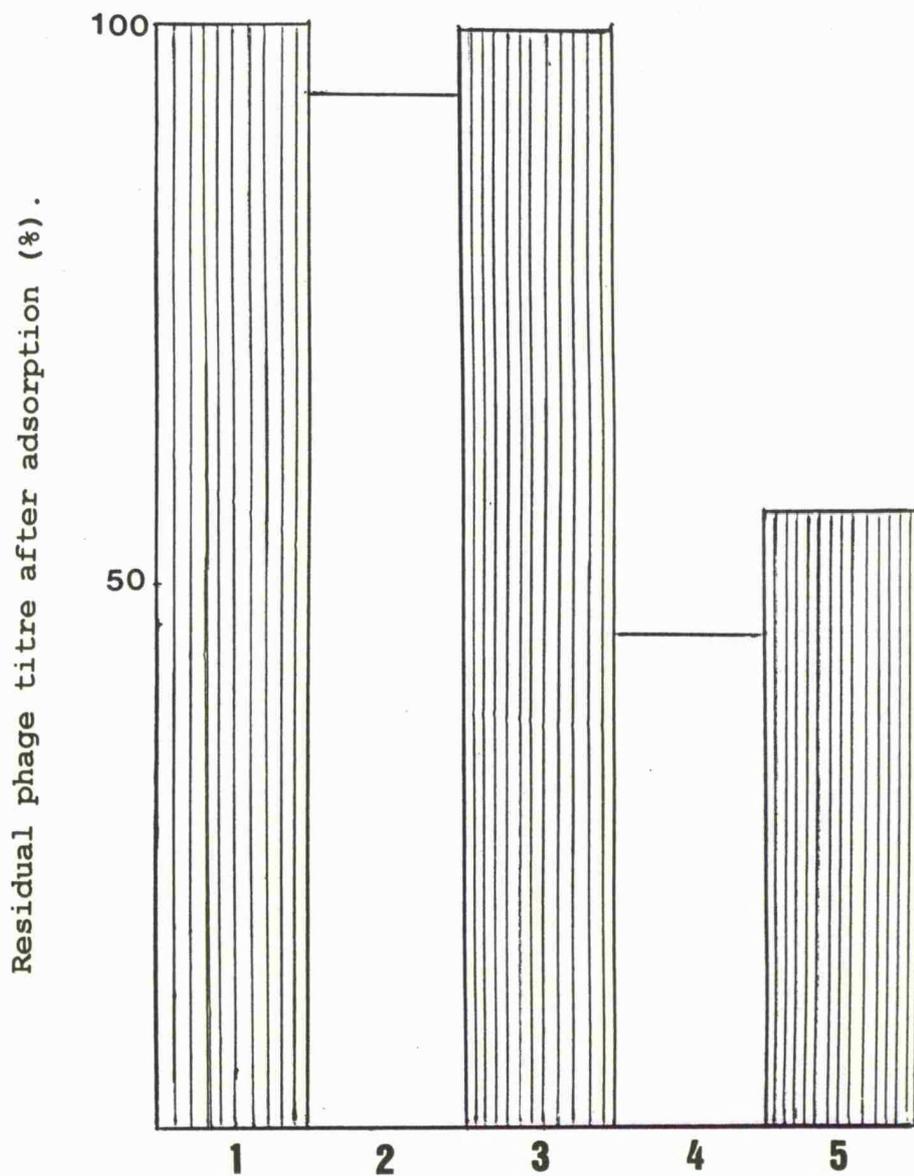


Figure 4.5: Cloacin DF13 inhibition to B74K adsorption of *E. coli* in the presence or absence of IutaA.

- 1: Phage titre (control).
- 2: Residual phage titre after W3110 adsorption.
- 3: Residual phage titre after W3110 (which has been treated with cloacin) adsorption.
- 4: Residual phage titre after W3110 (pLG141) adsorption.
- 5: Residual phage titre after W3110 (pLG141) (which has been treated with cloacin) adsorption.

this experiment are necessarily rather low. Strains W3110 and BZB1022 were more sensitive on M9 agar containing dipyriddy, while strains AN1937, LG1522 and LG1466 were equally sensitive to phage B74K on both nutrient agar and minimum agar containing dipyriddy and citrate. The ability of phage B74K to grow on *iutA* strains was not expected, because the phage was isolated using W3110(pLG141) rather than W3110.

4.3.4. B74K resistant mutants of *E. coli*.

On nutrient agar, the number of plaques of phage B74K on W3110 was significantly lower than on W3110 (pLG141), but the difference was less when they were grown on minimum medium agar containing dipyriddy. This suggests that besides using IutA as its receptor, phage B74K must also be able to use an alternative receptor(s).

In order to identify the receptor(s) of bacteriophage B74K, *E. coli* strains resistant to B74K were isolated; mutants MW4, B12, Mc34 are derivatives of strains W3110, BZB1022 and LG1703 respectively. The outer membrane proteins of these mutants were prepared and analyzed on SDS-PAGE (figure 4.6); the results showed that the mutants had both lost many proteins and gained same new protein bands. In addition, the sensitivity of the mutants to several colicins (table 4.4) and bacteriophages was also identified.

Mutant MW4 had lost OmpC and OmpF and many other proteins and gained several new proteins. This mutant was also resistant to bacteriophages BF23, TuIa, TuIb and T6 which have as receptors on BtuB, OmpF, OmpC and Tsx proteins respectively (Reeves, 1979). Mutant MW4 was resistant to colicins A, D, G, N and

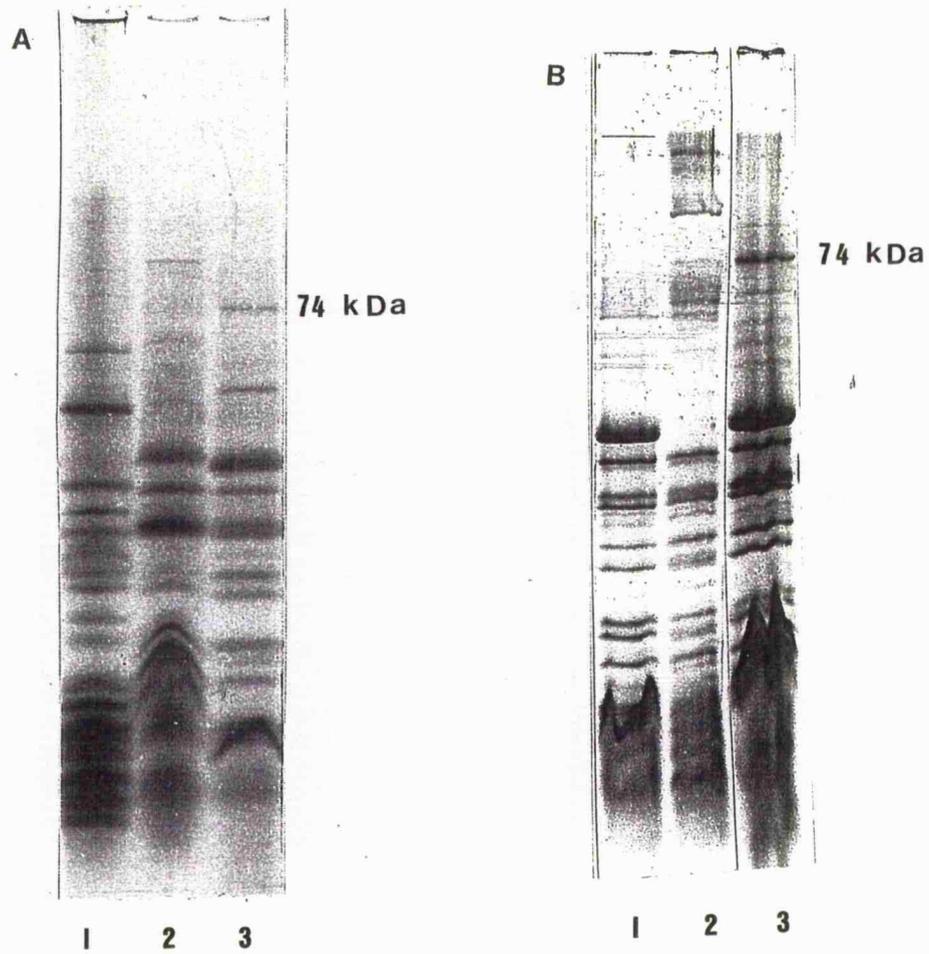


Figure 4.6: Outer membrane proteins profile on SDS-PAGE of B74K resistant mutant of *E. coli* strains.

A: 1: MW4; 2: B12; 3: W3110.

B: 1: MC34; 2: LG1703; 3: BZB (pABN1).

K. These results suggest either that this mutant has simultaneously lost the ability to make the outer membrane proteins that act as receptors of these bacteriophages and colicins, or more probably that the mutant is defective in transport systems required for bacteriophage and colicin infection.

Although mutant B12 was also resistant to the four bacteriophages used, the outer membrane profile was not identical with that of MW4. Mutant B12 was also resistant to all the colicins used except colicin G, suggesting that this mutant was probably not defective in the *tonB* gene, since *tonB* mutants are tolerant to colicin G. This mutant may either be defective in expression of many outer membrane proteins that normally act as receptors of these bacteriophages and colicins, or be defective in a transport mechanism other than the TonB system.

Mutant MC34 was isolated from strain LG1703, which is resistant to cloacin DF13 but has normal aerobactin uptake (Wooldridge, 1991). MC34 was resistant to colicins A, N and K but sensitive to colicins D and G; these data suggest that this mutant is not a *tonB* mutant, since such mutants are tolerant to colicins D and G. Besides this, mutant MC34 was also sensitive to bacteriophages BF23, TuIa, TuIb and T6 which are TonB-dependent. This indicates that BtuB, OmpF, OmpC and Tsx, the respective receptors of these bacteriophages, are present in the outer membrane of MC34. However, the mutant is resistant to colicins A, N and K, which normally utilize OmpF/BtuB, OmpF and Tsx respectively as their receptors. According to these results, therefore, it is likely that MC34 is not a *tonB* mutant, but is probably a *tol* mutant, since the products of *tol* genes are required for translocation of group A colicins (colicins A, E1, E2, E3, K, L, N and S4) across the outer membrane (Webster, 1991).

4.3.5. The sensitivity of MW4(pLG141) to B74K, BF23, Tu1a.

In order to confirm that phage B74K can use IutA as its receptor, plasmid pLG141, which carries the *iutA* gene, was introduced into mutant MW4 and its sensitivity to various bacteriophages was analyzed. In fact, MW4(pLG141) remained resistant to B74K but surprisingly it was sensitive to bacteriophages BF23 and Tu1a.

The resistance of MW4(pLG141) to B74K suggested that MW4 was defective in the transport system required for B74K infection. On the other hand, the sensitivity of MW4(pLG141) to phages BF23 and Tu1a does not automatically suggest that IutA is an alternative receptor for these phages. There are other possible explanations; firstly, MW4(pLG141) might be a partial revertant of MW4, so that receptors and/or proteins required for translocation of these phages (TonB system) across the outer membrane were present in this strain. Second, if MW4 had lost several outer membrane proteins but was not defective in the *tonB* system, it is possible that both bacteriophages were capable of using IutA as an alternative receptor. Since the presence of plasmid pLG141 in the *E. coli btuB* strain (RK4793) did not confer sensitivity to phage BF23, it is clear that IutA cannot replace BtuB as the receptor for BF23. Further observations were carried out using other *E. coli* strains, W3110, MH1160, MH760 and MH1460. The results revealed that the presence of plasmid pLG141 increased the number of plaques of phage BF23 on these strains (figure 4.7). In addition, there was no difference in BF23 adsorption before and after cloacin treatment of MW4 and of MW4 containing pLG141, but there was a significant difference in phage adsorption to MW4(pLG141) with or without antibodies raised against whole cells containing IutA (table 4.5). This phenomenon was also found in

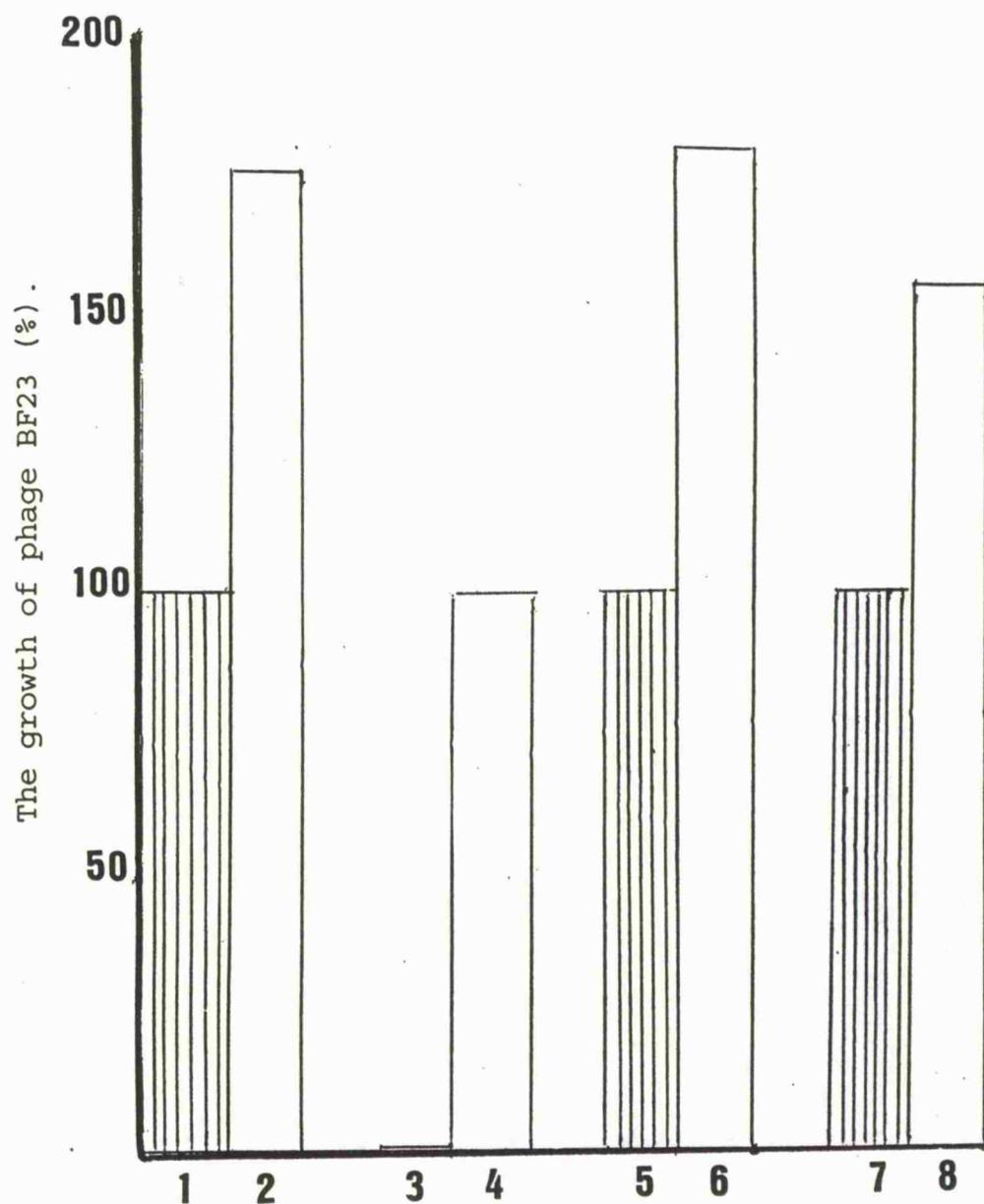


Figure 4.7: The presence of plasmid pLG141 increases the ability of *E. coli* strains to support BF23 growth in 1:W3110 (wild type), 2:W3110 (pLG141), 3:MW4 (W3110 B74K^R), 4:MW4 (pLG141), 5:MH1160 (*ompC⁻ ompF⁻*) 6:MH1160 (pLG141), 7:MH760 (*ompC⁻ ompF⁺*), 8:MH760 (pLG141)

Table 4.5: The inhibition by cloacin or antibodies raised against Triton/urea-soluble outer membrane of *E. coli* containing IutA to BF23 adsorption on B74K resistant mutant in the presence or absence of plasmid pLG141.

Strains	Serum/ cloacin Df13	10 ⁻² residual phage titre (p.f.u.)
MW4	-	26.5
MW4	serum	30
MW4	cloacin	26
MW (pLG141)	-	6.5
MW4 (pLG141)	serum	23
MW4 (pLG141)	cloacin	6.5

Table 4.6: The inhibition by antibodies raised against Triton/urea-soluble outer membrane of *E. coli* strain expressing IutA to phage Tu1a adsorption on MH146 (*ompF⁻ ompC⁺*) in the presence and absence of plasmid pLG141.

Strains	Serum/ buffer	10 ⁻² residual phage titre (p.f.u.)
MH1460	buffer	18
MH1460	serum	21
MH1460 (pLG141)	buffer	9
MH1460 (pLG141)	serum	19

phage TuIa adsorption on *E. coli* strain MH1460(pLG141) using the same antibodies (table 4.6).

According to these results, it is assumed that bacteriophage B74K infection is not TonB-dependent, but presumably it requires the products of *tol* genes. Besides using IutA as its receptor, B74K is probably also able to utilize other outer membrane proteins.

4.4. DISCUSSIONS

The concentration of bacteriophage in sewage was increased by adding samples to minimal medium containing dipyriddy and the *E. coli* strain W3110(CoIV-K30), and incubating at 37°C overnight. Bacteriophages in the supernatant were plated with the same strain on minimal medium agar containing dipyriddy to obtain single plaques. Bacteriophage B74K was isolated among single plaques selected for their ability to grow on *E. coli* strain W3110(pLG141) but not on W3110. A plaque with this phenotype was isolated and a phage lysate was prepared using W3110(pLG141) for use in further experiments. Although the initial screening showed that the phage did not grow on W3110, the phage lysate prepared on W3110(pLG141) was able to grow in W3110, albeit at an efficiency about 100-fold lower than on W3110(pLG141). On the assumption that the receptor of B74K was IutA, antibodies raised against whole cells of an *E. coli* strain expressing IutA were used to determine if adsorption of B74K was blocked by this serum. It was observed that adsorption of B74K was indeed significantly reduced when W3110(pLG141) cells were treated with

this antiserum compared to untreated cells. There was no significant difference in phage adsorption to serum-treated or untreated W3110 cells (Roberts *et al.*, 1989). Since IutA is the receptor of aerobactin and cloacin DF13, an experiment was carried out to determine whether either of these agents interfered with B74K adsorption. In this experiment aerobactin was spotted onto a lawn of strain W3110(pLG141) on nutrient agar, followed by a bacteriophage lysate; this showed that aerobactin did not inhibit phage adsorption. On the other hand, the results of another experiment suggested that endogenous aerobactin may interfere with B74K adsorption (figure 4.4). Possibly the latter experiment is correct, but the effect could not be observed in the first experiment due to the large number of phage particles on each spot. Additionally, the fact that phage B74K has alternative receptors may influence the results.

In an experiment to observe the influence of cloacin DF13 on adsorption of phage B74K, the results showed a small difference between phage adsorption to strain W3110 (pLG141) with or without cloacin treatment. There are two possible explanations; the first, as mentioned above, is that phage B74K is able to use protein receptors other than IutA, while the second concerns the mechanism of cloacin DF13 entry into bacterial cells. It is known that cloacin DF13 comprises two polypeptides, the active bacteriocin and an immunity protein. Krone *et al.* (1986) reported that when cloacin DF13 binds to the outer membrane protein receptor of susceptible *E. coli* cells, the immunity protein is released and the cloacin molecule is translocated into the cells. Thus there is a possibility that when the immunity protein is released from IutA, this receptor protein will be available for phage binding. Alternatively

B74K may recognize a site on the IutA protein which is distinct from the cloacin DF13/aerobactin binding sites.

Although it is generally assumed that phages recognize a single receptor protein, recent results suggest that there are exceptions. For examples, phage TC23 is capable of using three proteins, originally designated Ia, Ib and E, as receptors (Chai and Foulds, (1978); the recommended uniform nomenclature for these proteins is OmpF, OmpC and NmpA respectively (Osborn and Wu, 1980). Similarly it is suggested that phage B74K is also able to utilize receptor proteins in addition to IutA. Several B74K-resistant mutant *E. coli* strains isolated in this study had lost several outer membrane proteins, again suggesting that B74K may be able to use more than one outer membrane protein as its receptor. An exception is mutant MC34 which was only reduced in OmpF, as shown by the reduction of its susceptibility to phage TuIa. This does not mean that OmpF can act as a receptor for phage B74K, since OmpF⁻ *E. coli* strains were in fact sensitive to phage B74K (table 4.3). Mutant MC34 was also resistant to colicin A. It has been demonstrated that *ompF* cells were completely resistant to colicin A, whereas *btuB* cells were partially resistant (Chai *et al.* , 1982). In fact, MC34 mutant was sensitive to phages TuIa and BF23, which normally use OmpF and BtuB respectively as receptors, indicating that these proteins were still expressed in the MC34 mutant. Although there is a possibility that resistance of MC34 to colicin A is due to the reduction or modification of OmpF and BtuB, the fact that OmpF⁻ and BtuB⁻ mutants were also sensitive to phage B74K indicates that MC34 was probably defective in phage translocation. It is well known that translocation of colicins, bacteriophages and siderophores into the periplasm usually requires either TonB or TolA. A group colicins require the products of *tol* genes

(Webster, 1991). Thus it is most likely that mutant MC34 is defective in Tol functions.

Although the presence of IutA failed to overcome resistance of a BtuB⁻ strain to phage BF23, it increased the sensitivity of BtuB⁺ strains that were OmpC⁺F⁺ or OmpC⁻F⁻ or OmpC⁻F⁺ to phage BF23. Antibodies raised against Triton/urea-soluble outer membranes of *E. coli* cells expressing IutA reduced phage BF23 adsorption to MW4(pLG141) significantly. On the other hand, a *btuB* mutant carrying pLG141 was resistant to BF23. It is possible that the reduction of phage adsorption on MW4(pLG141) is due to the presence of BtuB on this strain as the result of reversion of the MW4 mutant carrying pLG141. This hypothesis could be tested by analyzing the outer membrane protein profile of this strain on SDS-PAGE, but unfortunately there was no time to do this experiment.

The influence of IutA on the sensitivity of bacterial strains to BF23 was shown by the increased number of plaques produced on strains containing pLG141. This phenomenon is similar to killing activity of colicin A in *E. coli* strains. The sensitivity to colicin A of an OmpF⁻BtuB⁺ strain was 320 times less than that of an OmpF⁺BtuB⁺ strain, but the OmpF⁺BtuB⁻ strain was only 50 times less sensitive. This showed that the presence of BtuB increased the sensitivity of *E. coli* strain to colicin A, although BtuB itself could not replace the function of OmpF as the receptor of colicin A (Chai *et al.*, 1982). In contrast, although MW4(pLG141) was sensitive to phage TuIa, and phage adsorption by the OmpF⁻C⁺ strain MH1460 carrying pLG141 was decreased after treatment with the antibodies raised against Triton/urea-soluble outer membrane of an *E. coli* strain expressing IutA, the ability of strains W3110 and W3110(pLG141) to support growth of phage TuIa is similar. The

possibilities are either that by chance strain MH1460(pLG141) has reverted to OmpF⁺ or that IutA can be used as an alternative receptor for TuIa in OmpF⁻ strains. These data show that bacteriophage B74K is TonB independent, that it probably requires the products of *tol* genes and that it is capable of using IutA and other outer membrane proteins as its receptors. Because of this, phage B74K could not be used for IutA identification. In addition, the data show that the presence of IutA increased the sensitivity of *E. coli* strains to phage BF23.

CHAPTER 5

GENERAL DISCUSSION

IutA is the outer membrane protein responsible for the uptake of ferric aerobactin (Bindereif *et al.*, 1982), the binding of the bacteriocin cloacin DF13 produced by strains of *Enterobacter cloacae* (de Graaf *et al.*, 1969, Van-Tiel-Menkveld *et al.*, 1982) and the binding of bacteriophage B74K (Roberts *et al.*, 1989). The migration rate of IutA proteins of different enterobacterial species in SDS-PAGE varies, from 74 kDa in *E. coli* strains harbouring plasmid ColV-K30 (Bindereif *et al.*, 1982), 76 kDa in *Shigella* (Derbyshire *et al.*, 1989; Griffiths *et al.*, 1985; Marolda *et al.*, 1987), 80 kDa in *Erwinia carotovora* (Ishimaru and Loper, 1992) to 85 kDa in *Enterobacter cloacae* (Crosa *et al.*, 1988; Krone *et al.*, 1985). However, although plasmid ColV-K30 and *Shigella* express IutA proteins with apparently different profiles on SDS-PAGE, this study has identified that the *iutA* gene from *Shigella flexneri* ser.6 consists of 2196 nucleotides, the same as the *iutA* gene of ColV-K30, and the similarity of the predicted amino acid sequences of both IutA proteins was 93%. Moreover, as well as the difference in the apparent molecular mass on SDS-PAGE, IutA_{Sh} differs biologically from the IutA of ColV-K30 in that it mediates lower susceptibility to cloacin DF13. On the other hand, IutA of *E. coli* 15972 (76 kDa) and IutA of ColV-K30 (74 kDa) are biologically indistinguishable in terms of aerobactin and cloacin DF13 uptake, although the restriction endonuclease maps are identical (Bouchet *et al.*, 1994).

Hybrids of two homologous genes were used to study structure-function relationships and to locate domains of the protein involved in certain functions. Previously, various *ompF::ompC*, *ompC::ompF* and *ompF::ompC::ompF* chimeric genes have been used to locate domains of these outer membrane proteins involved in cellular sensitivity to colicins and phages (Mizuno *et al.*, 1987; Fourel *et al.*, 1990). In this study, hybrid *iutA::iutA_{Sh}* genes were constructed to locate the domains of these proteins involved in aerobactin and cloacin uptake. The results demonstrated that the apparent molecular weights of the protein products of all constructs on SDS-PAGE depended on the origin of the 3'-terminus; constructs with the 3'-terminus from *iutA* expressed a 74 kDa protein, while constructs with the 3'-terminus from *iutA_{Sh}* expressed a 76 kDa protein. With regard to the cloacin binding function, the sensitivity to cloacin DF13 of strains carrying any of the constructs was higher than that of a strain carrying the cloned of *iutA_{Sh}* and lower than that of a strain expressing *IutA*, whatever the protein profiles on SDS-PAGE. This phenomenon was also observed with hybrid *ompC::ompF*; although many constructs which contain more than 50% of *ompF* gene as the 3'-terminus express OmpC type protein, strains carrying these constructs were still sensitive to phage TuIa which normally uses OmpF as the receptor (Fourel *et al.*, 1990; Datta *et al.*, 1977). These cases suggest that although the protein conformation has changed, binding sites are still functioning, providing they can be recognized.

Replacement of each of three regions in *iutA_{Sh}* (the 5' terminus upstream of the *ClaI* site; the central region between *ClaI* and *EcoRI*; the 3' terminus downstream of the *EcoRI* site) with the homologous fragment from *iutA* resulted in increased cloacin

sensitivity compared to that of a sensitive strain expressing normal IutA_{Sh}. These data suggest that sequences in all three regions of *iutA* are involved in cloacin sensitivity.

Products of the *tolAQR* genes are required for translocation of group A colicins, filamentous phages and cloacin DF13 from the relevant outer membrane binding receptors into the cell. Equivalent to the *tolA-tolQR* system is the *tonB-exbBD* system for group B colicins and for the infection by phages such as T1 and Φ 80. It has been proposed that interaction between TonB, ExbB and ExbD, and between TolA, TolQ and TolR, takes place in the cytoplasmic membrane; proper interaction is essential for the 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 (Kampfenkel and Braun, 1993). Constructs 2, 3 and 5, which all contain the *iutA*_{Sh} fragment between the *ClaI* and *EcoRI* sites, conferred a higher level of cloacin adsorption but the same or lower level of cloacin sensitivity than constructs 4 and 1, which contain the homologous fragment from *iutA*. These results suggest that perhaps proteins expressed by these first constructs (2, 3 and 5) could not interact properly with the complex TolA, TolQ and TolR, thus they could not transfer cloacin optimally.

The outer membrane proteins expressed by construct 5 (*iutA* in which the central *ClaI-EcoRI* fragment was replaced with the homologous fragment from *iutA*_{Sh}) including the 74 kDa protein and an additional band of 70 kDa, suggests that the protein expressed by construct 5 was not stable. The *ClaI-EcoRI* fragments of IutA and IutA_{Sh} differ in five amino acids (figure 5.1), one or more of which may be important for the stability of IutA.

IutA_{Sh}. IDESRQLQLI TQYYKSQGDD NYGLNLGKGF SAISGSSTPY VSKGLN
 . .ttBBBBBB BBBBttTTTT Tbbbb.tt. ...ttt... .tTT..

IutA. IDESRQLQLI TQYYKSQGDD **D**YGLNLGKGF SAIRGTSTPF VSINGLN
 b.ttBBBBBB BBBBttTTTT Tbbbb.tt.tt... .TTt..

Figure 5.1: Amino acid sequences between the *ClaI* and *EcoRI* sites of IutA_{Sh} and IutA and the predicted secondary structure according to the method of Chou and Fasman (1978). H indicates α -helix, B indicates β -strand and T indicates a potential reverse turn. Lower case equivalents indicate the same structure but predicted with less certainty. Bold letters indicate amino acid residues of IutA which differ from those of IutA_{Sh}.

There are two amino acid residues with very different properties, namely phenylalanine (non polar) and asparagin (polar) in IutA, while IutA_{Sh} has tyrosine and lysine at the same position. Tyrosine and lysine contain both polar and non polar substituents; this type amino acid residue adds greater flexibility to the interactions that become possible between amino acids in the folded state (Light, 1974). According to this theory, the protein expressed by construct 6 should be less stable than protein expressed by construct 5, but in fact, the condition was the other way around. Although there are differences in electrical charge among these amino acid residues (asparagine and serine are uncharged; aspartate is negatively charged; arginine and lysine are positively charged), these differences do not appear to influence protein

stability. Positively charged lysine or arginine in T4 lysozyme could be replaced with negatively charged glutamic acid with obviously changing the crystallographic structure of the enzyme (Dao-pin *et al.*, 1991).

There are two possibilities that may be influencing the protein stability. The first is hydrophobicity, which is generally the major factor in stabilizing the folded structures of globular proteins (Matthews, 1993). The different amino acid residues in IutA and in IutA_{SH} are hydrophilic except for the phenylalanine in IutA, which is hydrophobic and may be important for the stability of the IutA protein structure. The second is the difference in side chains among these different amino acid residues, because amino acid side chain interactions are important in the folding of a polypeptide chain from the disorganized state to the conformation typical of the biologically active molecule (Light, 1974). In this case, there are two amino acid residues in IutA, arginine and asparagine, which have very different side chains from two amino acid residues at the same position of the IutA_{SH}, serine and lysine respectively (figure 5.1). Therefore, interactions between amino acid residues among amino acid sequences in the regions upstream of *ClaI* site and downstream of *EcoRI* site with certain amino acid residues in the region between *ClaI* and *EcoRI* sites may result in different protein conformations.

Outer membrane proteins seem to be similar to the vast majority of polytopic membrane proteins. They have cell surface-exposed loops that can be specifically recognized as receptor sites by bacteriophages or bacteriocins, can be cleaved by exogenously added proteases, or can bind specific antibodies. Other exposed loops face the periplasm and are recognized by antibodies or cleaved by proteases only when the cells are lysed or the outer membrane is permeabilized. Studies have been

performed on several outer membrane proteins with the aim of determining their membrane organization, especially in relation to membrane insertion and assembly (Pugsley, 1993).

Two models have been proposed to explain how proteins are inserted into the outer membrane. In the first model, outer membrane proteins are partially assembled in the cytoplasmic membrane and migrate to the outer membrane via contact sites that are proposed to span the periplasm between the two membranes (Kellenberger, 1990). The first evidence that supports this model was the observation by Metcalfe and Holland (1980) who observed that newly synthesized OmpF protein is released into the medium by sphaeroplasts derived from *E. coli*. Similar observations on TonA were made by Jackson *et al.* (1986). The released soluble OmpF from sphaeroplasts was confirmed by Sen and Nikaido (1990) who showed that the released protein was monomeric and that it could trimerize and insert into *E. coli*-derived membranes when mixed with a very small amount of the detergent Triton X-100. Similar results were observed for the outer membrane protein PhoE (de Cock *et al.*, 1990a). Furthermore, it was observed that the absence of detergent prevented insertion into the membrane in a protease-resistant form without affecting trimerization and association with the membrane fragment. It seems that the detergent did not induce a conformational change, but rather assisted or facilitated insertion into the membrane (de Cock *et al.*, 1990b). Possibly the detergent acts in the same way *in vitro* as a lipid or other molecules with which outer membrane proteins might associate during transit through the periplasm (Pugsley, 1993).

The second model proposes the influence of LPS in the insertion of outer membrane proteins. Monoclonal antibodies have been developed as probes to study

the topology of outer membrane proteins and used to monitor conformational changes that occur during their export and assembly. These studies revealed that there were three groups of monoclonal antibodies. The first group recognized structures that appeared early during porin assembly, whereas the second group recognized the metastable trimeric intermediate stage and the third group recognized the fully inserted outer membrane trimer (Fourel *et al.*, 1992). Furthermore, it had been observed that the transition from metastable to stable trimer and full insertion into the outer membrane occur after the LPS association step (Reid *et al.*, 1990); trimerization occurred at the surface of or within the outer membrane, possibly via partially folded monomeric and dimeric forms (Reid *et al.*, 1988; de Cock *et al.*, 1990b).

The amino acid sequence of a protein dictates the three dimensional structure of the molecule (Light, 1974). Several results showed that structural alterations abolish outer membrane protein assembly; possibly, certain segments of outer membrane proteins are more crucial than others because they nucleate the folding reactions that eventually lead to the formation of the insertion-component state or interact with LPS or other envelope components that promote assembly (Klose *et al.*, 1988; Pugsley, 1993).

Outer membrane proteins often, although not always, fail to assemble in the outer membrane as a result of even relatively minor tinkering with the sequences of the transmembrane segments (Bosch *et al.*, 1986; Freudi *et al.*, 1985; Klose *et al.*, 1988). Models of bacterial outer membrane protein structure, which are largely based on analysis of porins and OmpA, emphasize the likelihood of membrane-spanning β -sheet that are perpendicular to the plane of the bilayer (Murphy, *et al.*, 1990).

It is most likely that the whole or a part of the region between *ClaI* and *EcoRI* sites of IutA is a transmembrane segment, since the protein structure prediction of this region showed β -sheet (figure 5.1) and reverse turn and replacement of this region with the homologous region of IutA_{Sh} may influence the protein stability, as shown by the outer membrane protein profile encoded by construct 5. The other possibility is that this region nucleates the folding reaction as mentioned above so that replacement of the *ClaI-EcoRI* fragment of *iutA* with the homologous fragment from *iutA*_{Sh} results in instability of the protein structure. However, the stability of the 74 kDa IutA protein structure not only depends on the presence of the *ClaI-EcoRI* fragment, since construct 3, which has the *iutA* fragment downstream of the *EcoRI* site, expressed a 74 kDa protein the same size as IutA. In this case, although the interaction between amino acid residues in the region downstream of the *EcoRI* site of IutA with amino acid residues in the region between the *ClaI* and *EcoRI* sites of IutA_{Sh} may not be stable, stable interactions between amino acid residues of IutA_{Sh} in the region upstream of the *ClaI* site and those in the region between *ClaI* and *EcoRI* sites may contribute to the stability of the whole protein structure.

On the other hand, construct 6, which is *iutA*_{Sh} with replacement of the region between *ClaI* and *EcoRI* sites with the homologous region from *iutA* expressed the 76 kDa protein the same extent as the original IutA_{Sh}. Possibly the centre region between the *ClaI* and *EcoRI* sites of IutA_{Sh} is not a trans membrane segment, or, alternatively, only a part of this region, possibly without the C terminus region, is a transmembrane segment, since the amino acid sequences in the *ClaI* site region are identical with those of the IutA, while most of the different amino acid residues are close to the *EcoRI* site.

Sequence changes affecting the extramembranous segments of outer membrane proteins appear to be relatively well tolerated. Comparison of the sequences of the OmpA from different species (Braun and Cole, 1984) and PhoE (van der Ley *et al.*, 1987) reveal that the majority of the sequence differences affect surface-exposed loops. Amino acids can be removed from or inserted into extramembranous segments without affecting outer membrane assembly (Agteberg *et al.*, 1989). Only some of the mutations in the short periplasmic loops of PhoE protein affected trimerization or outer membrane insertion (de Cock *et al.*, 1991).

It is most likely that the region downstream of *EcoRI* site of either IutA or IutA_{Sh} is located on the surface, especially the region close to the *EcoRI* site, because replacement of this region with the homologous region did not influence protein stability.

Concerning aerobactin uptake, the lowest level was shown by a strain carrying construct 5. The highest level of aerobactin uptake was shown by construct 2, which consists of the *iutA* fragment upstream of the *ClaI* site and the *iutA*_{Sh} fragment downstream of the *ClaI* site as the 3'-terminus. The aerobactin uptake of strains carrying constructs with the 3'-terminus of *iutA*_{Sh} was higher than that of strains carrying constructs with the 3'-terminus of *iutA*, indicating that the *iutA*_{Sh} fragment downstream of *ClaI* is more important in aerobactin uptake function.

Mouse monoclonal antibodies were generated against the 76 kDa IutA protein of pathogenic avian *E. coli* strain 15972. Six IutA-specific monoclonal antibodies (AB1 to AB6) recognized conformational epitopes in IutA proteins which are destroyed upon protein denaturation, and they did not significantly interfere with either the killing activity of cloacin DF13 or the uptake of aerobactin. Two monoclonal

antibodies (AB9 and AB10) recognize linear epitopes that remain buried in the native forms of IutA. The linear epitope recognized by AB9 is highly conserved in all IutA proteins examined. Therefore, this epitope may correspond to a region of the protein molecule essential for its structural or functional preservation (Bouchet *et al.*, 1994).

Although infection of bacteriophage B74K, which is able to use IutA and other proteins as receptors, was not inhibited by aerobactin in the spot test experiment, adsorption of this phage was significantly inhibited by antibodies raised against whole cells *E. coli* expressing IutA. With reference to the work cited above (Bouchet *et al.*, 1994), possibly the phage B74K binding site is on the epitope that was recognized by one of the monoclonal antibodies AB1-AB6, which did not significantly interfere with the killing activity of cloacin DF13 and the aerobactin uptake.

It has been mentioned that although the presence of IutA failed to overcome the resistance of a BtuB⁻ strain to phage BF23, it increased the sensitivity of BtuB⁺ strains OmpF⁺C⁻ or OmpF⁻C⁻ or OmpF⁻C⁺ to phage BF23. Bacteriophage BF23, which has BtuB as its receptor is TonB-dependent, like ferric-aerobactin which uses IutA as the receptor. As has been mentioned before, TonB, ExbB and ExbD interact as a complex with outer membrane proteins that are receptors for TonB-dependent uptake. Thus, the ability of IutA to increase the sensitivity of BtuB⁺ strains to phage BF23, is probably due to the interaction between the TonB-ExbB-ExbD complex and BtuB as well as with IutA to increase the efficiency of BF23 translocation.

In summary, experiments in this study demonstrate that the location of the cloacin DF13 uptake function in IutA is mainly in the 3'-terminus downstream of the *ClaI* and *EcoRI* sites. However, the presence of other fragments of *iutA*, upstream of the *ClaI* site and the fragment between *ClaI* and *EcoRI* of *iutA_{sh}* to replace the

homologous fragments are also able to increase cloacin sensitivity conferred by IutA_{Sh}. On the other hand, to locate the aerobactin uptake function is rather difficult because both proteins have the same capability in this function. Nevertheless, it seems that the main aerobactin uptake function of *iutA*_{Sh} is located in the 3'-terminus downstream of *Cla*I and *Eco*RI sites; it is probable that the main aerobactin uptake function in *iutA* is located in the 5'-terminus upstream of *Cla*I and *Eco*RI sites. Aerobactin uptake conferred by construct 2, which consist of the 5'-terminus of *iutA* and the 3'-terminus of *iutA*_{Sh} with the junction or the *Cla*I site, is the highest level for any of the other constructs; it seems that there is an accumulation of aerobactin uptake functions in both *iutA* genes, with two main functional domains in IutA and IutA_{Sh}, upstream of *Cla*I and downstream of *Eco*RI. The domains are linked by the small region between *Cla*I and *Eco*RI sites. Where most of the amino acid differences between the two proteins are encoded. Alternatively, the aerobactin uptake function in *iutA* is also in the 3'-terminus downstream of *Cla*I and *Eco*RI sites, but the conformation of proteins expressed by constructs with the 5'-terminus of *iutA* is capable to increase the aerobactin uptake. Bacteriophage B74K is capable of using IutA and other outer membrane proteins as its receptors. Because of this condition, phage B74K could not be used for IutA identification. In addition, the presence of IutA increased the sensitivity of *E. coli* strains to phage BF23.

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