THE AEROBACTIN-MEDIATED IRON UPTAKE SYSTEM IN *ESCHERICHIA COLI* K-12

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by

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For my Father and in memory of my Mother

Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Da	Daltons
DNA	Deoxyribonucleic acid
dATP	Deoxyribo-adenosine triphosphate
dCTP	Deoxyribo-cytosine triphosphate
ddNTP	Dideoxyribo-nucleoside triphosphate
dGTP	Deoxyribo-guanidine triphosphate
DTT	Dithiothreitol
dTTP	Deoxyribo-thymidine triphosphate
dNTP	Deoxyribo-nucleoside triphosphate
EDTA	Diaminoethanetetra-acetic acid
ELISA	Enzyme-linked immuno-sorbant assay
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IPTG	Isopropylthio-β-D-galactoside
IROMP	Iron-repressible outer membrane protein
kb	Kilobase
kDa	Kilodalton
keV	Kiloelectron volt
LPS	Lipopolysaccharide
MDH	Malate dehydrogenase
MECAM	1,3,5-N,N',N''-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzine
MOPS	3-(N-morpholino)propane-sulphonic acid
OM	Outer membrane
PBS	Phosphate buffered saline
RNA	Ribose nucleic acid
SDS	Sodium dodecyl sulphate (Sodium laurel sulphate)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLT	Shiga-like toxin
TCA	Trichloroacetic acid
TBE	Tris-borate-EDTA
TE	Tris-EDTA
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XP	5-bromo-4-chloro-3-indolyl phosphate

Publications arrising from this work

1. **Roberts, M., K.G. Wooldridge, H. Gavine, S.I. Kuswandi, and P.H. Williams.** 1989. Inhibition of biological activities of the aerobactin receptor protein in rough strains of *Escherichia coli* by polyclonal antiserum raised against native protein. Journal of General Microbiology, **135**:2387-2398.

2. **Wooldridge, K.G., and P.H. Williams.** 1991. Sensitivity of *Escherichia coli* to cloacin DF13 involves the major outer membrane protein OmpF. Journal of Bacteriology, **173**:2420-2424

3. **Brock, J.H., P.H. Williams, J. Liceaga, and K.G. Wooldridge.** 1991. Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. Infection and Immunity, **59**:3185-3190.

Contents.

Abstract

The Aerobactin-Mediated Iron Uptake System in *Escherichia coli*. Karl G. Wooldridge.

Some strains of *E. coli* secrete a low molecular weight compound, aerobactin, with a high affinity for ferric iron. After complexing ferric iron aerobactin may be transported back into the cell and used as a source of iron. The ability to secrete aerobactin and utilize iron derived from this source has been demonstrated to be an important virulence determinant for bacteria causing extraintestinal disease. The genes encoding the aerobactin system are often associated with large plasmids and are arranged in an operon consisting of five genes. Four genes encode enzymes responsible for the biosynthesis of aerobactin, while the fifth encodes an outer membrane receptor specific for ferric aerobactin.

Antibodies against the outer membrane aerobactin receptor protein were raised in an attempt to evaluate the prospect of using the aerobactin receptor as a protective antigen. Antiserum was shown to react specifically in ELISA assays with whole cells of *E. coli* K-12 expressing the receptor and to inhibit uptake of [^{14}C]aerobactin. The antiserum did not, however, react with the aerobactin receptor protein in a range of *E. coli* isolates from representative *E. coli* serotypes isolated from human extraintestinal infections. This was shown to be due to shielding of the receptor protein by lipopolysaccharide molecules on the surface of these strains.

The presumed role of a number of chromosomally encoded gene products in the uptake of aerobactin across the outer and cytoplasmic membranes of *E. coli* has never been directly demonstrated. Their functions were confirmed experimentally, and entry of aerobactin into the cytoplasm of an uptake proficient cell was demonstrated for the first time. Inhibition of uptake into the cytoplasm of aerobactin, but not aerobactin-derived iron, was demonstrated in the presence of endogenous aerobactin.

The aerobactin receptor protein also acts as a receptor for the bacteriocin cloacin DF13. No other envelope-associated proteins were known to be involved in sensitivity to cloacin DF13. The major outer membrane protein OmpF was demonstrated to be required in addition to the aerobactin receptor for sensitivity to cloacin DF13. It was shown not to be required for initial binding of the bacteriocin to the receptor and a role in subsequent translocation across the outer membrane was suggested.

Studies on a number of mutant aerobactin receptor proteins revealed that structural information necessary for stable association with the outer membrane was present throughout most of the length of the polypeptide. A region involved in binding and/or translocation of aerobactin was identified and another region was shown to be involved in cloacin DF13 binding and/or translocation. A mutation which altered the signal sequence such that it more closely resembled the consensus structure for signal peptides increased the efficiency of processing of the precursor protein.

Chapter 1

General Introduction

1.1. The role of iron in biological systems. That the lactic acid bacteria do not seem to require iron for their metabolism (Archibald, 1983) would appear to be the exception that proves the rule that biological systems have an absolute requirement for this trace metal (Weinberg, 1978). Iron, the most abundant transition metal in living organisms (Brock, 1989), is so widely employed in metabolism because of its two stable valencies, which confer a very considerable range on its potential for oxidation/reduction reactions and chemical reactivities (Byers, 1987).

The systems in which iron plays an essential role in biology are diverse. The iron-containing ribonucleotide reductase is involved in transformation of ribonucleotide diphosphates to their corresponding deoxy-derivatives required for DNA synthesis (Reichard and Ehrenberg, 1983). RNA polymerase III requires iron for its activity (Shoji and Ozawa, 1985; 1986). Iron is required in electron transfer, where it is necessary for the action of cytochromes, hydrogenase, ferridoxin and succinate dehydrogenase (Neilands, 1981, Dallman, 1986). In certain groups of organisms iron is involved in nitrogenase function in nitrogen fixation and in chlorophyl synthesis (Neilands, 1981). Iron is involved in reversable oxygen binding in both the haemoglobins and myoglobins of vertebrates (Dickerson and Geis, 1983), haemerythrin (containing non-haem iron) of invertebrates (Crichton and Charloteaux-Wauters, 1987), and microbial haemoglobin-like proteins (Lascelles, 1964) and leghaemoglobin (Ellfolk, 1972). In addition, iron is required for the activity of a number of enzymes in microbial and mammalian organisms which participate in oxygen metabolism such as superoxide dismutase, catalase and peroxidase (Halliwell and Gutterige, 1985), and various amino acid hydroxylases and dioxygenases (Feigelson and Brady, 1974; Nozaki and Ishimura, 1974; Wigglesworth and Baum,

1980). In vertebrates iron is required for several innate and aquired immune functions including neutrophil function, T- and B-lymphocyte activity and natural killer cell function (Brock and Mainou-Fowler, 1986; Bryan *et al.*, 1986; Dallman, 1986; Sherman and Lockwood, 1987).

While some of the processes in which iron plays an essential role (such as DNA synthesis and electron transfer) are found universally, others (e.g. nitrogen fixation or photosynthesis) are restricted to certain groups of organisms. Despite such diversity, however, the actual amount of iron required by cells for metabolism, with the exception of the *lactobacilli*, is relatively uniform at aproximately 0.4-4.0 μM for plant cells (Price and Carell, 1964), prokaryotes (Weinberg, 1974) and animal cells (Higuchi, 1970).

The above examples by no means provide a comprehensive list of the biochemical functions in which iron plays a vital role. They merely serve to illustrate the importance of this trace element to both eukaryotic and prokaryotic organisms.

1.2. The toxicity of iron. Iron is an excellent biological catalyst because of its two stable oxidation states. This means that, as well as being very useful to biological systems, it may also participate in detrimental reactions that can damage biomolecules. Iron is a central component participating in Haber-Weiss-Fenton chemistry where it catalyses the production of the hydroxide radical from the superoxide anion and hydrogen peroxide ($O_2^- + Fe^{3+} = O_2 + Fe^{2+}$; $2O_2^- + 2H^+ = H_2O_2 + O_2$; $H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH\cdot$) (Flitter *et al.*, 1983; Griffiths, 1987a). The highly reactive hydroxide radical reacts with most biological molecules (Griffiths, 1987a) and has in particular been implicated in the destruction of biological membranes via peroxidation of lipids, and scission of DNA (Weinberg, 1989). As a consequence, aerobic organisms have evolved the enzymes glutathione peroxidase and catalase to remove hydrogen peroxide, and superoxide dismutase to remove the superoxide anion (Griffiths, 1987a).

Nontheless, most biological systems produce detectable levels of the superoxide anion and hydrogen peroxide which could lead to the production of the hydroxyl radical and, therefore, the need to limit the levels of free iron available for Haber-Weiss-Fenton chemistry summarized above is apparent.

1.3. Availability of iron to microorganisms in the environment. Iron is the fourth most abundant element and the second most abundant metal, after aluminium, in the earth's crust (Crichton and Charloteaux-Wauters, 1987). In aerobic conditions, however, the element exists almost exclusively in the ferric (Fe^{3+}) state of oxidation. At physiological pH, ferric iron forms hydroxides and oxyhydroxides which are virtually insoluble, and consequently the levels of iron in solution and available to biological systems in aerobic environements, in the order of 10^{-18} M (Bullen *et al.*, 1978; Spiro and Saltman, 1969), are many orders of magnitude below levels required by microorganisms for growth (Weinberg, 1974).

1.4. Availability of iron to the microbe *in vivo*. The blood, tissues and tissue fluids of animals necessarily contain concentrations of iron sufficient to supply the needs of metabolizing cells. The problems of the low solubility of ferric iron and, furthermore, the requirement of living systems to limit the levels of free iron such that it is not available to participate in potentially damaging Fenton chemistry, means that higher organisms have evolved mechanisms for reducing the "free" iron concentration in an essentially iron-rich milieu to levels well below those required for microbial growth.

In man, the majority of iron is intracellular (Beinert, 1973), either complexed with metaloproteins such as haemoglobin (74.3%), myoglobin (3.3%), catalase (0.11%), cytochrome c (0.08%), etc., or stored in the iron-storage protein ferritin (16.4%) or its insoluble degradation product haemosiderin (Deis, 1983; Theil, 1987). In addition, in the blood of

vertebrates the protein haptoglobin binds to free haemoglobin (Muller-Eberhard, 1970), which may be released upon lysis of erythrocytes. The haemoglobin-haptoglobin complex normally accounts for approximately 0.2% of the total iron in man (Beinert, 1973); it is rapidly removed from the circulation by cells of the reticuloendothelial system (Arceleo and Greer, 1982; Kluger and Bullen, 1987). Similarly, if haemoglobin molecules are oxidized and dissociate into ferrihaem and globin (a situation which may occur after the saturation of circulating haptoglobin) the serum proteins, haemopexin and, with somewhat lower avidity, albumin are able to bind haem (Muller-Eberhard, 1970). Complexes of haem with haemopexin are also removed from the circulation by hepatic parenchymal cells (Kluger and Bullen, 1987; Koskelo and Muller-Eberhard, 1977; Seery and Muller-Eberhard, 1973). Complexes of haem with albumin (two molecules of haem bind to one molecule of albumin to form methalbumin) are not cleared and will continue to circulate until the haem can be transferred to apo-haemopexin as it becomes available (Kluger and Bullen, 1987). The synthesis of haemopexin, an acute-phase reactant, is increased during inflammation (Konijn *et al.*, 1981), therefore increasing the host's ability to bind free haem which may be liberated as a result of tissue damage during inflammation.

Transport and recycling of iron in vertebrates is achieved via the iron binding glycoprotein transferrin and its cognate receptor (Bezkorovainy, 1987; Brock, 1989; Weinberg, 1989). Human serum transferrin has a molecular weight (based on amino acid sequence) of 79,550 (Bezkorovainy, 1987; MacGillivray *et al.*, 1982). The molecule is thought to have arisen as a result of a gene duplication and thus has two functionally similar domains (Arosio *et al.*, 1989; Bezkorovainy, 1987). Each domain has the capacity to bind one ion of ferric iron, and to do so must also bind an anion which *in vivo* is usually carbonate or bicarbonate (Bezkorovainy, 1987). In man, transferrin-iron accounts for approximately 0.07% of total iron (Beinert,

1973). However serum transferrin is normally only about 30% saturated; therefore, since transferrin has a high affinity for ferric iron (approximately 1-6 x 10^{22} M^{-1}, Crichton and Charloteaux-Wauters, 1987), the levels of iron in low molecular weight complexes in equilibrium with the transferrin-iron complex are very low (Bullen *et al.*, 1978; Bullen, 1981).

The transferrin receptor is composed of two 93 kDa disulphide linked proteins whose expression is regulated at the transcriptional level according to the iron requirements of the cell (Rao *et al.*, 1986). Uptake of transferrin, cellular removal of its iron, and recycling of the apotransferrin molecule occurs by a process of endocytosis of multiple transferrin-receptor complexes within a clathrin coated pit/vesicle, acidification of the vesicle, reduction and transport of the iron to the cytoplasm, and return of the apotransferrin-receptor complex to the cytoplasmic membrane (Nunez *et al.*, 1990). The apotransferrin is released from the receptor due to a reduced affinity for the receptor with respect to iron-transferrin (Young *et al.*, 1984).

A protein closely related to transferrin, called lactoferrin, is found in mucosal secretions such as milk, tears, nasal mucus, saliva, bronchial mucus, gastrointestinal fluid, hepatic bile, cervical mucus, and seminal fluid (Weinberg, 1984; Bezkorovainy, 1980). It differs from transferrin in that it has no known role in iron recycling, and it retains a high affinity for iron at pH values below 4.5 (unlike transferrin, which loses its affinity for iron at pH values below about 5.0). This property may be of particular relevance at sites of sepsis where the pH may be significantly lower than normal due to the metabolic activities of invading microorganisms and/or metabolically stimulated leukocytes (Weinberg, 1984). Lactoferrin is also present in the specific granules of polymorphonuclear leukocytes (PMNs) (Briggs *et al.*, 1983; Leffel and Spitznagel, 1972).

1.5. The hypoferrraemia of infection. Iron levels in blood and tissue fluids may be reduced still further in response to the presence of invading microorganisms. This reaction of the host is refered to as the

hypoferraemic response (Weinberg, 1978; 1984). The mechanisms involved in this response are not fully understood, but are thought to be mediated by the cytokine interleukin 1 (Il-1). The response can be induced in the absence of infection by administration of recombinant human or murine Il-1 (Westmacott *et al.*, 1986). Il-1 may be released by macrophages or monocytes after stimulation by microorganisms or their products (Weinberg, 1984).

One suggested mechanism for the reduction in serum iron levels in response to Il-1 is the degranulation of PMNs, releasing lactoferrin (Webster *et al.*, 1980) which at reduced pH may remove iron from serum transferrin (Van Snick *et al.*, 1974; Klempner *et al.*, 1978). Lactoferrin itself is removed from circulation by cells of the reticuloendothelial system. While this mechanism may be important at local sites of inflammation, however, it is not thought that it could account for the systemic reduction in serum iron concentrations observed. Even minute amounts of endotoxin or Il-1 cause a fall in serum iron without a corresponding reduction in pH, and at physiological pH iron is not readily exchangable between transferrin and lactoferrin (Konijn and Hershko, 1989; Westmacott *et al.*, 1986).

The reduction in serum iron levels in response to infection is likely to be due mainly to a reduction in the release of tissue iron to serum transferrin (Lee, 1983; Roeser, 1980). This seems to be the result of increased ferritin synthesis (Konijn and Hershko, 1989) which may be in direct response to stimulation by Il-1. In addition, the rate at which ferritin is denatured to form haemosiderin is increased during infection, probably due to the effect of intracellular inflammatory mediators on the lysosomal membrane (Hershko *et al.*, 1974; Roeser 1980). Both the increase in levels of ferritin synthesis and its conversion to haemosiderin will make iron less available to the intracellular "labile pool" and thus to serum transferrin (Konijn and Hershko, 1989).

A further mechanism which may reduce serum iron levels in response to inflammation is reduced uptake of iron from the intestine. Intestinal absorption of iron has been reported to be reduced early in infection (Cartwrite *et al.*, 1946), but whether this response is mediated by Il-1 is unknown.

Whatever the mechanism(s) responsible, there is no doubt that serum iron levels are reduced in response to infection, and this has been suggested as a non-specific defence mechanism, termed "nutritional immunity" (Kochan, 1973; Weinberg, 1978; 1984). Whether reducing the already vanishingly small levels of free iron in normal human serum still further has any adaptive value in terms of increased resistance to infection has, however, been questioned (Brock and Mainou-Fowler, 1986). Some extracellular pathogens have been shown to use iron from intracellular host iron stores, however (Brock *et al.*, 1991), and the reduction in the intracellular labile pool observed during the hypoferraemic response might thus be of greater significance.

1.6. Strategies of microbial iron acquisition. In the face of the extremely low availability of iron both in the environment and *in vivo*, microorganisms have evolved a number of different strategies to obtain sufficient quantities of this essential trace element to support their metabolism and growth. At least five have been identified (Weinberg, 1989), avoidance of a requirement for iron, reduction of ferric iron and transport of ferrous iron, occupation of an intracellular niche and utilization of intracellular host iron compounds, utilization of extracellular host iron compounds, and transport of low molecular weight ferric chelates (siderophores).

1.6.1. Avoidance of a requirement for iron. Total abstinance from utilizing ferric iron has, thus far, only been identified in non-

pathogenic *lactobacillus* species (Archibald, 1983). The *lactobacilli* are apparently able to use cobalt as the metal cofactor for the enzyme ribonucleotide reductase, and manganese as the cofactor for such enzymes as catalase and RNA polymerase. The mechanisms by which they obtain these metals have not been studied (Weinberg, 1989).

1.6.2. Reduction of ferric iron and ferrous iron transport. Reduction of ferric iron at the cytoplasmic membrane and subsequent transport of ferrous iron into the cytoplasm is the method by which some microorganisms obtain sufficient iron for their metabolism and growth. This strategy has been adopted by the intracellular pathogen *Listeria monocytogenes* which is apparently able to remove iron from transferrin by secreting a 9 kDa reductant (Cowart and Foster, 1985). This may be of significance during periods of extracellular existence. The micro-aerophilic anaerobe *Bifidobacterium bifidus* actively acquires ferrous ions by using an ATPase in the cytoplasmic membrane (Bezkorovainy *et al.*, 1986). This organism is found in the infant intestine where the pH is around 5.0, so that ferric and ferrous iron are both relatively soluble. At higher pHs the organism relies on a high affinity ferric iron transport system (Bezkorovainy *et al.*, 1986). The dental plaque organism *Streptococcus mutans* acquires only ferrous iron via a membrane flavin reductase (Evans *et al.*, 1986). Tooth plaque is anaerobic due to the metabolic activity of other microorganisms present (Weinberg, 1989). Similarly the yeast *Saccharomyces cerevisiae* employs a cytoplasmic membrane reductase to reduce ferric iron and subsequently transports ferrous iron (Lesuisse *et al.*, 1987) although this organism is also able to transport ferric iron, with a higher affinity, if it is supplied as a ferric chelate (Lesuisse and Labbe, 1989). *Escherichia coli* does not apparently have an extracellular iron reductase system but if ferrous iron is present, for example under anaerobic conditions, it is able to transport it via a specific transport system which is

inducible under conditions of iron restriction (Hantke, 1987). Paradoxically this system is derepressed under aerobic conditions in which ferrous iron would be expected to be rapidly oxidized to the ferric form (Hantke, 1987).

1.6.3. Occupation of an intracellular niche. Some parasitic microorganisms acquire sufficient iron by occupying an intracellular niche where iron appears to be relatively freely available. *Listeria* (Cowart and Foster, 1985), *Yersinia* (Robins-Browne *et al.*, 1987), *Salmonella* (Benjamin *et al.*, 1985) and *Shigella* (Lawlor *et al.*, 1987) species are representative prokaryotic parasites which occupy intracellular sites where they may obtain iron from ferritin or haem (Weinberg, 1989). For example, mutants of *Shigella flexneri* unable to obtain iron from high affinity siderophore-mediated systems (discussed below) were unable to grow in the presence of transferrin but could, nontheless, utilize haem and obtain sufficient iron for growth in various experimental intracellular environments (Lawlor *et al.*, 1987).

1.6.4. Utilization of host iron compounds. A fourth strategy involves utilization of host iron binding compounds present in the extracellular milieu. A number of pathogenic microorganisms are able to utilize haem or haemoglobin present in trace quantities in serum due to lysis of erythrocytes (Morgan, 1981). *E. coli* (Griffiths, 1987b) is able to use the iron in haem to multiply in an otherwise iron-restricted environment in host tissues. *Yersinia* species have been shown to utilize haem as an iron source (Perry and Brubaker, 1979) and *Neisseria meningitidis*, *N. gonorrhoeae* (Dyer *et al.*, 1987), *Haemophilus influenzae* (Pidcock *et al.*, 1988) and *Vibrio cholerae* (Stoebner and Payne, 1988) utilize both haem and haemoglobin. The genetic determinants of a 51 kDa haem-binding membrane-associated lipoprotein have recently been cloned from *Haemophilus influenzae* type b and expressed in *E. coli* (Hanson and Hansen, 1991).

As mentioned in section 1.4, free haemoglobin is rapidly bound by haptoglobin, and free haem by haemopexin and albumin (Muller-Eberhard, 1970). It has been suggested that the iron in bound haemoglobin or haem may not be available to microorganisms capable of utilizing the free forms of these iron compounds (Eaton *et al.*, 1982; Dyer *et al.*, 1987). Eaton *et al.* (1982) showed that haptoglobin blocked the ability of haemoglobin to donate iron to a pathogenic *E. coli* strain, suggesting that, as well as facilitating the rapid clearance of free haemoglobin from the circulation, haptoglobin also sequesters haemoglobin in a form that is not capable of providing iron for microbial growth. All strains of *Haemophilus influenzae* tested by Pidcock *et al.* (1988), however, were able to acquire iron from haemoglobin or haem regardless of whether they were bound (by haptoglobin or haemopexin respectively). Both *N. meningitidis* and *N. gonorrhoeae* are capable of using haemoglobin or haem as a source of iron and some strains of both species can use haemoglobin bound to haptoglobin (Dyer *et al.*, 1987). None of the strains tested, however, were able to use haem bound either to haemopexin or serum albumin (Dyer *et al.*, 1987).

In normal serum the levels of free haem are rather low (Morgan, 1981), and it has been suggested that they would not support the growth of most pathogens (Griffiths, 1987b). Some pathogenic microorganisms, however, are able to increase the availability of free haemoglobin (and thus haem) in blood by secreting toxins capable of erythrocyte lysis. Many invasive strains of pathogenic *E. coli* for example are able to secrete α-haemolysin (Aumont et al., 1989; Cavalieri *et al.*, 1984; Valvano *et al.*, 1986). In experimental infections with a mixed inoculum of Hly^+ cells and an Hly^- mutant cells led to an enhanced growth of the latter than when the infection was performed with the Hly^- strain alone (Waalwijk *et al.*, 1983). Furthemore the expression of haemolysin genes has been shown to be derepressed under conditions of iron-limitation (Gruenig *et al.*, 1987).

Similarly *Vibrio cholerae* strains are able to secrete haemolysin in response to iron restricted conditions and to utilize released heme and haemoglobin as a source of iron (Stoebner and Payne, 1988) and aerolysin, a haemolysin produced by *Aeromonas sobria*, seems to play a role in systemic infections by this organism (Goebel *et al.*, 1988).

In addition to utilizing haem compounds, some pathogenic microorganisms are capable of extracting iron directly from iron binding glycoproteins. *Neisseria gonorrhoeae* and *N. meningitidis* are able to scavenge sufficient iron for growth from transferrin even at only 4% saturation (Mickelsen and Sparling, 1981). The process is highly specific; these organisms were unable to utilize chicken ovotransferrin (Mickelsen and Sparling, 1981) which shows a high degree of homology with human transferrin (Bezkorovainy, 1987). Utilization of transferrin-iron by gonococci or meningococci was shown to be induced by iron restriction and dependent upon a functional electron transport chain (Simonson *et al.*, 1982; McKenna *et al.*, 1988). In addition, both pathogenic *Neisseria* species have been shown to utilize lactoferrin as an iron source (McKenna *et al.*, 1988; Dyer *et al.*, 1988). Non-pathogenic *Neisseria* species able to utilize transferrin or lactoferrin are less frequently encountered (approximately 25% of strains tested) (Mickelsen and Sparling, 1981; Mickelsen *et al.*, 1982; Simonson *et al.*, 1982). Dyer *et al.* (1988) have described a pleiotropic iron uptake mutant of *N. meningitidis* that was unable to utllize transferrin as a source of iron and lacked a 70 kDa outer membrane protein. This protein was not, however, the receptor for transferrin as the mutant bound transferrin as efficiently as the wild type (Tsai *et al.*, 1988). A mutant of *N. gonorrhoeae* defective in uptake of iron from transferrin and haemoglobin has been described that is avirulent in mouse subcutaneous chambers (Genco *et al.*, 1991).

Pathogenic strains of *Haemophilus influenzae* are also able to utilize

iron bound to transferrin but not lactoferrin as a source of iron (Herrington and Sparling, 1985; Pidcock et al., 1988). This specificity was further investigated by Morton and Williams (1990) who observed that *H. influenzae* type b could utilize transferrin of human, bovine and rabbit origin, but not from 6 other mammalian sources, nor chicken ovotransferrin, nor lactoferrin of human or bovine origin. They also identified a 72 kDa outer membrane protein which was iron repressible and which bound transferrin after separation in a denaturing electrophoresis gel and transfer to nitrocellulose. A number of pathogenic non-typable *H. influenzae* have also been shown to utilize transferrin-iron but the normally non-pathogenic *H. parainfluenzae* was unable to utilize this iron source (Herrington and Sparling, 1985).

In contrast to the specificity for iron binding proteins exhibited by the *Neisseria* and *Haemophilus* species, *Bordetella pertussis* is apparently able to utilize transferrin, ovotransferrin or lactoferrin in a process which involves direct binding of the proteins to the bacterial surface, and in which the different proteins are mutually competitive, suggesting a common receptor (Redhead *et al.*, 1987). Five *Bacteroides* species were shown to use transferrin as an iron source (Verweij-Van Vught *et al.*, 1988), and one virulent strain of the fish pathogen *Aeromonas salmonicida* has also been shown to use either transferrin or lactoferrin as an iron source (Chart and Trust, 1983).

1.6.5. Uptake of iron by means of siderophores. The final strategy employed by microorganisms to obtain iron for growth involves the uptake of siderophores (Lankford, 1973; Neilands, 1981). These compounds are defined as low molecular weight compounds (500-1000 Da) possessing a high affinity for ferric iron ($K_{aff} > 10^{30}$), whose biosynthesis is regulated by iron levels, and whose function is to supply iron to the cell (Neilands, 1981). Siderophores are normally either catechols or hydroxamic acids (Hider,

1984; Neilands, 1981). They are taken up by the utilizing strain via specific outer membrane receptor proteins (Neilands, 1982; Hider, 1984).

1.7. Siderophore systems in *E. coli*. In *E. coli* at least five new outer membrane proteins in the molecular weight range 74-83 kDa are observed when the cells are grown in iron-limiting media (Hider, 1984; Hantke, 1983), Cir (74 KDa), FhuE (76 kDa), FhuA (78 kDa), FepA (81 kDa) and Fiu (83 kDa). In addition, in the presence of 0.1 mM citrate a sixth outer membrane protein, FecA (80.5 kDa) is derepressed in response to iron limitation (Wagegg and Braun, 1981). FecA is only easily observed in outer membrane preparations of mutant strains lacking the similarly sized FepA protein (Hancock *et al.*, 1976).

The 81 kDa outer membrane protein FepA is the receptor for the endogenous catechol siderophore enterochelin (Hollifield and Neilands, 1978). This siderophore, independently isolated in the same year from *E. coli* (O'Brien and Gibson, 1970) and *Salmonella typhimurium* (Pollock and Neilands, 1970) and called enterobactin by the latter workers, is produced by wild type strains of *E. coli* (O'Brien and Gibson, 1970; Rogers, 1973; Rogers *et al.*, 1977) and other enteric bacteria (Lawlor and Payne, 1984; Perry and San Clemente, 1979; Pollock and Neilands, 1970). The enterochelin molecule is a cyclic triester of 2,3-dihydroxy-*N*-benzoyl serine, and has the highest affinity for ferric iron of any compound that has been tested (Harris *et al.*, 1979; Hider, 1984).

The 78 kDa FhuA protein has been identified as the receptor for ferrichrome (Wayne and Neilands, 1975), a cyclic trihydroxamate siderophore produced by many fungal species including *Ustilago sphaerogena* and all penicillia (Hider, 1984). The closely related hydroxamate siderophores ferricrysin and ferricrosin, both produced by *Aspergillus* species (Hider *et al.*, 1984), are also taken up via the FhuA

receptor (Hider *et al.*, 1984). Similarly the 76 kDa FhuE protein has been identified as the receptor for the linear hydroxamate siderophores coprogen and rhodotorulic acid (Hantke, 1983) produced by *Penicillium* and *Neurospora* species and by *Rhodotorula*, *Sporobolomyces* and *Leucosporidium* species respectively (Hider, 1984). The ecological significance of the possession of receptors by enteric bacteria for siderophores produced by these soil fungi is not clear (Hider *et al.*, 1984).

The 80.5 kDa protein FecA is required for the utilization of ferric dicitrate as an iron source (Hancock *et al.*, 1976; Wagegg and Braun, 1981). Citrate, unlike true siderophores, binds iron relatively weakly; a 30-fold molar excess of citrate is required to bind ferric iron as a mononuclear complex (Hider, 1984). It is often treated as a siderophore, however, since uptake of its ferric iron complex by enteric bacteria follows a similar pathway to the uptake of true siderophores. That citrate is only efficient in delivery of iron when present at relatively high concentrations is reflected by the regulation of its receptor which, unlike the other iron-repressible outer membrane receptor proteins, also requires the presence of citrate in the extracellular milieu to achieve derepression (Hancock *et al.*, 1976; Zimmermann *et al.*, 1984).

The roles of the 74 kDa Cir protein and the 83 kDa Fiu protein were until recently an enigma (Hantke, 1983; Nau and Konisky, 1989). Working independently, however, two groups demonstrated a role for these proteins in the susceptibility of *E. coli* K-12 to catechol substituted cephalosporins (Curtis *et al.*, 1988) and β-lactams (Nikaido and Rosenberg, 1990), suggesting a physiological role in the transport of monocatecholic iron complexes. Subsequently Hantke (1990) demonstrated that both of these proteins, as well as FepA, were capable of transporting iron complexes of the breakdown product of enterochelin dihydroxybenzoylserine (DBS), and the enterochelin precursor dihydroxybenzoate (DHB). However the

observation that Cir was less efficient than either FepA or Fiu in transporting DBS, and that FepA was as efficient as Fiu in transporting DBS and only slightly less efficient than Cir or Fiu in transporting DHB suggests that these receptors might have other specificities for as yet unidentified monocatechol-iron complexes.

In 1979 Williams reported a high affinity iron uptake system in *E. coli* strains harbouring the plasmid ColV-K30. This system was shown to be independent of the enterochelin-mediated iron uptake system (Williams, 1979) and depended on the excretion of a hydroxamate siderophore (Williams and Warner, 1980) which was later found to be aerobactin (Warner *et al.*, 1981). This bacterial siderophore had previously been recognized in some strains of *Aerobacter* (now *Enterobacter*) *aerogenes* (Gibson and Magrath, 1969). The system was derepressible under conditions of iron stress and was associated with a new outer membrane protein (IutA) with a similar electrophoretic mobility as the 74 kDa outer membrane protein Cir (Bindereif *et al.*, 1982; Grewal *et al.*, 1982). The genetic determinants of the aerobactin-mediated iron uptake system were cloned (Bindereif and Neilands, 1983) and found to consist of five genes arranged in an operon, four (*iucABCD*) encoding the enzymes required for the biosynthesis of aerobactin, and the fifth (*iutA*) encoding the outer membrane receptor protein IutA (Carbonetti and Williams, 1984).

In addition to the requirement for outer membrane receptors, uptake of iron via siderophores requires several other envelope-associated proteins. Some of these are involved in transport of the siderophore across the outer membrane, and others are required to transport either the intact siderophore or just the iron it carries across the periplasm and cytoplasmic membrane into the cytoplasm. These functions do not exibit the same degree of specificity as outer membrane receptors and may be involved in uptake of several siderophores.

1.7.1. TonB-dependence of siderophore uptake across the outer membrane. Uptake across the outer membrane of *E. coli* of all siderophores of both hydroxamate and catechol classes and of ferric dicitrate requires the participation of the cytoplasmic membrane protein TonB (Frost and Rosenberg, 1975; Hantke and Braun, 1978; Postle, 1990b; Schoffler and Braun, 1989; Williams, 1979). This protein is thought to provide energy derived from a cytoplasmic membrane proton gradient to the outer membrane proteins involved in active transport across the outer membrane (Hancock and Braun, 1976; Hantke and Braun, 1978; Postle, 1990b). In addition to iron-chelates, vitamin B_{12} is also actively transported across the outer membrane in a process that is dependent on an energised cytoplasmic membrane and a functional TonB protein (Kadner, 1990). Outer membrane proteins which are dependent on TonB have been shown to contain a consensus pentapeptide called the "TonB box" (Postle, 1990b; Nau and Konisky, 1989; Kadner, 1990). Mutations affecting this region of a particular receptor result in a $TonB^-$ phenotype for the cognate ligand (Postle, 1990b). Direct physical interaction of TonB-dependent outer membrane receptor proteins and the TonB protein is strongly suggested by observations that mutations in the *tonB* gene itself partially suppress mutations affecting the TonB box of the vitamin B_{12} receptor BtuB (Heller *et al.*, 1988) and the ferrichrome receptor FhuA (Schofler and Braun, 1989). Products of the *exb* locus (the proteins ExbB and ExbD) are also somehow involved in TonB-dependent uptake across the outer membrane, but the requirement for Exb functions is variable depending on the particular siderophore, and is less stringent than the requirement for TonB (Fischer *et al.*, 1989; Postle, 1990b). The ExbB protein has been shown to stabilize the TonB protein, and *exbB* mutants can be bypassed by overexpression of TonB (Fischer *et al.*, 1989). The function of ExbD has not been resolved.

1.7.2. Uptake of siderophores into the cytoplasm. Once

siderophores have been transported across the outer membrane, uptake across the periplasm and cytoplasmic membrane into the cytoplasm occurs via periplasmic-binding-protein-dependent mechanisms similar to those that have been described for a number of nutrients (Ames, 1986; Higgins *et al.*, 1988). This is despite the fact that, unlike the siderophores and vitamin B_{12}, nutrients are generally able to cross the outer membrane by diffusion through non specific aqueous channels formed by the major outer membrane proteins OmpF and OmpC (Benz, 1988), or by facilitated diffusion through more specific channels such as LamB (Wandersman *et al.*, 1979). Periplasmic-binding-protein-dependent systems typically consist of a hydrophilic periplasmic binding protein, one or two very hydrophobic integral membrane proteins of the cytoplasmic membrane, and a relatively hydrophilic protein which is, nontheless, associated with the cytoplasmic membrane and contains regions of strong homology to nucleotide binding proteins (Ames, 1986; Higgins, 1988). Despite the overall organizational similarity of these systems, with the exception of the conserved nucleotide binding domains of the peripheral membrane proteins, there is in general little sequence similarity (Ames, 1986). In contast the components of the periplasmic transport systems of the TonB-dependent class exibit regions of extensive amino acid homology (Shea and McIntosh, 1991) suggesting that these systems are a closely related subset of the periplasmic transport systems of *E. coli*.

Hydroxamate-mediated uptake of iron across the periplasm and cytoplasmic membrane requires the products of the genes *fhuBCD* (Braun *et al.*, 1983; Hantke, 1983; Prody and Neilands, 1984; Wookey *et al.*, 1981). FhuD is the hydrophilic periplasmic protein (Koster and Braun, 1989), FhuB is a very hydrophobic cytoplasmic membrane protein which displays internal homology suggestive of a gene duplication (Koster and Braun, 1989) and FhuC is a hydrophilic cytoplasmic membrane protein with

homology to ATP-binding proteins (Burkhardt and Braun, 1987).

Enterochelin-mediated iron uptake across the periplasm and cytoplasmic membrane requires the products of the *fepBCDEFG* genes (Chenault and Earhart, 1991; Elkins and Earhart, 1989; Ozenberger, 1987; Pierce and Earhart, 1986; Pierce *et al.*, 1983; Shea and McIntosh, 1991). FepB is the hydrophilic putative periplasmic binding protein (Elkins and Earhart, 1989), and FepC is a cytoplasmic membrane protein that corresponds to the conserved nucleotide binding protein (Elkins and Earhart, 1989; Shea and McIntosh, 1991). The *fepD*, *fepE*, *fepF*, and *fepG* genes have been less vigorously studied because of difficulty in identifying their cognate polypeptides (Ozenberger *et al.*, 1987). By analogy with other systems they are anticipated to be highly hydrophobic membrane proteins (Elkins and Earhart, 1989). 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).

Uptake of iron from ferric dicitrate across the periplasm and cytoplasmic membrane requires the products of the *fecBCDE* genes (Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989). FecB is the putative periplasmic binding protein (Pressler *et al.*, 1988). The *fecC* and *fecD* genes encode very hydrophobic polypeptides which localized in the cytoplasmic membrane and exibited homologies to each other and to the N- and C-terminal halves of the FhuB protein of the hydroxamate transport system (Staudenmaier *et al.*, 1989). The product of the *fecE* gene is a hydrophilic, cytoplasmic membrane-associated protein containing regions of homology to ATP-binding proteins (Staudenmaier *et al.*, 1989).

The similarity of vitamin B_{12} transport across the outer membrane

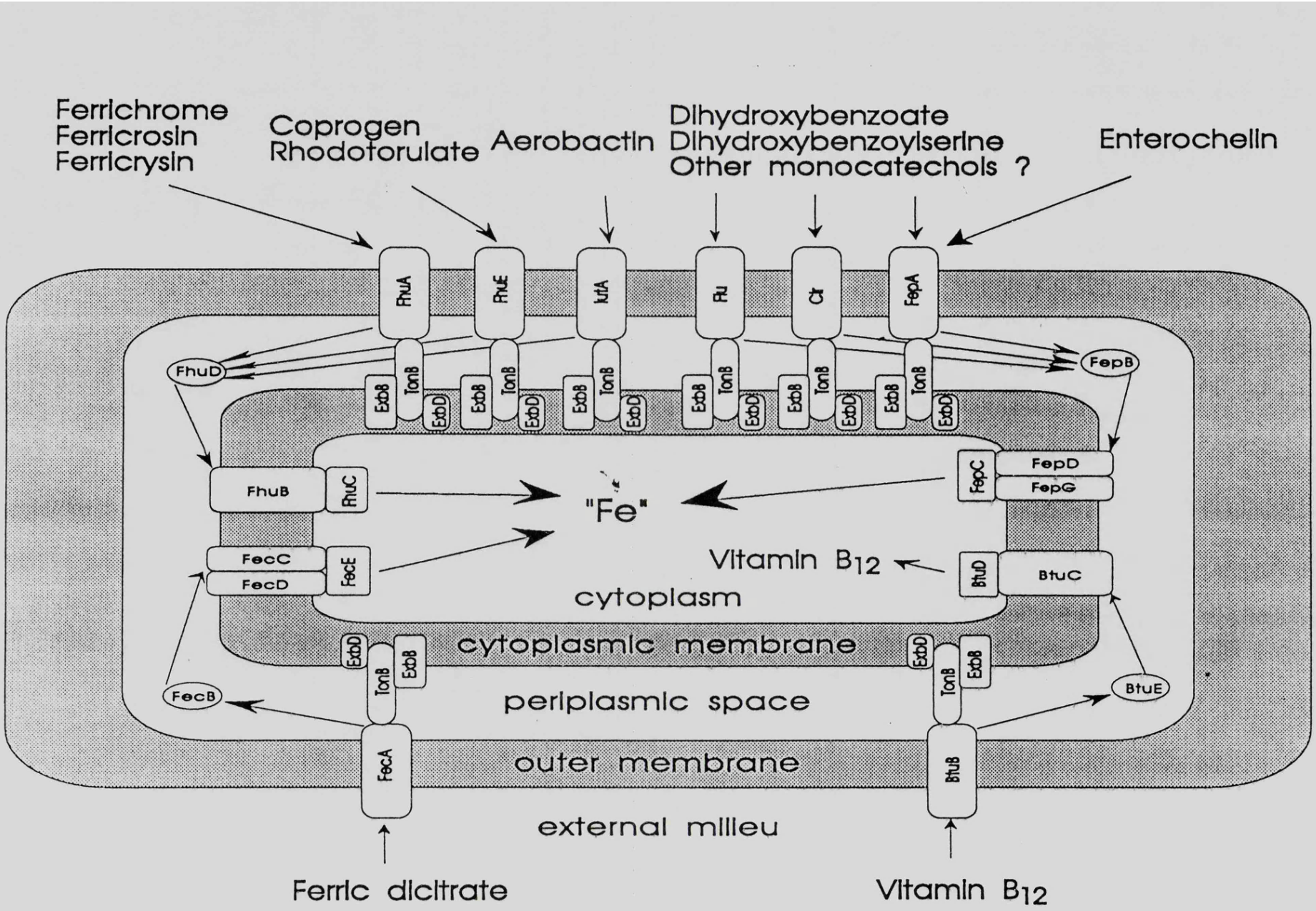


Figure 1.1. TonB-dependent transport systems of *Escherichia coli*.

to the transport of iron-chelates extends to the transport across the periplasm and cytoplasmic membrane. The products of the *btuCED* region of the *E. coli* chromosome participate in the transport of vitamin B_{12} across the cytoplasmic membrane (Freidrich *et al.*, 1986). The BtuE protein appears to have a periplasmic location, the BtuC protein is highly hydrophobic and cytoplasmic membrane-associated, and the BtuD protein is relatively polar, membrane-associated and contains segments bearing homology to the ATP-binding peripheral membrane constituents of periplasmic-binding-protein-dependent systems (Freidrich *et al.*, 1986). Interestingly the cytoplasmic membrane-associated BtuD protein also has regions of homology with the outer membrane vitamin B_{12} receptor BtuB (Freidrich *et al.*, 1986). Dependence on a periplasmic binding protein in this system, however, has not yet been determined. The TonB-dependent transport systems of *Escherichia coli* are sumarised in Fig. 1.1

1.8. **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. Enterochelin is synthesized by *Salmonella*, *Klebsiella* and *Shigella* species (Byers, 1987; Payne, 1980; Perry and San Clemente, 1979; Pollock and Neilands, 1970; Schmitt and Payne, 1988; Williams *et al.*, 1987). The aerobactin-mediated iron uptake system is very widely distributed within the enterobacteriaceae and is represented 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; Williams *et al.*, 1987).

One species of enteric bacteria, *Serratia marcescens*, has an unusual high affinity iron uptake system that has been cloned and is functional in *E. coli* (Zimmermann *et al.*, 1989). The genes involved have been sequenced and closely resemble the periplasmic transport components of the TonB-

dependent siderophore-mediated transport systems described above (Angerer *et al.*, 1990). No siderophore has been detected, however, and no potential siderophore biosynthetic enzymes or an outer membrane protein are encoded by the cloned material. In addition the system is not dependent on TonB/Exb functions (Zimmerman, 1989). Transport of an iron compound through the non-specific porins was excluded as the system was functional in an *ompF ompC* double mutant (Zimmermann *et al.*, 1989). How the iron is solubilized and transported across the outer membrane by this system is an enigma. The system does, however, require participation of functions encoded by the *E. coli* chromosome (Zimmermann *et al.*, 1989). Cloning and analysis of these genes will perhaps provide clues to the mechanism of outer membrane iron transport.

No bacteria belonging to families other than the enterobacteriaceae have been shown to synthesize aerobactin or enterochelin. However certain non-enteric bacteria can utilize these siderophores when available exogenously. *Neisseria gonorrhoeae* for example is able to use aerobactin (West and Sparling, 1987). Furthermore, a gene cloned from this organism complements *E. coli fhuB* mutations, suggesting that at least the periplasmic transport system for aerobactin in this organism is homologous with that of *E. coli* (West and Sparling, 1987). Similarly *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus* (Williams et al., 1990), and *Pseudomonas aeruginosa* (Pool *et al.*, 1990) have been shown to utilize enterochelin.

Siderophore-mediated uptake systems of non-enteric bacteria have not been studied as extensively as those of the enteric bacteria. As with *E. coli* many non-enteric bacteria synthesize new envelope proteins in conditions of iron limitation (Weinberg, 1989). In most cases, however, a role in siderophore-dependent iron acquisition has not been determined (Weinberg, 1989). Siderophore production by non-enteric bacteria has been

most extensively studied in *Vibrio*, *Pseudomonas* and *Mycobacteria* species. Pathogenic strains of the fish pathogen *Vibrio anguillarum* produce the catecholic siderophore anguibactin (Crosa, 1984). The ability to synthesize and utilize anguibactin may be associated with large plasmids, the prototype of which is pJM1 (Crosa, 1984) or may be chromosomally encoded (Toranzo *et al.*, 1983). *V. cholerae* strains secrete the catecholamine siderophore vibriobactin (Payne and Finkelstein, 1978; Sigel and Payne, 1982; Griffiths *et al.*, 1984), and the opportunist pathogen *V. vulnificus* apparently produces siderophores of both catechol and hydroxamate classes (Simpson and Oliver, 1983).

Pseudomonas aeruginosa produces two siderophores, pyoverdin and pyochelin (Cox and Adams, 1985). Pyoverdin is a large siderophore with a hexapeptide backbone, a dihydroxyquinoline moiety and two N-hydroxyornithine residues (Ankenbauer *et al.*, 1985). Pyochelin is a salicylic acid substituted cysteinyl peptide (Cox *et al.*, 1981). The receptor for pyochelin has been identified as a 14 kDa outer membrane protein (Sokol and Woods, 1983).

The mycobacteria synthesize two iron binding compounds in response to iron stress, mycobactin and exochelin (Barclay and Ratledge, 1983; Hall and Ratledge, 1987; Ratledge *et al.*, 1982). Exochelin is water soluble, is excreted and acts to solubilize and transport iron, whereas mycobactin is hydrophobic and is retained within the cell wall where it is thought to act as an iron storage compound (Ratledge *et al.*, 1982). Production of both mycobactin and exochelin, as well as four envelope proteins was shown to be coordinately regulated by the iron status of cells of *M. neoaurum* (Sritharan and Ratledge, 1988). A 29 kDa iron-repressible envelope protein has been tentatively identified as the exochelin receptor (Hall *et al.*, 1987).

Aeromonas salmonicida has been shown to have an inducible siderophore-dependent iron uptake system capable of removing iron from iron-binding proteins (Chart and Trust, 1983). *Corynebacterium diphtheriae* also obtains iron via a siderophore-dependent system (Russell and Holmes, 1983; Russel *et al.*, 1984). The nature of both these siderophores is, however, unknown.

1.9. High affinity iron uptake systems and virulence. Since the levels of free iron *in vivo* are well below microbial requirements, it might be expected that possession of high affinity iron scavenging systems would constitute important virulence determinants. Certainly the presence of exogenous siderophores supplied to hosts infected with pathogenic strains of *Salmonella* (Jones *et al.*, 1977; Kochan *et al.*, 1978), *Vibrio* (Write *et al.*, 1981) or *Yersinia* (Melby *et al.*, 1982; Robins-Brown and Prpic, 1985) markedly enhanced the virulence of these pathogens (Weinberg, 1989). Two siderophore-mediated iron uptake systems have been shown conclusively to constitute important virulence determinants. The aerobactin-mediated system of the plasmid ColV-K30 was shown to enhance the virulence of laboratory strains of *E. coli* in mice and this enhancement was abolished if the mice were burdened with excess iron (Williams, 1979). The cloned aerobactin determinants alone, separated from other ColV-K30 genes were sufficient to restore full virulence to a wild type veterinary isolate of *E. coli* cured of its native ColV plasmid (Roberts *et al.*, 1989). Furthermore, epidemiological studies have shown that the aerobactin-mediated iron uptake system is far more prevalent in *E. coli* isolates 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). The virulence of *Klebsiella pneumoniae* has also been shown to be enhanced by the possession of the aerobactin determinants in a mouse peritonitis model (Nassif and

Sansonetti, 1986). By contrast, insertion mutations using either Tn*5* (Lawlor *et al.*, 1987) or Tn*10* (Nassif *et al.*, 1987) within the aerobactin gene clusters of *Shigella flexneri* impaired growth of these organisms *in vitro* in low iron conditions but did not affect their ability to grow intracellularly and kill HeLa cells, produce lethal infections in chicken embryos, or produce keratoconjunctivitis in guinea pigs (Lawlor *et al.*, 1987). A dose-dependent effect was, however, observed by Nassif *et al.* (1987) in a rabbit ligated ileal loop model, and in the ability of these bacteria to produce keratoconjunctivitis in guinea pigs. These data suggest that the production of aerobactin was important during extracellular multiplication but was unimportant during intracellular stages of infection where the bacteria perhaps satisfy their iron requirements by utilizing haem compounds (Lawlor *et al.*, 1987).

A second siderophore-mediated iron uptake system that has been convincingly demonstrated to constitute an important virulence determinant is the anguibactin-mediated iron scavenging system of *Vibrio anguillarum* (Crosa, 1984), an organism which causes a fatal septicaemia in marine fish (Harbell *et al.*, 1979; Ransom *et al.*, 1984). The high virulence phenotype correlates with the presence of a 65 kb plasmid which is absent from avirulent strains (Crosa *et al.*, 1977). Furthermore, curing virulent strains of this plasmid renders them avirulent (Crosa *et al.*, 1980). The plasmid specifies anguibactin synthesis and the ability of plasmid-bearing strains to multiply *in vitro* in the presence of non-utilizable iron chelators (Crosa, 1979; 1980). Furthermore an 82 kDa outer membrane protein is encoded by this plasmid and is expressed under conditions of iron stress (Crosa and Hodges, 1981). In some highly virulent strains of *V. anguillarum* the plasmid is absent, but in these cases hybridization studies have shown the genetic determinants for siderophore production to be present on the chromosome (Toranzo *et al.*,. 1983; Crosa, 1984).

There is conflicting evidence for a role for enterochelin in virulence. Yancey *et al.* (1979) reported that *Salmonella typhimurium* mutants unable to synthesize enterochelin were very much less virulent for mice. More recently, however, Benjamin *et al.* (1985) found that, although such mutants were unable to multiply in mouse serum, their virulence was not reduced in several mouse strains. As with *Shigella flexneri* mentioned above, *Salmonella typhimurium* is primarily an intracellular pathogen, and enterochelin may be important only during periods of extracellular multiplication. A possible role for enterochelin as a virulence determinant for extracellular pathogens such as of *E. coli* has not been investigated, although production of this siderophore *in vivo* during fatal infections with *E. coli* has been described (Griffiths and Humphreys, 1980).

Sigel *et al.* (1985) have evaluated the importance of vibriobactin as a virulence factor of *Vibrio cholerae* in an infant mouse infection model. In their experiments, two mutants of *V. cholerae* defective in the vibriobactin-mediated iron transport system retained their ability to produce disease in this model. Doubt has been cast on the authors conclusion that vibriobactin is not an important virulence factor, however, due to the high inocula of iron-replete bacteria used in these experiments (Griffiths, 1987b). Another group have reported reduced virulence in a new born mouse model of a Tn*phoA* mutant defective in expression of a 77 kDa iron regulated outer membrane protein (Goldberg *et al.*, 1990b). This protein has been shown to be homologous to the *E. coli* enterochelin receptor and is thought to be the vibriobactin receptor, suggesting a role in virulence for this siderophore (Goldberg *et al.*, 1990a). An alternative explanation for these findings, however, is that the protein was involved in virulence by translocating exogenous siderophores (for example enterochelin itself) produced by the normal gut flora of the mice.

1.10. Distribution and genetic location of aerobactin determinants. The aerobactin genetic determinants were first identified on plasmid ColV-K30 (Williams, 1979; Warner *et al.*, 1981). The genes have since been found on other ColV plasmids (Gross *et al.*, 1984; 1985; Williams and George, 1979) and on non-ColV plasmids in *E. coli*, *Aerobacter* (*Enterobacter*) *aerogenes* 62-1 and *Salmonella* species (Elena-Fernandez-Beros *et al.*, 1988; Colonna *et al.*, 1985; Gonzalo *et al.*, 1988; McDougall and Neilands, 1984; Roberts *et al.*, 1986b). Wherever the incompatibility group has been identified, the plasmids have all been found to belong to IncF1 (Colona *et al.*, 1985; Crosa, 1985; Williams and George, 1979; Waters and Crosa, 1986). In addition the aerobactin iron uptake 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).

The widespread distribution of the aerobactin-iron uptake system genes suggests that the aerobactin operon may be genetically mobile. The finding that the operon in ColV-K30 is flanked by inverted IS*1* elements (Lawlor and Payne, 1984; McDougall and Neilands, 1984; Perez-Casal and Crosa, 1984) and two distinct replication regions, REPI and REPII (Perez-Casal and Crosa, 1984) lends weight to this hypothesis. The probability that the genetic elements between the IS*1* elements behave as a conventional transposon has, however, been questioned. The insertion sequences are not in the orientation that is usual for composite transposons, and the region bound (approximately 18 kb) is large, giving a low probability of transposition (Waters and Crosa, 1986). Also conservation of sequences between different replicons in some instances extends beyond these sequences (Waters and Crosa, 1986) and in others conservation beyond the aerobactin genes but between the IS*1* sequences, or indeed the insertion sequences themselves, may be lost (Roberts *et al.*, 1986b). Transposition has

proved difficult to demonstrate in the laboratory (Roberts *et al.*, 1986b; Waters and Crosa, 1986), although one group has reported a transposition-like event under the strong selective pressure of antibiotic resistance (de Lorenzo *et al.*, 1988b). This event appeared to involve IS*1*-mediated replicon fusion and subsequent resolution which may or may not have been a *recA*-dependent process. Roberts *et al.* (1986b) suggest that the sequences surrounding the aerobactin genes may represent the remnants of an "extinct transposon", whereas Waters and Crosa (1986) suggest that the region bound by the IS*1* elements may be part of a larger "virulence factor replication unit" including the aerobactin genes and REPI sequences. Whatever the nature of the transposition events, it seems likely that the flanking IS*1* elements and/or the upstream REPI element have at least in the past been instrumental in the spread of the aerobactin genes between many different genera.

In addition to the finding of genetic determinants for the production and utilization of aerobactin which cross-hybridize to DNA probes derived from prototypical aerobactin operon sequences, an aerobactin system has been identified and cloned from *Enterobacter cloacae* which shows no homology detectable by DNA hybridization experiments (Crosa *et al.*, 1988). Whether this system is a divergent relative of the ColV-type aerobactin operon or has arisen independently is not known. Nucleotide sequencing of the genes and the enzymology of aerobactin biosynthesis in this system may be informative on this matter. Similarly, some strains of *Klebsiella pneumoniae* which do not produce aerobactin nevertheless express an aerobactin receptor with an apparent molecular weight of 76 Kda, the gene for which does not hybridize with a probe derived from the aerobactin receptor gene of ColV-K30 (Williams *et al.*, 1989). In both cases, the receptor in these bacteria is recognized, albeit poorly, by antiserum raised against the ColV-K30-encoded receptor (Crosa *et al.*, 1988; Williams *et al.*, 1989).

1.11. Genetics and biochemistry of siderophore biosynthesis in *E. coli*. The biosynthesis of enterochelin and aerobactin in *E. coli* have been extensively studied. The genes encoding enzymes required for enterochelin biosynthesis and transport are clustered around 13 min on the *E. coli* chromosome in a region encompassing approximately 22 kb (Crosa, 1989; Elish *et al.*, 1988; Nahlic *et al.*, 1989; Ozenberger *et al.*, 1989; Staab *et al.*, 1989). The initial stage of enterochelin synthesis is the production of 2,3-dihydroxybenzoic acid from the aromatic amino acid precursor chorismic acid. This step requires the products of *entC* (isochorismate synthetase, Crosa, 1989), *entB* (2,3-dihydro-2,3-dihydroxybenzoate synthetase, Nahlic *et al.*, 1989) and *entA* (2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, Liu *et al.*, 1989). The second step involves the production of one molecule of enterochelin from three molecules each of 2,3-dihydroxybenzoate and L-serine. This process is thought to be catalysed by a multienzyme complex refered to as enterochelin synthetase and composed of the products of the *entD*, *entE*, *entF*, and *entG* genes (Crosa, 1989; Greenwood and Luke; 1976; Staab *et al.*, 1989). The genes required for enterochelin synthesis and transport are transcribed from two sets of bidirectional promotors (Pettis and McIntosh, 1987; Pettis *et al.*, 1988; Elkins and Earhart, 1988; 1989; Brickman *et al.*, 1990).

The genes for aerobactin biosynthesis, *iucABCD* first found on the plasmid ColV-K30 (Warner *et al.*, 1981; Williams, 1979) are arranged as a single transcriptional unit which also includes the *iutA* gene specifying the outer membrane aerobactin receptor (Carbonetti and Williams, 1984). The genes encode polypeptides of 63 kDa (synthetase), 33 kDa (acetylase), 62 kDa (synthetase) and 53 kDa (oxygenase) respectively (de Lorenzo and Neilands, 1986). The precursors of aerobactin are L-lysine and citrate and the reactions involved in the biosynthesis include hydroxylation (catalysed by IucD) and acetylation (catalysed by IucB) of lysine, condensation of N^6-

acetyl-N^6-hydroxylysine with citric acid (catalysed by IucA) followed by the condensation of the resulting N^2-citryl-N^6-acetyl-N^6-hydroxylysine with a further molecule of N^6-acetyl-N^6-hydroxylysine to produce aerobactin (catalysed by IucC) (Bagg and Neilands, 1987a; de Lorenzo and Neilands, 1986; de Lorenzo *et al.*, 1986; Ford *et al.*, 1986; Gross *et al.*, 1985). The order in which the acetylation and hydroxylation of lysine occur is, however, in dispute. Most groups propose that the first reaction is the hydroxylation of lysine (de Lorenzo and Neilands, 1986; de Lorenzo *et al.*, 1986; Gross *et al.*, 1986). This is consistent with the findings that *in vitro* the purified acetylase uses N^6-hydroxylysine but not lysine as a substrate (Coy *et al.*, 1986), and that cells harbouring a plasmid containing only the *iucD* gene produce N^6-hydroxylysine (Bagg and Neilands, 1987a). On the other hand Ford *et al.* (1986) propose that acetylation precedes hydroxylation. This is based on the observation that cells harbouring plasmid pABN1 (carrying all five genes of the aerobactin operon) with Tn*1000* insertions within *iucD* (the hydroxylase gene) incorporated ^{14}C-lysine into a compound with identical electrophoretic mobility to acetyllysine. In addition the only plasmid-bearing strains that did not accumulate a radioactive compound when supplied with ^{14}C-lysine were those containing Tn*1000* insertions within the *iucB* (acetylase) gene.

1.12. Iron-responsive genetic regulation in microorganisms.

In *E. coli* virtually all the genes involved in siderophore biosynthesis and transport are coordinately negatively regulated in response to the iron status of the cell (Bindereif and Neilands, 1985; Braun and Burkhardt, 1982; Fleming *et al.*, 1983; Griggs and Konisky, 1989; Hantke, 1981; 1983; 1987; Postle, 1990a; Roberts *et al.*, 1986; Zimmerman *et al.*, 1984). Hantke (1981) isolated a mutant which expressed iron regulated genes at a high and constitutive level. The mutation mapped to a locus which was designated *fur* (*f*erric *u*ptake *r*egulation) and the gene was subsequently cloned

(Hantke, 1984), sequenced (Schaffer *et al.*, 1985), and found to encode a 17 kDa polypeptide. The purified Fur protein and a plasmid encoding a *lacZ* fusion to *iucA* (the first gene in the aerobactin operon) was used in conjunction with an *in vitro* coupled transcription/translation system to demonstrate that the Fur protein acts as a repressor employing Fe(II) (or certain other divalent metal ions) as a corepressor (Bagg and Neilands, 1987b). The operator sequences involved in Fur binding have been identified in the aerobactin operon (de Lorenzo *et al.*, 1987) and the *cir* gene promotors (Griggs and Konisky, 1989) by DNA footprinting. Comparison of sequences upstream of the coding regions of many other iron regulated genes has led to proposals for a consensus sequence for Fur binding sites (de Lorenzo *et al.*, 1987; Pressler *et al.*, 1988). Confirmation of this consensus was provided by inserting a synthetic oligonucleotide based on the consensus between the *ompF* promotor (which is not normally iron-responsive) and the *lacZ* structural gene. In this construction β-galactosidase activity was iron regulated, confirming that the consensus sequence was sufficient to function as an operator site (Calderwood and Mekalanos, 1988).

The *fur* gene itself is autoregulated; that is Fur represses transcription of the *fur* gene in a metal-dependent manner (de Lorenzo *et al.*, 1988a). The Fur binding site of the *fur* operator has been defined by footprinting experiments and conforms to the Fur binding consensus (de Lorenzo *et al.*, 1988a), although protection was much weaker than that afforded to the aerobactin operator. Transcription of *fur* is also subject to catabolite repression, significant stimulation of *fur* gene expression being observed in the presence of cyclic-AMP and catabolite activator protein (de Lorenzo *et al.*, 1988a). The significance of such dual control of the *fur* gene is not obvious but it clearly points to a relationship between iron regulation and metabolic status of the cells.

Similar iron responsive regulation systems are assumed to operate

in other microorganisms. A *fur*-like regulatory gene has been cloned from *Yersinia pestis* (Staggs and Perry, 1991). An iron-regulated gene cloned from *Vibrio cholerae* containing a sequence in the vicinity of the promotor with homology to the Fur binding consensus is iron regulated in an *E. coli* background. Similarly, the iron regulated *E. coli* gene *slt-1A* (Shiga-like toxin-1A) is iron regulated in a *V. cholerae* background, suggesting that a system homologous with *fur* exists in this organism (Goldberg *et al.*, 1990). Several iron-regulated loci in *Vibrio anguillarum* are modulated by a *trans*-acting transcriptional activator encoded by *angR* (Farrel *et al.*, 1990). This gene is itself regulated by iron, and a region upstream from *angR* is similar to Fur-binding operators of *E. coli* (Farrel *et al.*, 1990)

In addition to genes related to acquiring iron other genes may be regulated by the iron status of some microorganisms. In *E. coli*, for example, the genes encoding the iron- and manganese-dependent superoxide dismutase enzymes are under the control of the Fur-regulatory system (Niederhoffer *et al.*, 1990). In addition a number of bacterial toxins including the diphtheria toxin of *Corynebacterium diphtheriae* (Boyd *et al.*, 1990; Cryz *et al.*, 1983), Shiga toxin of *Shigella dysenteriae* (Dubos and Greiger, 1946) and *E. coli* shiga-like toxins SLT-I and SLT-II (Calderwood and Mekalanos, 1987) are derepressed in low iron conditions, and in the last case expression has been shown to be mediated by the Fur system (Calderwood and Mekalanos, 1987). It has been suggested that low iron conditions may constitute an environmental signal to bacteria that they have entered a mammalian host, and thus a number of virulence determinants may be coordinnately regulated by Fur or Fur-like systems (Calderwood and Mekalanos, 1988; Staggs and Perry, 1991).

1.13. Aerobactin versus enterochelin as *in vivo* iron scavengers. It is interesting to speculate as to why possession of the aerobactin system should provide selective advantage to bacteria growing in

the iron deficient milieu of infected tissues, and thus constitute a virulence determinant, when virtually all enteric bacteria possess the highly effective enterochelin-mediated iron scavenging system. The thermodynamic stability constant for ferric-enterochelin is many orders of magnitude above that of ferric-aerobactin (10^{52} for enterochelin compared to 10^{23} for aerobactin, Bagg and Neilands, 1987a). However, these values are for the fully deprotonated ligands and probably do not reflect the relative ability of the two ligands to compete for iron at physiological pH (Hider, 1984; Harris *et al.*, 1979). Harris *et al.* (1979) calculated the negative logarithms of the concentrations of free iron in equilibrium with a series of iron chelators in defined conditions to produce what they refere to as p[M] values. For example at pH 7.4 with a ligand concentration of 10^{-3}M and a metal concentration of 10^{-6}M the calculated p[M] value for enterochelin was 37.6 compared to 25.37 for aerobactin. Under the same conditions the value for transferrin was 25.62, approximately the same as for aerobactin (Harris *et al.*, 1979).

Even comparing these more physiologically relevant p[M] values, enterochelin is still by far superior as a chelator. There are, however, other factors to be considered when comparing the relative efficiency of the two systems. A number of physical properties of the molecules themselves have been suggested as reasons for the apparent effectiveness of aerobactin *in vivo*. At neutral pH enterochelin deferrates transferrin at a faster rate than aerobactin in HEPES buffer 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. As a consequence, enterochelin molecules attached to proteins in serum appear to behave as haptens, since the presence of antibodies against the enterochelin molecule have been detected in normal human serum (Moore *et al.*, 1980), and such antibodies inhibit enterochelin uptake

(Moore and Earhart, 1981). In addition, the enterochelin molecule is relatively unstable (Kochan *et al.*, 1978) and is poorly soluble (Pollock and Neilands, 1970).

As well as the physical properties of the molecules themselves, differences in the mechanisms by which the respective siderophore-dependent transport systems operate might also contribute to an increased efficiency of aerobactin compared with enterochelin. Aerobactin has been shown to stimulate growth at concentrations some 500-fold lower than enterochelin (Williams and Carbonetti, 1984). This may be because aerobactin may be repeatedly recycled (Braun *et al.*, 1984), whereas an enterochelin molecule can only deliver a single ion of ferric iron and is subsequently hydrolysed by the cytoplasmic enzyme ferrienterochelin esterase (O'Brien *et al.*, 1971). Also aerobactin apparently does not release iron to an intracellular "labile pool", but channels it directly to iron-dependent metabolism (Williams and Carbonetti, 1984). In response to iron stress *in vitro*, pathogenic *E. coli* have been shown to excrete aerobactin more rapidly than enterochelin, the latter accumulating for several hours in the periplasm before being detected extracellularly (de Vartanion, 1988). Finally *E. coli* cells producing aerobactin have been shown to accumulate iron preferentially from a cell-derived source rather than from transferrin in *in vitro* conditions designed to mimic an extraintestinal extracellular environment that extracellular pathogens might encounter (Brock *et al.*, 1991). Bacterial cells were enclosed within a dialysis membrane which in turn was immersed in tissue culture medium containing 30% saturated transferrin and K562 erythroleukemia cells. In some experiments the iron within the cells was labeled while in others the iron within the transferrin was labelled. Enterochelin-producing cells aquired predominantly transferrin-bound iron, while aerobactin producing cells preferentially acumulated iron from a cellular source (Brock *et al.*, 1991). Thus bacteria

producing both siderophores would have access to both of these sources of iron.

1.14. Scope of this project. The aims of this project were to examine the molecular machinery involved in the binding and translocation of aerobactin. In addition the binding and translocation of the bacteriocin cloacin DF13, which shares the aerobactin receptor, was examined and prospects for utilizing the aerobactin receptor as a protective antigen were investigated.

Chapter 2

The Aerobactin Receptor Protein as a Candidate Protective Antigen: Inhibition of aerobactin uptake by antibodies and shielding of the receptor by smooth lipopolysaccharide.

2.1. INTRODUCTION.

The iron-repressible outer membrane proteins (IROMPs) of Gram-negative bacterial pathogens are often suggested as candidate immunogens that may be useful in immunoprophylactic therapy (Byers, 1987; de Lorenzo and Martinez, 1988; Griffiths *et al.*, 1985; Johnson, 1991; Weinberg, 1989). Outer membrane proteins, including IROMPs, have been shown to be highly conserved among Gram-negative bacteria with respect to their antigenicity (Chart and Griffiths, 1985; Henrikson and Maeland; 1987, Hofstra and Dankert, 1979). Furthermore, there is direct evidence that several Gram-negative species express IROMP receptors for ferric siderophores *in vivo* since such proteins can be demonstrated in outer membrane preparations of bacteria isolated without subculture from patients or infected animals. Brown *et al.* (1984) demonstrated the expression of IROMPS in mucoid strains of *Pseudomonas aeruginosa*, which have more effective iron aquisition systems than do non-mucoid strains (Boyce and Miller, 1980). Similarly IROMPs have been identified in *E. coli* from the peritoneal cavity of guinea pigs (Griffiths *et al.*, 1983), in *Vibrio cholerae* from the intestinal tracts of infant rabbits (Sciortino and Finkelstein, 1983), and in Gram-negative bacteria from the urine of patients with urinary tract infections (Lam *et al.*, 1984; Shand *et al.*, 1985). In addition, convalescent sera often contain antibodies to IRMOPs (Weinberg, 1984). Convalescent sera from

patients with disseminated gonorrhoea contained immunoglobulins to *Neisseria gonorrhoeae* IROMPs of 19, 36, 70 and 84kDa (McKenna *et al.*, 1988) and covalescent sera from patients with typhoid fever contained immunoglobulins to the 78 and 81kDa IROMPs of *Salmonella typhi* (Gonzalez *et al.*, 1987). Similarly sera from patients with meningococcal meningitis contained both IgG and IgM specific for a 70kDa IROMP and IgG specific for an IROMP of 94kDa of *Neisseria meningitidis* which were absent in acute phase and normal sera (Black *et al.*, 1986).

The aerobactin receptor protein is a particularly tempting candidate as a protective antigen in view of its widespread distribution among pathogenic members of the family Enterobacteriaceae (Braun, 1981; Roberts *et al.*, 1986b; Martinez *et al.*, 1987; Warner *et al.*, 1981). In addition, a role in virulence for the aerobactin system has been strongly suggested by several epidemiological studies in which the incidence of the aerobactin system was consistently shown to be significantly higher among bacteria isolated from extraintestinal infections than among faecal isolates from healthy individuals (Carbonetti *et al.*, 1986; Jacobson *et al.*, 1988; Linggood *et al.*, 1987; Montgomerie *et al.*, 1984). Such evidence is backed up by data from experimental infections, where the presence of the aerobactin system has been shown to increase the virulence of *E. coli* K-12 strains (Williams, 1979), a clinical isolate of *E. coli* isolated from a case of chicken septicaemia (Roberts *et al.*, 1989) and *Klebsiella pneumoniae* strains of serotypes K1 and K2 (Nassif and Sansonetti, 1986). In addition, the aerobactin system in some pathogenic isolates is encoded by plasmids which also encode multiple antibiotic resistance (Colonna *et al.*, 1985; Gonzalo *et al.*, 1988); thus, passive immunization with antisera to the aerobactin receptor may be a useful tool to the clinician in cases where antibiotic therapy is not promising.

The rationale for investigating the use of the aerobactin receptor as

an immunogen that might elicit protective antibodies has two aspects. First, specific binding of immunoglobulins to IutA may inhibit its interaction with ferric aerobactin, thus disabling an important virulence determinant. Second, antibodies bound to the aerobactin receptor may lead to mediation of immune mechanisms by opsonization or activation of the complement cascade. Coulton (1982) reported reduction of ferrichrome binding to the *E. coli* FhuA protein by polyclonal sera raised against the receptor. Similarly, blockage of ferripyochelin uptake by polyclonal and monoclonal antibodies against the ferripyochelin binding protein of *Pseudomonas aeruginosa* has been reported (Sokol and Woods, 1984; 1986). Moreover, these antibodies caused a tenfold increase in the number of *P. aeruginosa* cells ingested by polymorphonuclear leukocytes (Sokol and Woods, 1986), and mutant strains not expressing the receptor protein were avirulent (Sokol, 1987). In an attempt to evaluate the prospect of exploiting the aerobactin receptor protein as a protective antigen, antisera to the protein in both native and denatured forms were raised and their activity against native protein in ELISA assays and in aerobactin binding inhibition assays was assessed for both laboratory and clinical isolates of *E. coli.*

2.2. MATERIALS AND METHODS.

2.2.1. Bacterial strains and plasmids. Strain BZB1022 is a *cir* (colicin I-resistant due to loss of the chromosome-encoded 74 000 Da outer-membrane protein, Cir), *gyrA* (naladixic acid-resistant) derivative of the standard *E. coli* laboratory strain W3110 (Pugsley, 1985). Strain AN1937 is a $FepA^+$ strain of E. coli (Williams, 1979) while strain LG1522 is a $FepA^-$ derivative of AN1937 carrying an iuc^- derivative of plasmid ColV-K30 (Carbonetti and Williams, 1984). Strain D551 is an aerobactin-producing *E. coli* $ColV^+$ isolate ($O78:H^-$) from a case of chicken septicaemia (from the collection of Unilever Research, Colworth House, Sharnbrook, England);

strain LG1695 is a plasmid-cured derivative of D551 (ColV$^-$, unable to make aerobactin or express an aerobactin receptor protein) selected after prolonged growth in the presence of 1 % (w/v) sodium dodecyl sulphate (SDS). *E. coli* strains of serotypes O1:K1:H7, O4:K12:H5, O6:K12:H1, O18ac:K5:H7 and O75:K5:H$^-$ are representatives of some of the major clonal groups associated with human urinary tract infections (Vaisanen-Rhen *et al.*, 1984); a strain of serotype O$^-$:K1:H33 was from a case of septicaemia in an infant less than 21 days old (Korhonen *et al.*, 1985). *Klebsiella pneumoniae* strain M5a1 expresses a receptor protein for type 1 klebicins [of which cloacin DF13 is an example (Cooper and James, 1985)] and for aerobactin, but does not itself synthesise this siderophore.

Recombinant plasmid pABN1 contains a 16.3 kb *Hind*III fragment of the prototype plasmid ColV-K30 which expresses the complete aerobactin system in an iron-regulated fashion (Bindereif and Neilands, 1983) and was a gift from J.B. Neilands. Plasmid pLG141 contains a 6.5 kb *Bam*HI fragment of pABN1 which expresses the aerobactin receptor protein (IutA) constitutively, but lacks most of the genetic determinants of aerobactin biosysnthesis (Carbonetti and Williams, 1984) cloned into vector pACYC184 (Chang and Cohen, 1978).

2.2.2. Culture media. Bacteria were grown in either nutrient broth (Oxoid no. 2) or in M9 minimal salts medium (Roberts *et al.*, 1963) containing 0.2% (w/v) glucose, 0.5% (w/v) casamino acids and 20 μgml^{-1} L-tryptophan. Ampicillin (100 μgml^{-1}) or chloramphenicol (10 μgml^{-1}) were included in media to select for the presence of pABN1 or pLG141 respectively. α,α'-Dypyridyl (200 μM) was added as required to limit availability of iron. Solid media contained 1.5% (w/v) agar (Difco).

2.2.3. Preparation of outer membrane protein antigens. Strain BZB1022(pABN1) was grown overnight in 250 ml of M9 medium containing

dipyridyl at 37°C with aeration. Bacteria were harvested by centrifugation at 10,000 x *g* for 10 min at room temperature, resuspended in 10 ml Tris-HCl (pH7.8), 10 mM $MgCl_2$, and disrupted by sonication. Non-disrupted cells were removed by centrifugation at 9,000 x *g* for 10 min at 4°C, and the supernatant fraction was subjected to ultracentrifugation at 100,000 x *g* for 40 min at 4°C. Pellets containing the total membrane fraction were resuspended in 1 ml of the Tris-$MgCl_2$ buffer containing 2% (v/v) Triton X-100 to disolve inner membrane proteins (Schnaitman, 1971) and again subjected to ultracentrifugation at 100,000 x *g* for 40 min at 4°C. Pellets containing outer membranes were resuspended as before, incubated at room temperature for 30 min and again subjected to ultracentrifugation at 100,000 x *g* for 90 min at 4°C. Soluble outer membrane protein preparations for inoculation of rabbits were prepared by resuspending and homogenizing the final pellets in 50 mM sodium phosphate buffer (pH7.2) containing 4 mM EDTA, 2% (v/v) Triton X-100 and 1.5 M urea. Remaining insoluble material was removed by ultracentrifugation as described above, and supernatant fractions were extensively dialysed against sodium phosphate-EDTA-Triton X-100 buffer to remove the urea before inoculation into a rabbit.

Purified denatured IutA was prepared by mixing outer membrane protein preparations with an equal volume of 0.25 M Tris/HCl (pH8.8) containing 2% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol, incubated at 65°C for 30 min and loaded onto a preparative 11% SDS-polyacrylamide gel for electrophoresis to separate the constituent polypeptides. The gel was briefly stained in Coomassie brilliant blue and a gel slice containing the IutA protein was excised, soaked in water for 30 min to remove methanol and acetic acid, and then macerated. A small volume of PBS (140 mM NaCl, 27 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) was added before inoculation into a rabbit.

2.2.4. Antisera. Strain BZB1022(pABN1) was chosen as the

vaccinating strain to raise antibodies against native aerobactin receptor because it lacks the similarly sized Cir protein (Pugsley, 1985). Bacteria were grown overnight in M9 medium containing dipyridyl and ampicillin. Rabbits were given 5 intravenous inoculations at 5 day intervals with 0.1, 0.2, 0.4, 1.0 and 1.5 ml of bacteria harvested by centrifugation at 2,000 x *g* and resuspended in the same volume of PBS. Rabbits were bled 7 days after the last inoculation. For antisera raised against denatured IutA, 1 mg of solubilized membrane protein or protein from SDS-PAGE purification as described above was mixed with an equal volume of Freund's complete adjuvant for primary inoculations, and with Freund's incomplete adjuvant for subsequent inoculations, and administered subcutaneously at six sites on the backs of the rabbits. Secondary inoculations were given after a 1 month interval, and rabbits were bled 14 days later.

2.2.5. Enzyme-linked-immunosorbent-assay (ELISA). Antisera were diluted 1:50 in PBS and absorbed three times for 20 min each at room temperature and with continuous mixing with 2% (w/v) suspensions of cells of appropriate strains grown overnight in M9 medium containing dipyridyl. Cells for sensitizing ELISA plates were harvested by centrifugation at 12,000 x *g* for 5 min from overnight M9 cultures containing dipyridyl, washed and resuspended in PBS to a density of 10^8 cells ml^{-1}. For removal of lipopolysaccharide (LPS), cells were resuspended after washing in 20 ml 1% (w/v) trichloroacetic acid (TCA) in 0.14 M NaCl, and incubated at 4°C for 1 h with continuous agitation before being pelleted by centrifugation at 2,000 x *g* for 10 min and washed again as described above. Cell suspensions (50 µl per well) were used to coat ELISA plates (Dynatech Microelisa) overnight at 4°C. Liquid was removed and replaced by PBS containing 10% (v/v) foetal calf serum and 0.5% (w/v) casein, and the plates were incubated at room temperature for 2 h before being washed three times with PBS containing 0.05% (v/v) Tween-20. Absorbed antisera were diluted in PBS

containing foetal calf serum and casein, and applied to appropriate ELISA plates at 50 µl per well. Plates were incubated at 37°C for 30 min, washed twice in PBS-Tween, and then secondary antiserum (Dakopatts; goat immunoglobulins to rabbit immunoglobulins conjugated with horseradish peroxidase) diluted 1:1000 in PBS-Tween was applied and incubated as described above. Plates were washed twice with PBS-Tween and once with 0.1M sodium citrate buffer (pH4.5), before addition to each well of 50 µl of chromophore solution (1 $mgml^{-1}$ *o*-phenylendiamine dihydrochloride in 0.1 M citrate buffer, pH4.5, to which 0.4 μlml^{-1} 30% (v/v) H_2O_2 had been added immediately before use). The colour that developed within 5 min was quantified by measuring the absorbance at 450 nm with a Titertech Multiskan Plus™ ELISA reader (Flow Laboratories).

2.2.6. Aerobactin binding inhibition assay. [^{14}C]Aerobactin was prepared as described in section 3.2.3. To assess the effect of antisera on aerobactin binding to receptors, cells from 5 ml of overnight nutrient broth cultures of W3110, W3110(pLG141) or D551 were harvested by centrifugation at 2,000 x *g* for 10 min, washed in PBS and resuspended in either antiserum (heated to 56°C for 30 min to destroy complement activity) appropriately diluted in PBS, cloacin DF13 (prepared as described in section 4.2.3) in PBS, or in PBS alone. Mixtures were incubated at room temperature with continuous agitation for 30 min. Cells were recovered by centrifugation at 10,000 x *g* for 10 min, washed twice and resuspended in one tenth the original volume of PBS. [^{14}C]Ferriaerobactin (1 µl; approximately 1500 cpm, corresponding to 1 pmol aerobactin) was added to each sample, and incubation was continued for a further 30 min. The cells were harvested by centrifugation at 10,000 x *g* for 10 min, resuspended in 200 µl PBS and, after addition of 2 ml Packard Emulsifier Safe scintillation fluid and vortexing, the radioactivity associated with the cell pellets was determined in a Packard Tri-Star liquid scintilation spectrometer.

2.3. RESULTS.

2.3.1. Effect of antisera against native IutA. An ELISA system using whole cells expressing the aerobactin receptor protein IutA as the sensitizing antigen was used to determine the activity of antisera against native protein. Antiserum raised against whole BZB1022(pABN1) bacteria expressing the IutA protein, and then absorbed with cells of the IutA$^-$ parent (BZB1022) displayed activity against BZB1022 carrying pABN1 but not against the plasmid-free strain, indicating specific interaction with native receptor (Fig. 2.1a). Antiserum raised against IutA protein recovered from denaturing gels displayed a similar profile, albeit with a lower titre, but antiserum raised against Triton/urea solubilized outer membrane proteins showed no activity specifically against the pABN1-containing strain (data not shown).

2.3.2. Effect of LPS on antibody binding to IutA. None of the antisera, including that raised against whole IutA$^+$ cells, gave a significant reaction in an ELISA with the clinical isolate D551 (Fig. 2.1b). To determine if this was due to steric hindrance of antibody access to the IutA protein, cells were treated with TCA to remove LPS. After TCA treatment, D551 cells gave a positive reaction with antiserum raised against whole bacteria, while TCA-treated LG1695, the ColV plasmid-cured derivative of D551, did not, indicating that the observed activity was specific for the aerobactin receptor (Fig. 2.1b). A selection of aerobactin producing clinical isolates with serotypes representing some of the major clonal groups associated with human urinary tract infections were also tested and produced similar results (Fig. 2.2a-e); increased immunoglobulin binding to strains of serotypes O1:K1:H7, O4:K12:H5, O6:K2:H1, O18ac:K5:H7 and O75:K5:H$^-$ was observed after TCA treatement of the bacteria, although the enhancement was variable from serotype to serotype. Significant reaction of antiserum was observed in an ELISA with a rough clinical isolate of

serotype O⁻:K1:H33 (Fig.2.2f); in this case TCA treatment caused a marked reduction in the level of immunoglobulin binding.

2.3.3. Effect of LPS on antibody binding to FepA. To determine if shielding of the aerobactin receptor from access by antibodies was due to a specific interaction of IutA with LPS molecules, or if it reflected a more general protection of outer membrane proteins by LPS, an ELISA assay was used to study antibody-accessibility of another iron regulated outer membrane protein, the enterochelin receptor FepA. As the antiserum raised against native IutA in whole cells was raised against a FepA⁺ strain [BZB1022(pABN1)] it was reasoned that this antiserum could be used as a source of antibodies against native FepA. By using strain AN1937 to sensitize ELISA plates and an isogenic FepA⁻ strain LG1522 to preabsorb the antiserum it was possible to demonstrate specific activity against native FepA (Fig. 2.3a). When the LG1522-absorbed antiserum was titrated against LG1695, the plasmid-cured derivative of D551, some specific activity was detectable against FepA without prior TCA treatment but this was greatly enhanced by treatment with TCA (Fig. 2.3b).

2.3.4. Effect of antisera on aerobactin uptake. To determine whether any of the antisera would interfere with the physiological function of the aerobactin receptor, uptake of [^{14}C]aerobactin into live cells expressing the receptor was measured after pretreatment with the various antisera. Only antiserum raised against whole IutA⁺ cells had a significant inhibitory effect on the binding of [^{14}C]aerobactin to whole bacteria expressing the receptor (Table 2.1). Siderophore binding to W3110(pLG141) was reduced by approximately 40% when the cells were pretreated with antiserum diluted 1:10. To determine if this was the maximum inhibition achievable [^{14}C]aerobactin uptake was measured in cells treated with a range of antiserum dilutions from 1:2 to 1:100 (Fig. 2.4). The graph displays two plateaux, one representing maximal levels of uptake and occurring at

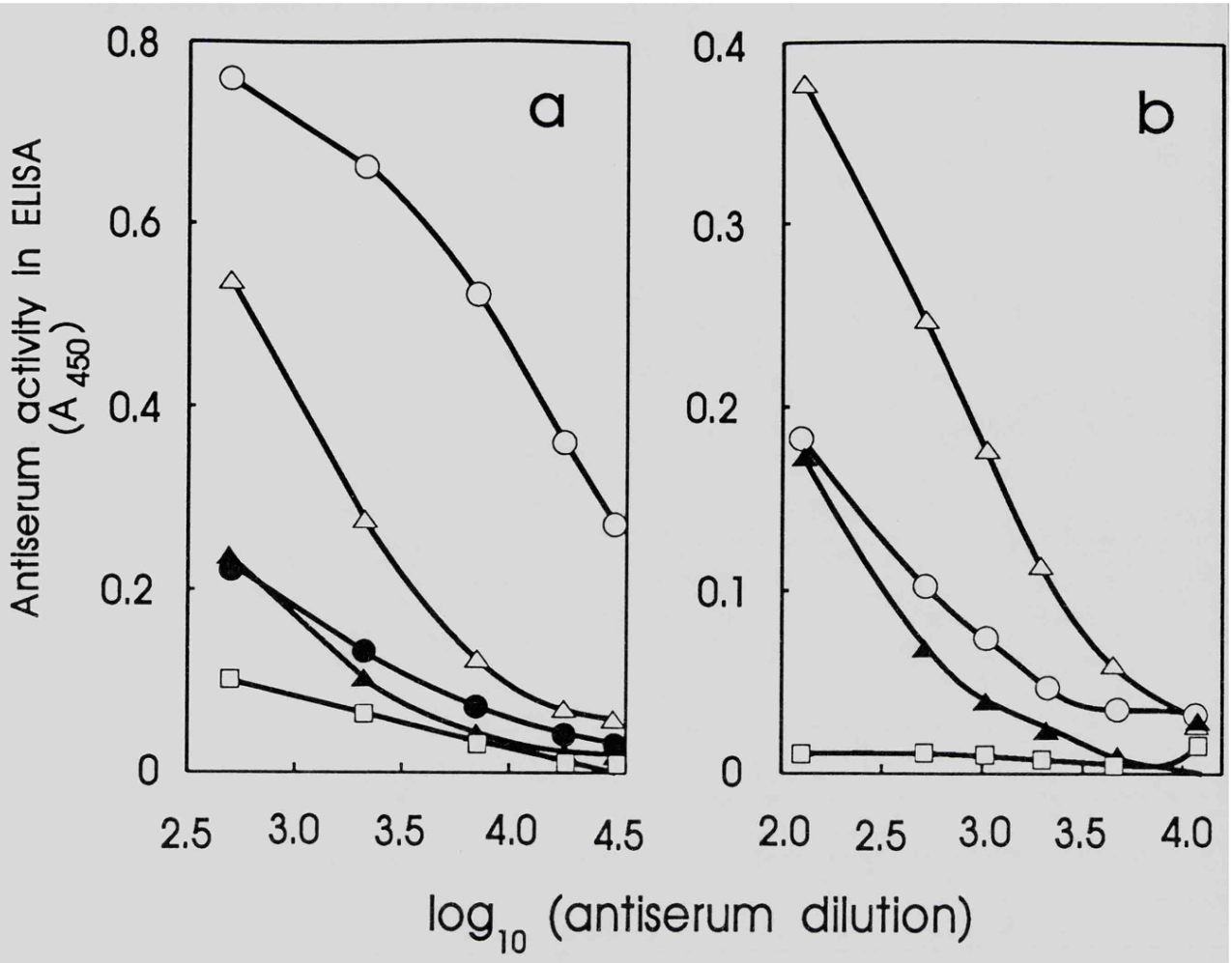
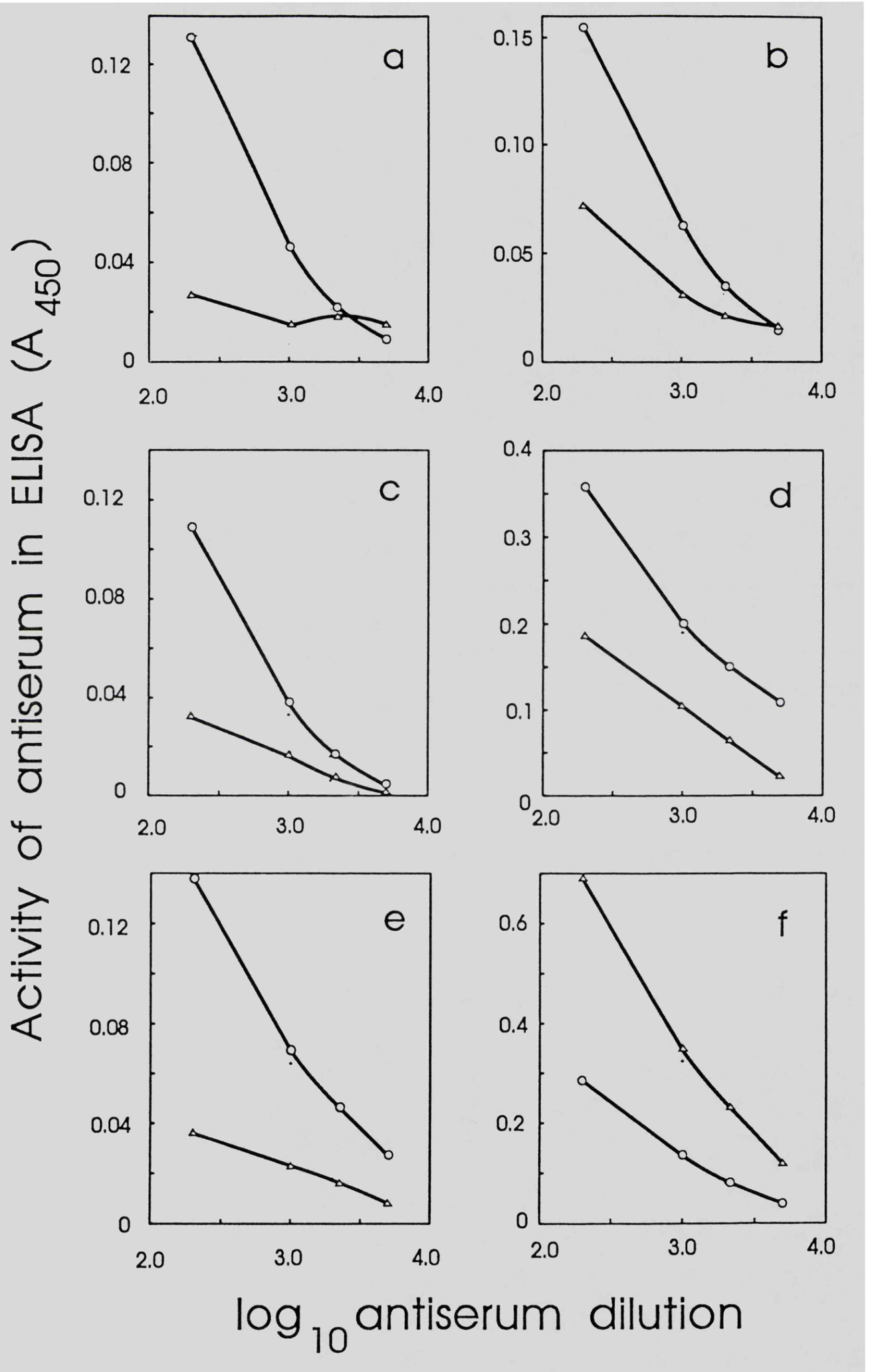


Figure 2.1. Activity of antisera against native IutA in ELISA. (a) Microtiter plates were coated with whole bacteria of strain BZB1022(pABN1) (open symbols) or BZB1022 (filled symbols) and reacted with dilution series of antiserum against whole IutA$^+$ bacteria (○,●), or of antiserum against denatured IutA protein recovered from an SDS-PAGE gel (△,▲), or of pre-immune serum (□). (b) Dilutions of antiserum against whole IutA$^+$ were added to wells of microtiter plates coated with whole bacteria of strain D551, untreated (▲) or treated with TCA to remove LPS (△), or whole TCA-treated bacteria of strain LG1695 (○). Plates coated with TCA-treated D551 were also reacted with dilutions of pre-immune serum (□). A_{450} measurements were recorded after processing of plates as described in Materials and Methods. The effect of each treatment was determined four times; 1 SD was always less than 0.05 A_{450} units.

Figure 2.2. Activity of antiserum against the aerobactin receptor protein in human clinical isolates. Microtiter plates coated with whole bacteria untreated (△) or treated with TCA to remove LPS (○) were reacted with dilution series of antiserum raised against whole IutA$^+$ bacteria. Bacteria used to coat plates were of strains of serotype (a) O1:K1:H7, (b) O4:K12:H5, (c) O6:K2:H1, (d) O18ac:K5:H7, (e) O75:K5:H$^-$, or (f) O$^-$:K1:H33. A_{450} measurements were recorded after processing of plates as described in Materials and Methods.


a
b
c
d
e
f
0.12
0.08
0.04
0
0.15
0.10
0.05
0.4
0.3
0.2
0.1
0.6
2.0
3.0
4.0
Activity of antiserum in ELISA (A_{450})
$\log_{10}$ antiserum dilution

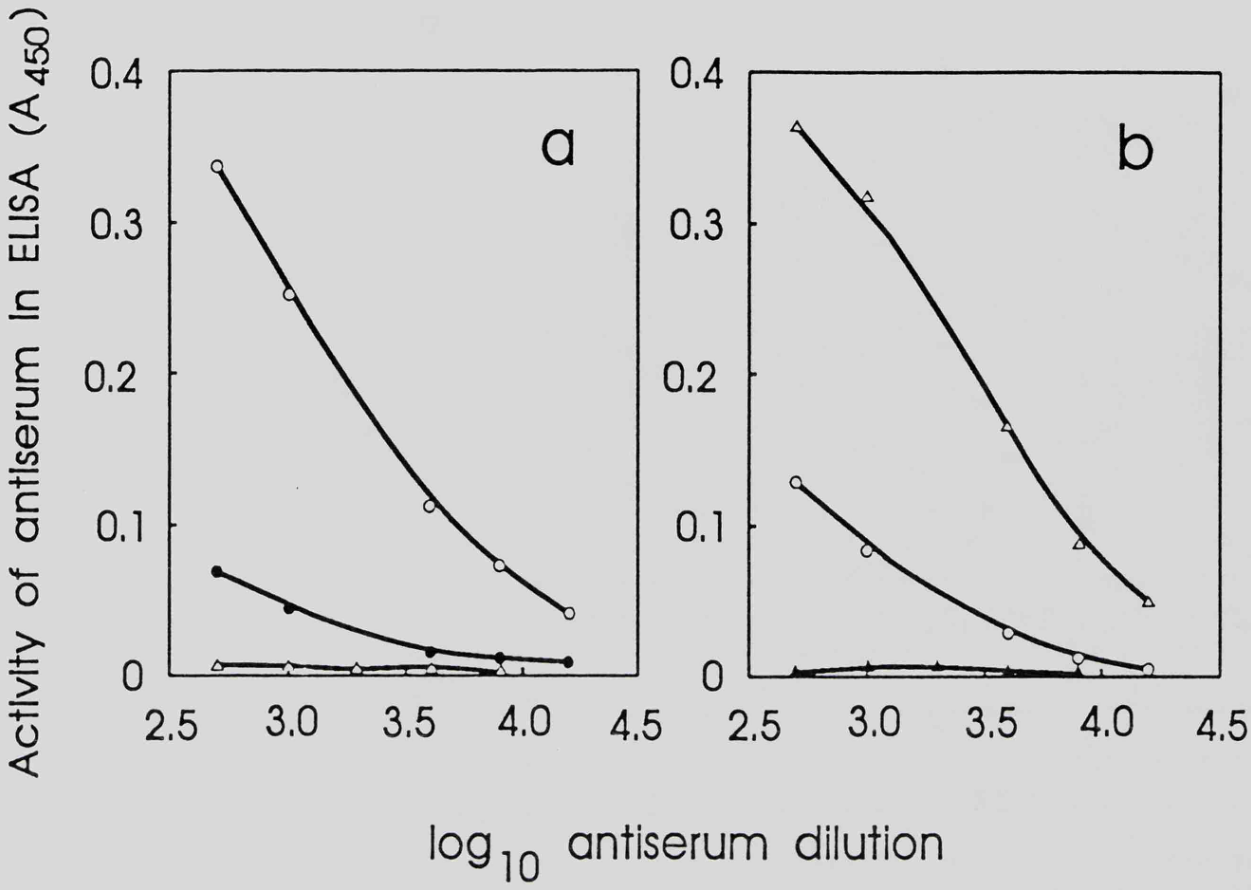


Figure 2.3. Activity of antiserum against native FepA in ELISA. (a) Microtiter plates were coated with whole bacteria of strain AN1937 (open symbols) or LG1522 (filled symbols) and reacted with dilution series of antiserum against whole FepA$^+$ bacteria (○ , ●), or of pre-immune serum (△). (b) Dilutions of either antiserum against whole FepA$^+$ bacteria (open symbols) or pre-immune serum (filled symbols) were added to wells of microtiter plates coated with whole bacteria of strain LG1695, untreated (○) or treated with TCA to remove LPS (△ , ▲). A_{450} measurements were recorded after processing of plates as described in Materials and Methods.

Table 2.1. Effect of antisera and cloacin DF13 on [14C]aerobactin uptake by whole bacteria expressing the receptor.

Bacterial Strain	Pre-treatment[a]	[14C]aerobactin uptake[b]
W3110	-	8.0%
W3110(pLG141)	-	100.0%
W3110(pLG141)	Pre-immune serum	97.2%
W3110(pLG141)	Anti-whole cell serum	59.7%
W3110(pLG141)	Anti TSOMF[c]	96.8%
W3110(pLG141)	Anti IutA (SDS-PAGE-purified)[d]	98.5%
W3110(pLG141)	Cloacin DF13	27.0%
D551	-	100.0%
D551	Pre-immune serum	75.3%
D551	Anti-whole cell serum	84.0%
D551	Cloacin DF13	100.0%

[a] Bacteria were incubated with the competitors indicated before addition of [^{14}C]aerobactin.

[b] Uptake is expressed as a percentage of the activity associated with each IutA$^+$ strain in the absence of pre-treatment [563 cpm or 540 cpm for strains W3110(pLG141) and D551 respectively].

[c] Antiserum raised against Triton-Urea-EDTA-solubilized IutA protein.

[d] Antiserum raised against IutA protein recovered from an SDS-PAGE gel slice.

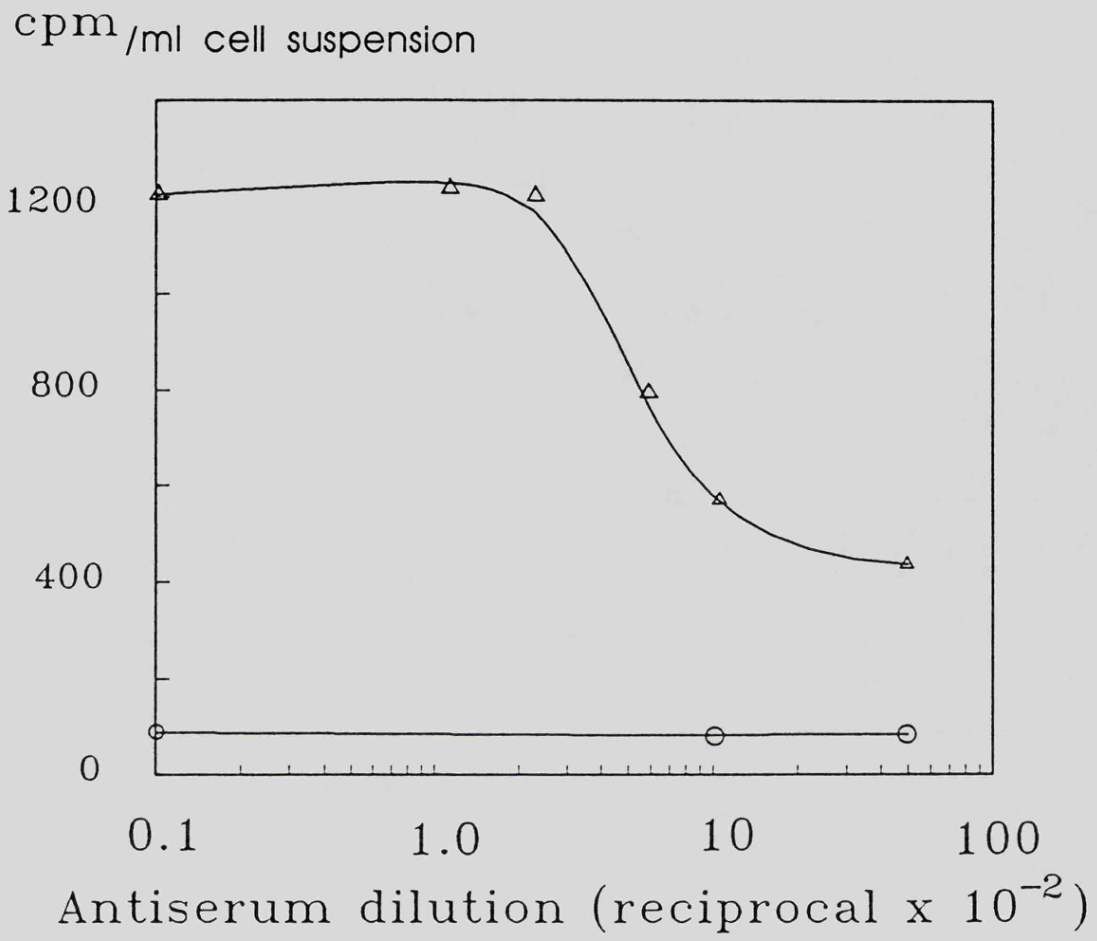


Figure 2.4. Inhibition of [^{14}C]aerobactin uptake by antiserum raised against whole IutA^{+} bacteria. Bacteria of strain W3110(pLG141) (△) or W3110 (○) were incubated with various dilutions of antiserum before addition of [^{14}C]aerobactin.

antiserum dilutions of 1:50 or above, and a second at approximately 25% of maximal levels occurring at antiserum dilutions of 1:10 and below. The *Enterobacter cloacae* bacteriocin cloacin DF13, which also binds to aerobactin receptor proteins (de Graaf *et al.*, 1969; Bindereif *et al.*, 1982; Carbonetti and Williams, 1984; Chapter 4 of this thesis), reduced siderophore binding to W3110(pLG141) by more than 70% (Table 2.1). Neither cloacin DF13 nor any of the antisera raised against IutA displayed significant inhibition of aerobactin uptake into cells of strain D551 (Table 2.1).

2.4. DISCUSSION.

Antibodies raised against native IutA protein in whole cells reacted against native protein in an ELISA assay with whole cells and caused significant inhibition of aerobactin uptake by live cells expressing the aerobactin receptor. Antiserum raised against SDS-denatured IutA displayed some activity in an ELISA against native protein but with a rather lower titre than the antiserum raised against native protein, and no specific activity could be detected in an antiserum raised against a urea-denatured outer membrane protein preparation containing IutA. Neither of the antisera raised against denatured preparations of IutA displayed any activity in an aerobactin uptake inhibition assay, and thus antisera raised against denatured IutA are unlikely to be effective in immunotherapy. These results agree with the observations of others that antisera raised against a protein in denatured form often react poorly or not at all with the native protein, and vice versa (Benjamin *et al.*, 1984; Gabay *et al.*, 1985). The majority of antibodies raised against native proteins recognize conformational epitopes, but such epitopes are often irreversibly destroyed during denaturation and thus would not be presented to the immune

systems of animals immunized with denatured protein preparations. Antibodies raised against denatured proteins recognize sequential epitopes, which may be distorted by secondary structure or buried within the native protein, and therefore not accessible to immunoglobulins. There are, however, reports of inhibition of biological activity by antisera raised against denatured proteins; antisera raised against SDS-denatured FhuA protein of *E. coli* blocked adsorption of phage T5 and partially inhibited ferrichrome-mediated iron uptake by the biologically active native protein (Coulton, 1982). Morover, it has been claimed that antisera raised against SDS-denatured IROMPs of a virulent *E. coli* strain passively protected turkeys in experimental infections (Bolin and Jenson, 1987) and similar results were obtained with antibodies against denatured OmpF protein of *Pseudomonas aeruginosa* in a burned mouse model (Matthews-Greer and Gilleland, 1987).

The observation that saturation binding of immunoglobulins raised against the native protein or of cloacin DF13 does not completely inhibit aerobactin binding and uptake by live cells expressing the receptor suggests that immunoglobulins and cloacin DF13 do not bind to the site at which aerobactin binds. It has been suggested that the majority of binding sites within proteins, including immunoglobulins, are cavities or grooves (Novotny *et al.*, 1987); thus epitopes on the surface of a protein are presumably convex to fit the concave paratopes of immunoglobulins. If this is also the case with the aerobactin biding site of IutA, it is likely to be non-immunogenic. The incomplete inhibition of aerobactin binding to IutA by immunoglobulin and cloacin DF13 is thus probably due to steric hindrance of access to the binding site by these ligands. Alternatively, binding of immunoglobulin or cloacin DF13 to the IutA receptor at sites distant from the aerobactin binding site might induce conformational changes within the protein which reduce its affinity for aerobactin. A further possibility is that

immunoglobulins or cloacin DF13 bound to the IutA receptor may inhibit conformational changes necessary for translocation of aerobactin across the outer membrane. The cell associated radioactivity that is detected in this assay is predominantly intracellular and the level of aerobactin associated with the outer membrane itself is too low to be detected by this assay (see Chapter 3).

Although the antiserum raised against whole bacteria had a high degree of activity against native protein expressed on the surface of laboratory strains of *E. coli*, similar activity was not observed against several clinical isolates of *E. coli* which harbour the aerobactin system. Nevertheless, the degree of aerobactin uptake was similar in the pathogenic isolate D551 and in BZB1022(pABN1), indicating comparable expression of receptor molecules. Thus, either the aerobactin receptor in strain D551 lacks immunological homology with that encoded by pABN1 in the immunizing strain, or it is masked by surface structures such as LPS, which covers about 45% of the surface area of Gram-negative bacteria (Gmeiner and Schlect, 1980). While small molecules such as aerobactin (molecular mass 536) would not be significantly inhibited from gaining access to receptors in the outer membrane by LPS, it is likely that large proteins such as cloacin DF13 or immunoglobulin (with molecular masses of 70,000 and greater) would be sterically excluded from the bacterial surface by LPS.

Certainly the presence of O-antigens on *E. coli*, *Klebsiella*, and *Citrobacter* cells prevented binding of polyclonal and monoclonal antibodies raised against the *E. coli* K-12 PhoE protein (van der Ley *et al.*, 1986b), and the binding of phages and colicins to *E. coli* may also be abolished (Van der Ley *et al.*, 1986a). Recently Bently and Klebba (1988) used a series of *rfaE* mutants of *E. coli* and *Salmonella typhimurium* to demonstrate that anti-porin monoclonal antibodies bind more effectively the shorter the LPS chain. On the other hand, Griffiths *et al.* (1983, 1985) showed that IROMPs

of smooth *E. coli* strains of serotypes O111 and O18 readily labeled by a lactoperoxidase-radioiodination technique, indicating that they were surface-exposed and accessible to at least some large protein molecules. TCA has been used to remove LPS from the surface of *Coxiella burnetii* cells to expose antigens that are normally shielded (Hackstadt, 1988). When a modification of this extraction procedure was used to treat smooth *E. coli* cells, antibodies raised against whole IutA$^+$ bacteria were able to bind specifically, confirming that the aerobactin receptor proteins in these strains can be recognized by antibodies raised against the prototype receptor, but are normally masked by LPS molecules. This masking, however, does not seem to reflect a specific interaction between the aerobactin receptor and LPS as similar inhibition of binding was also observed with antibodies raised against native FepA protein. That the observed masking is associated with LPS rather than the capsule antigen may be inferred from the observation that immunoglobulin binding to an O1:K1 strain can be demonstrated only after TCA treatment of the cells while specific binding of immunoglobulins to a rough K1 strain can be demonstrated without prior TCA treatment, and indeed in the later case TCA treatment actually inhibited binding, suggesting that some of the epitopes may have been destroyed by the TCA treatment. It has recently been shown that the presence of LPS encoded by chromosomal genes of *Shigella flexneri* inhibited binding of cloacin DF13 to the 76 kDa *S. flexneri* aerobactin receptor in *E. coli-S. flexneri* hybrid cells (Derbyshire *et al.*, 1989). These results, and the observation reported here that smooth LPS from the O-serogroups most commonly associated with extraintestinal infections all afforded protection of the aerobactin receptor protein from access of immunoglobulins suggest that the prospect of using IutA as the basis for immunotherapy, at least for *E. coli* infections, is not promising.

Chapter 3

Transport of Ferric Aerobactin into the Periplasm and Cytoplasm of Escherichia coli K-12: Role of Envelope-Associated Proteins and Effect of Endogenous Siderophores.

3.1. INTRODUCTION.

Siderophore-mediated uptake of iron by *Escherichia coli* is initiated by binding of ferric-siderophore complexes to cognate outer membrane receptor proteins (Braun and Hantke, 1981; Neilands, 1982). Transport of iron into the periplasmic space then requires the activity of the cytoplasmic membrane protein TonB and, to a lesser and variable extent, products of the *exb* locus (Fischer *et al.*, 1989; Neilands, 1982). The TonB protein has been thought for some years to provide energy to the outer membrane receptor proteins (Hankock and Braun, 1976; Hantke and Braun, 1978; Postle, 1990b). Recent evidence that some mutations in the "TonB box", a short consensus sequence found in all TonB dependent outer membrane proteins (Coulton *et al.*, 1986; Heller and Kadner, 1985; Lundrigan and Kadner, 1986; Pressler *et al.*, 1988; Sauer *et al.*, 1987), can be genetically suppressed by mutations in the *tonB* gene itself (Heller *et al.*, 1988; Schoffler and Braun, 1989) suggest a direct interaction between TonB and such proteins. The *exb* locus comprises two genes *exbB* and *exbD* (Eick-Helmerich and Braun, 1989). The ExbB protein has been shown to stabilize both TonB and ExbD, and partial phenotypic supression of *exb* mutations has been observed in cells overexpressing TonB (Fischer *et al.*, 1989). The function of ExbD has not been resolved, but it is assumed to act with ExbB to enhance TonB-dependent uptake across the outer membrane (Eick-Helmerich and Braun, 1989).

Subsequent to transport across the outer membrane, passage of iron

through the periplasm and across the cytoplasmic membrane requires the participation of several gene products which resemble the periplasmic transport mechanisms described for a number of nutrients which do not require TonB to cross the outer membrane (Ames, 1986; Higgins, 1988). These systems comprise a hydrophilic protein located in the periplasm, one or two very hydrophobic proteins in the cytoplasmic membrane, and a relatively hydrophilic protein, which is nonetheless also associated with the cytoplasmic membrane and contains regions of extensive homology with ATP-binding proteins. In *E. coli*, specific systems of this general type are involved in the transport of iron by enterochelin (Chenault and Earhart, 1991; Elkins and Earhart, 1989; Ozenberger *et al.*, 1987; Shea and McIntosh, 1991), ferric-dicitrate (Staudenmaier *et al.*, 1989), and hydroxamate siderophores (Burkhardt and Braun, 1987; Köster and Braun, 1989), as well as of vitamin B_{12} (Friedrick *et al.*, 1986) which, in common with the iron chelates, is transported across the outer membrane of *E. coli* via a specific outer membrane protein (BtuB) in a TonB-dependent process (White *et al.*, 1973; Bassford *et al.*, 1976).

Aerobactin-mediated uptake of iron into *E. coli* cells requires the specific outer membrane receptor protein IutA (Bindereif *et al.*, 1982; Grewal *et al.*, 1982) for initial binding of the ferric siderophore complex, and the common inner membrane protein TonB (Williams, 1979) and its functionally associated protein ExbB (Braun *et al.*, 1982) for release of iron into the periplasm. Analysis of mutants (Braun *et al.*, 1982; Braun *et al.*, 1983; Hantke, 1983), and characterization and localization of proteins (Fecker and Braun, 1983; Köster and Braun, 1989), suggest that the products of the *fhuB*, *fhuC*, and *fhuD* genes form a common periplasmic binding system for uptake of iron via various hydroxamate siderophores, including aerobactin. FhuD is located in the periplasm, while the hydrophobic protein FhuB and the hydrophilic putative ATP-binding

protein FhuC are located in the cytoplasmic membrane and are thought to comprise a cytoplasmic membrane permease complex. However there are no reports in the literature of direct measurements of the uptake of any siderophore into well-defined cellular compartments of *E. coli*, nor physical confirmation of the assumed role of periplasmic binding protein systems in the translocation of siderophores to the cytoplasm. Indeed similar genetic and physical evidence exists for the citrate-mediated iron uptake system of *E. coli* (Frost and Rosenberg, 1973; Staudenmaier *et al.*, 1989; Wagegg and Braun, 1981), and yet citrate appears not to be transported across the cytoplasmic membrane (Hussein *et al.*, 1981).

IutA activity has been demonstrated using a sensitive binding assay involving radiolabelled aerobactin (Roberts *et al.*, 1989 and Chapter 2 of this thesis). Here, experiments to determine the subsequent subcellular localization of receptor-bound aerobactin in wild type *E. coli* and in mutants lacking each of the necessary envelope-associated functions are described. Furthermore, the rate of uptake of aerobactin, but not that of ferric iron supplied as an aerobactin chelate, is shown to be markedly reduced by the presence of endogenous aerobactin.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and plasmids. Characteristics of all bacterial strains and plasmids used in this study are described in Table 3.1. Recombinant plasmid pABN1 contains the entire iron regulated aerobactin operon of the prototype ColV plasmid ColV-K30 cloned in the vector p*Plac* (Bindereif and Neilands, 1983). Plasmid pLG141 is a subclone of pABN1 carrying the receptor gene *iutA*, but lacking most of the siderophore biosynthesis genes (*iuc*) genes, cloned in the vector pACYC184 (Carbonetti and Williams, 1984); *iutA* is expressed constitutively. ColV-K30*iuc* was isolated after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis of a strain

harbouring plasmid ColV-K30 (Williams and Warner, 1980). Plasmid pFB102 contains a *PstI-EcoRI* fragment of *E. coli* chromosomal DNA that includes the entire *fhu* region containing a Tn*5* insertion in *fhuD*, cloned into the ampicillin resistance gene of pBR322 (Fecker and Braun, 1983). Plasmid pBR322 was introduced by transformation into plasmid-bearing strains as a source of periplasmic β-lactamase (Sutcliffe, 1979).

3.2.2. Media and growth conditions. Bacteria were grown either in nutrient broth (Oxoid no. 2) or in M9 minimal salts medium (Roberts *et al.*, 1963) supplemented with 0.2% (w/v) glucose, 0.5% (w/v) casaminoacids and 20 μgml^{-1} L-tryptophan, and containing 200 μM 2,2'-dipyridyl to induce iron-regulated genes. Ampicillin (100 μgml^{-1}) was included in media for the growth of strains carrying plasmids pABN1 and pBR322, tetracycline (15 μgml^{-1}) was added to maintain plasmid pFB102 in strain LF940, and chloramphenicol (20 μgml^{-1}) was added to maintain strains harbouring pLG141. Cultures were grown aerobically with vigorous agitation at 37°C.

3.2.3. Preparation of [^{14}C]aerobactin. ^{14}C-labelled aerobactin was purified from culture supernatants of *Aerobacter aerogenes* strain 62-1. Bacteria were grown overnight in 10 ml of M9 salts medium, 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 [^{14}C]lysine (specific activity 11.99 $GBqmmol^{-1}$, Amersham). The suspension was incubated at 37°C for a further 4 h before bacteria were again pelleted by centrifugation, and the supernatant fluid lyophilyzed. 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 again lyophilyzed, 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 rf=0.53 (Gibson and Magrath, 1969) was eluted in 1 ml distilled water. To determine the concentration, 50 µl of the preparation was added to 50 µl of 10 mM $FeCl_3$ in 0.1 M HCl and diluted to 0.5 ml in 10 mM Tris-HCl (pH 7.6). Absorbance at 399 nm was determined against an appropriate blank and the concentration was determined using the molar extinction coefficient for ferric aerobactin reported by Harris *et al.* (1979). The solution was found to be 890 µM and the specific activity was calculated to be 414 $MBqmmol^{-1}$.

3.2.4. Preparation of [^{59}Fe/^{14}C]aerobactin. [^{14}C]apo-aerobactin was prepared as described above for aerobactin except that the sample was redissolved in 1 mM EDTA before chromatography and was found to migrate at rf=0.3. The concentration was determined as above and found to be 783 µM with a specific activity of 147 $MBqmmol^{-1}$. To 400 µl (313 nmol at 147 $MBqmmol^{-1}$) of this solution were added 40 µl of 1 M Tris-HCl (pH 7.6), 4 µl of 20 mM 2,2'-dipyridyl, 3.7 µl of ^{59}Fe (3.45 nmol; 46 kBq, as Fe(II)SO_4 in 0.05 M H_2SO_4, Dupont de Nemours, Germany), and 31 µl of 10 mM $FeCl_3$. The mixture was incubated at room temperature for 30 min before use to allow chelation of iron by aerobactin.

3.2.5. Uptake of labelled aerobactin and cell fractionation. Cells were washed and resuspended in PBS containing 200 µM dipyridyl to an optical density at 600nm of 1. [^{14}C]aerobactin (5µl; approximately 4.5 nmol) was added to each 1 ml sample and incubated at room temperature for 90 min. In double label experiments cells of strains W3110, W3110(pLG141) and W3110(pABN1) were grown overnight in nutrient broth containing dipyridyl, washed and resuspended in PBS containing dipyridyl to an optical density of 1; 13 ml aliquots were placed in flasks and pre-incubated for 5 min at 37°C before addition of 120 µl of doubly labelled aerobactin prepared as described above. At intervals 2 x 1 ml aliquots were

removed. After incubation with [^{14}C]aerobactin or [^{59}Fe/^{14}C]aerobactin cells were separated into periplasmic and cytoplasmic fractions by a modification of the cold osmotic shock method of Nossal and Heppel (1966), and outer and inner membrane fractions were separated on the basis of differential solubility of the two membranes in the non-ionic detergent Triton X-100 (Schnaitman, 1971). Bacteria from 1 ml reaction mixtures were pelleted by centrifugation, resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA containing 25% (w/v) sucrose, and washed twice in 1 ml amounts of the same buffer. Bacteria were harvested by centrifugation, resuspended in 0.4 ml of ice cold water and incubated on ice for a further 10 min, before again being recovered by centrifugation. The supernatant (periplasmic) fraction was removed, and the spheroplast pellet was resuspended in 0.4 ml of 10 mM Tris-HCl (pH 7.5). Cells were disrupted by sonication, non-disrupted cells were removed by low speed centrifugation, and the supernatant fraction was removed and subjected to ultracentrifugation (100,000 x *g*, 10 min). The supernatant (cytoplasmic) fraction was removed, and the pellet was resuspended in 0.4 ml 10 mM Tris-HCl (pH 7.5) containing 2% Triton X-100 and incubated at room temperature for 30 min. After further ultracentrifugation the supernatant fraction (cytoplasmic membranes) was removed and the pellet (Triton-insoluble outer membranes) was resuspended in 0.4 ml of the same buffer. Radioactivity associated with each fraction was measured, after addition of 4 ml of Optiphase X scintillation cocktail (Pharmacia), in a Packard Tri-Carb 2000A liquid scintillation analyser. In single label experiments, ^{14}C was determined at 0-156 keV; in double label experiments, ^{59}Fe was recorded at 80-2000 keV, and 0-10 keV was used to calculate ^{14}C activity, after taking into account the contribution of ^{59}Fe in this window.

3.2.6. Enzyme assays of sub-cellular fractions. To determine the purity of periplasmic and cytoplasmic fractions, enzyme activities

characteristic of the two cellular compartments were determined. The periplasmic enzyme β-lactamase, encoded by the *bla* gene of pBR322, was assayed by enzymic conversion of the chromogenic substrate nitrocefin (Glaxo) to a product quantifiable by absorbance measurement at 482 nm (A_{482}). Stock nitrocefin solution was prepared by dissolving 5 mg in 0.5 ml dimethylsulphoxide and adding 9.5 ml of 0.05 M phosphate buffer (pH 7.0). Periplasmic and cytoplasmic preparations were diluted 1:20 in the same buffer, and 0.5ml samples were added to 0.5ml of nitrocefin solution. Mixtures were incubated at 22°C for 5 min during which A_{482} measurements were recorded at 30 sec intervals. Activity was calculated from the slope of the plot of A_{482} against time; cytoplasmic fractions routinely contained less than 8% of total cellular β-lactamase activity. The cytoplasmic marker was the chromosomally encoded enzyme malate dehydrogenase (MDH). 0.1 ml samples of periplasmic and cytoplasmic extracts were added to 1 ml reaction mixtures containing 0.1 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$ and 80 mg of NADPH. Reactions were started by the addition of oxaloacetic acid to 1 M; incubation was at 22°C, and A_{340} measurements were made at 30 sec intervals for 7 min. Activity was calculated from the slope of the plot of A_{340} against time; periplasmic fractions consistently contained approximately 3% of total cellular MDH activity. These data indicate that the intracellular distribution of labelled aerobactin observed in the experiments described below is unlikely to be due to significant cross-contamination of compartmental contents.

Table 3.1. Bacterial strains and Plasmids

Strain/Plasmid	Relevant properties	Reference
Bacteria		
E. coli K-12		
W3110	Wild type	Bachmann, 1972
W3110-6	As W3110 but *exb*	Fischer *et al.*, 1989
AN1937	*ara entA lac leu mtl proC rpsL supE thi fhuA xyl*	Williams, 1979
LG1316	As AN1937 but *tonB*	Williams, 1979
LF940	*recA hsdM hsdR supE lacY leu thi pro aro fhuB fhuD*	Fecker & Braun, 1983
LF947	*recA minA minB lacY xyl thi mtl hsdM hsdR fepA fhuC12*	Fecker & Braun, 1983
BU736	*aroB cir tsx malT thi fhuB* ColV-K229	Braun *et al.*, 1982
LG1706	As BU736 but cured of plasmid ColV-K229	This study
Aerobacter aerogenes		
62-I	Aerobactin producer	Gibson & Magrath, 1969
Plasmids		
ColV-K30	$iutA^+$ iuc^+ cva^+	Williams, 1979
ColV-K30*iuc*	$iutA^+$ *iuc* cva^+	Williams & Warner, 1980
pABN1	$iutA^+$ iuc^+ bla^+ (Ap^R)	Bindereif & Neilands, 1983
pLG141	$iutA^+$ cat^+ (Cm^R)	Carbonetti & Williams, 1984
pBR322	bla^+	Sutcliffe, 1979
pFB102	*fhuB fhuC fhuD*::Tn*5*	Fecker & Braun, 1983

3.3.RESULTS

3.3.1. Cellular localization of aerobactin. Uptake of radiolabelled aerobactin into the four cellular fractions of strains with various defined mutations affecting utilization of aerobactin as an iron source was determined as described in Materials and Methods (Table 3.2). Radioactivity associated with membrane fractions of all strains tested was negligible, suggesting only transient association of aerobactin with membrane compartments. However, significant levels of radioactivity were observed in both the periplasmic and cytoplasmic fractions of the wild type strain W3110(pLG141), but not of strain W3110, which lacks IutA activity, nor of the *tonB* mutant strain LG1316(pLG141). These data confirm the requirement of aerobactin iron transport for IutA and TonB functions (Bindereif *et al.*, 1982; Braun *et al.*, 1982; Williams, 1979). Uptake into both cellular compartments of the *exbB* mutant W3110-6(pLG141) was also markedly reduced compared with wild type, but was nonetheless significantly higher than into *tonB* cells. This is consistent with earlier findings that requirement for *exb* gene products is less stringent than for TonB in TonB-dependent uptake mechanisms (Fischer *et al.*, 1989).

Accumulation of [^{14}C]aerobactin in the periplasmic compartments of the three *fhu* mutants LF940(pFB102, pLG141), LG1706(pLG141) and LF947(pLG141) was similar to that in the periplasm of W3110(pLG141). However, uptake into the cytoplasm of the *fhuD* mutant LF940(pFB102, pLG141) was negligible, confirming that the periplasmic binding protein FhuD is absolutely required for translocation of aerobactin across the cytoplasmic membrane. By contrast, *fhuB* and *fhuC* strains LG1706 and LF947 harbouring pLG141 consistently showed low (compared with wild type) but significant levels of uptake of aerobactin into the cytoplasmic fraction. It is possible that defects in either of the cytoplasmic membrane functions involved in periplasmic transport of ferric-hydroxamates are

partially complemented by equivalent components of other systems. Alternatively this may be explained if these mutant genes express proteins with some residual activity.

3.3.2. Effect of endogenous siderophores on aerobactin uptake. Strain AN1937 is an *entA* mutant and so is unable to synthesize enterochelin. A derivative of AN1937 harbouring plasmid pLG141 accumulated levels of radioactive aerobactin in the periplasmic and cytoplasmic fractions similar to those observed for the Ent^+ strain W3110(pLG141) (Table 3.2). Thus, the ability to make enterochelin has no effect on the rate or extent of uptake of aerobactin from the medium. On the other hand, the presence of endogenous aerobactin appears to inhibit markedly the uptake of exogenously supplied aerobactin; strain W3110 carrying plasmid pABN1, which specifies aerobactin biosynthesis as well as receptor activity (iuc^+iutA^+), showed significantly lower levels of [^{14}C]aerobactin in both cellular compartments compared with W3110(pLG141) (Table 3.2). Similar effects were observed with a strain containing the entire aerobactin plasmid ColV-K30; ^{14}C levels associated with the periplasm and cytoplasm of strain W3110(ColV-K30) were two-thirds and half, respectively, of those observed for the corresponding compartments of strain W3110(pLG141) (data not shown). It should be stressed, however, that these plasmids are not strictly comparable. First, the aerobactin operon in plasmids pABN1 and ColV-K30 is iron regulated (Braun *et al.*, 1984, Roberts *et al.*, 1986) while *iutA* is expressed constitutively from pLG141. Second, ColV-K30 has a copy number of one or two per cell, while pLG141 and pABN1 are multicopy plasmids, and so express higher levels of the receptor protein.

Therefore, to determine whether variations in *iutA* gene dosage or expression could account for these observations, uptake of radiolabelled siderophore into isogenic W3110 strains containing either the prototype

aerobactin plasmid ColV-K30, or a mutant derivative ColV-K30*iuc* (Williams *et al.*, 1980) was compared. Bacteria were grown overnight in minimal medium containing dipyridyl to induce maximal transcription of the aerobactin operon, and full expression of the *iutA* gene in both plasmids; cells were incubated with [^{14}C]aerobactin for 90 min as described above. The *iuc* mutant consistently accumulated significantly more exogenously supplied aerobactin in both cellular compartments than did the wild-type iuc^{+} strain ($P<0.025$), 378 cpm compared with 135 cpm into the periplasm, and 314 cpm compared with 139 cpm into the cytoplasm. (Figures obtained for each uptake into each compartment were means of readings from 3 independent experiments and were compared for significance using a test based on Student's T test).

A possible explanation for this difference is that the specific activity of [^{14}C]aerobactin was reduced by unlabelled siderophore secreted by producing strains. Since less than 2.5% of the total radioactivity in a reaction mixture is actually taken up by receptive bacteria, the level of residual radioactivity in the reaction mixture is not measurably altered, and buffers recovered after uptake assays can be reused. Incubation buffers used to determine [^{14}C]aerobactin uptake by strains W3110(ColV-K30) and W3110(ColV-K30*iuc*) were therefore subsequently used to label nutrient broth cultured W3110(pLG141) cells over a further 90 min incubation period. Virtually identical levels of uptake were observed [740 cpm in cells incubated in buffer pre-incubated with W3110(ColV-K30) compared with 654 cpm in cells incubated in buffer pre-incubated with W3110(ColV-K30*iuc*)], indicating that reduced accumulation of radioactivity by the strain carrying ColV-K30 was not due to a significant changes in specific activity of external [^{14}C]aerobactin.

3.3.3. The fate of iron taken up as ferric-aerobactin. To determine whether uptake of iron complexed with aerobactin, like uptake of

aerobactin itself, was inhibited by the presence of endogenous siderophore, double label experiments using [^{59}Fe/^{14}C]aerobactin were performed. Duplicate 1-ml aliquots were removed at intervals for cell fractionation and determination of ^{59}Fe and ^{14}C activity associated with subcellular compartments (Fig. 3.1.). Accumulation of ^{59}Fe into the cytoplasmic fraction of W3110(pABN1) cells exceeded that of [^{14}C]aerobactin by more than twofold over a 2 h period, while in the periplasm the reverse was the case (Fig. 3.1a). This is consistent with a model in which aerobactin is rapidly recycled in aerobactin producing cells; iron is removed, possibly at the cytoplasmic membrane, and the siderophore returns via the periplasm to the external medium. By contrast, uptake of aerobactin and iron into the non-aerobactin producing cells of strain W3110(pLG141) occurred at similar levels into both compartments (Fig. 3.1b). These data strongly suggest that ferric-aerobactin enters the cytoplasm as an intact complex in the absence of endogenous aerobactin. Interestingly, at later time points, when significant levels of exogenously supplied aerobactin may have accumulated in the cytoplasm, similar patterns of differential uptake of the two labels were observed in these cells also, albeit at a much lower magnitude than with the aerobactin producing strain. No significant uptake of ^{59}Fe into either compartment of cells of strain W3110 was observed (data not shown), ruling out the possibility that enterochelin (which all these strains are able to make) may be contributing to the observed assimilation of iron.

Table 3.2. Uptake of [^{14}C]aerobactin into *E. coli* K-12 strains lacking specific gene products involved in aerobactin uptake.

Strain	Relevant Characteristics	[^{14}C]aerobactin uptake (cpm ml^{-1})[a]			
		Periplasm	Cytoplasm	inner membranes	outer membranes
W3110(pLG141)	wild type	771	465	15	20
W3110	IutA$^-$	58	34	43	21
LG1316(pLG141)	*tonB*	65	23	33	39
W3110-6(pLG141)	*exb*	161	136	38	20
LF940(pFB102,pLG141)	*fhuD*	661	24	7	15
LG1706(pLG141)	*fhuB*	503	113	13	37
LF947(pLG141)	*fhuC*	678	114	50	60
AN1937 (pLG141)	*entA*	870	527	14	16
W3110(pABN1)	*iuc*$^+$	352	101	_[b]	_[b]

[a] ^{14}C emissions were measured in a window of 0-156 keV.

[b] Not determined.

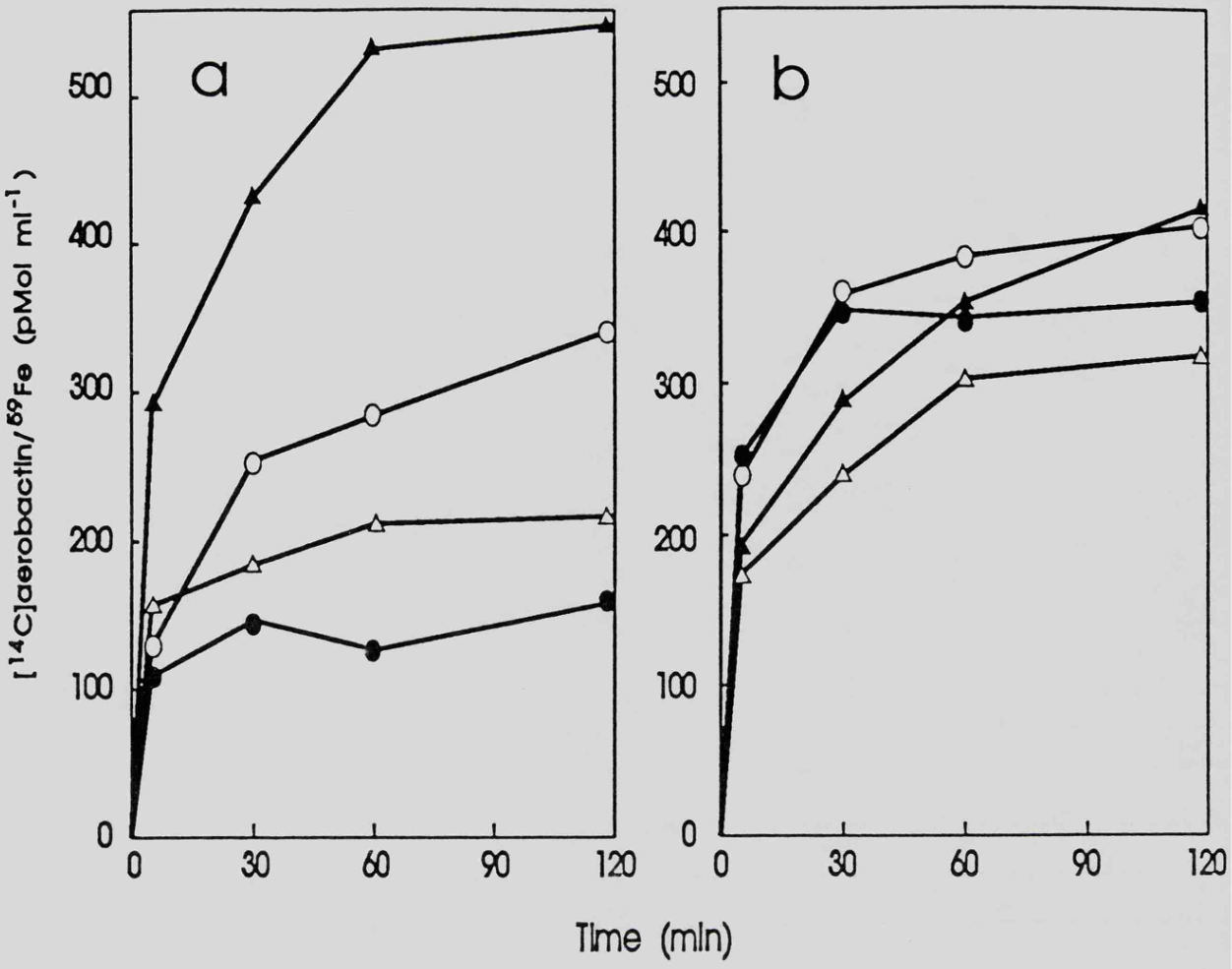


Figure 3.1. Uptake of [^{59}Fe/^{14}C]aerobactin by bacteria expressing the aerobactin receptor IutA, (a) the aerobactin producing strain W3110(pABN1), and (b) aerobactin deficient strain W3110(pLG141). Accumulation of ^{14}C (open symbols) and ^{59}Fe (filled symbols) activity in the periplasmic (○, ●) and cytoplasmic (△,▲) fractions were determined at intervals during incubation at 37°C as described in Materials and Methods. ^{59}Fe activity was calculated from emmisions measured in a window of 80-2000 KeV, acounting for 25% of total ^{59}Fe emissions. ^{14}C activity was calculated from the emissions measured in a window of 0-10 KeV which, after subtraction of the ^{59}Fe contribution (12% of total ^{59}Fe emissions), accounts for 25 % of ^{14}C emissions.

3.4. DISCUSSION

The genes whose products make up periplasmic iron transport systems have been identified on the basis of the inability of mutants to use a particular siderophore as a sole iron source (Braun *et al.*,1982; Braun *et al.*, 1983; Hantke, 1983; Ozenberger *et al.*, 1987; Pierce *et al.*, 1983; Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989). In general, constituent proteins were localized by analysis of particular subcellular compartments (Fecker and Braun, 1983; Köster and Braun, 1989; Ozenberger *et al.*, 1987; Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989). Despite apparent similarities in genetic and physical organization, however, the fate of the siderophore-iron complex as it traverses the cell envelope is not necessarily the same in each case. On the one hand, with ferric-dicitrate iron accumulates in *E. coli* cells but citrate does not, suggesting that the complex is not transported intact into the cytoplasm, but rather that iron is removed within the cell envelope (Hussein *et al.*, 1981). Supporting this view is the observation that most strains of *E. coli* are able to use ferric dicitrate as a source of iron but are unable to use citrate as a source of carbon (Wagegg and Braun, 1981). On the other hand, the observation that some siderophores are chemically modified and then excreted after delivering their iron to *E. coli* cells provides indirect evidence for uptake into the cytoplasm. The hydroxamate siderophore ferrichrome, for example, is acetylated after reduction (and removal) of the iron (Hartmann and Braun, 1980). However, the acetylation activity was subsequently demonstrated in cell free membrane fractions (Schneider et al., 1981) and it was suggested that transport of the ligand across the cytoplasmic membrane may not be necessary (Schneider *et al.*, 1981). In addition ^{55}Fe uptake from polymer bound ferricrosin in a FhuA/TonB-dependent manner was observed in *E. coli* (Coulton *et al.*, 1979). The polymer-bound ferrichrocin was assumed to be unable to penetrate into the cell and no ligand was found associated with cells. It was

concluded that sufficient amounts of iron could be released from the polymer complex to satisfy growth requirements (Coulton *et al.*, 1979). In more recent papers, however, this group has apparently assumed that ferrichrome is in fact transported across the cytoplasmic membrane (Fischer et al., 1989; Köster and Braun, 1989), although direct evidence has not been provided.

The catechol siderophore enterochelin is hydrolyzed by a cytoplasmic esterase (O'Brien *et al.*, 1971), possibly as a prerequisite for release of iron, the reduction potential of ferric-enterochelin at neutral pH being outside the range of biological reductants (O'Brien *et al.*, 1971). It has also been suggested, however, that the relatively low pH of the periplasmic compartment may allow reductive removal of iron from enterochelin before passage into the cytoplasm (Ecker *et al.*, 1986). Indeed, in faintly acidic solutions, the siderophore itself tends to reduce ferric iron (Hider, 1984). Further support for the suggestion that cytoplasmic ferric-enterochelin esterase may not in fact be required for removal of iron *in vivo* comes from the observation that synthetic analogues of enterochelin that are not susceptible to hydrolysis, such as 1,3,5-N,N',N''-tris-(2,3dihydroxybenzoyl)-triaminomethylbenzine (MECAM), are nonetheless biologically active in growth promotion assays (Heidinger *et al.*, 1983). Thus, it is still unclear whether ferric-enterochelin is delivered as an intact complex to the cytoplasm, or if iron is removed by reduction in the periplasm.

Unlike ferrichrome and enterochelin, aerobactin seems not to be modified after assimilation, and may be recycled for subsequent rounds of iron uptake (Braun *et al.*, 1984). Here direct evidence is provided for accumulation of aerobactin into the periplasm and cytoplasm of non-growing bacteria that do not themselves make aerobactin. The physiological relevance of this observation is confirmed by the behaviour of various mutants unable to grow with aerobactin as an iron source. Thus, *iutA*, *tonB*

and *exb* mutants exhibited significantly reduced levels of uptake of radiolabelled aerobactin across the outer membrane; on the other hand, *fhuB*, *fhuC* and *fhuD* mutants showed normal accumulation of aerobactin in the periplasm, but were defective in transport of the siderophore across the cytoplasmic membrane. Presumably aerobactin bound to IutA is released into the periplasm by a TonB(Exb)-dependent mechanism that does not require the periplasmic FhuD protein. FhuD subsequently binds aerobactin in the periplasm and delivers it to the cytoplasmic membrane permease complex FhuBC, which in turn catalyzes transport into the cell. This is in contrast to the uptake of maltodextrins which are translocated across the outer membrane of *E. coli* by a process of facilitated diffusion via the outer membrane protein LamB (Wandersman *et al.*, 1970). In this case release of maltodextrins from LamB seems to be dependent on a specific interaction between the periplasmic binding protein MalE and LamB (Bravoil and Nikaido, 1981; Wandersman *et al.*, 1979).

Uptake of aerobactin by aerobactin-producing strains was significantly lower than by strains that expressed only the aerobactin receptor gene *iutA*. A similar observation was previously reported for schizokinin-mediated iron uptake into the Gram positive organism *Bacillus megaterium* (Arceneaux *et al.*, 1973); exogenously supplied ferric-schizokinin remained associated with bacteria in which intracellular siderophore concentrations were low, but was rapidly lost from schizokinin-producing cells. By contrast, however, uptake into the cytoplasm of the iron component of ferric-aerobactin was unaffected by the presence of endogenous siderophore. Preferential accumulation of iron over aerobactin in the cytoplasm might result from rapid re-excretion of siderophore after removal of iron within the cytoplasm. If this were the case, however, one would expect a similar effect in cells that do not produce aerobactin; however, iron and aerobactin accumulate at comparable levels in the

cytoplasm of strain W3110(pLG141). A further possibility is that iron is removed from labelled exogenous aerobactin by endogenous aerobactin within the periplasmic space. This seems unlikely, however as kinetic exchange between siderophore molecules has been reported to be extremely slow, the half time for exchange of ^{59}Fe between desferrioxamine B and ferrichrome A (4mM, pH7.4) being over 200 h (Tufano and Raymond, 1981). The rate of exchange can, however be incresed by acidification and so, in the mildly acidic conditions of the periplasm this remains a formal possibility.

I propose that when endogenous aerobactin levels are high, some of the receptor-bound exogenous aerobactin is released from the cytoplasmic membrane, perhaps following reduction of ferric iron, or exchange of ferric ions with internal siderophore molecules. An exchange between permease-associated iron(II)-enterochelin and cytoplasmic apo-enterochelin has been suggested by Hider *et al.*, 1979; Hider, 1984). The mechanism by which such an exchange could occur, however, is not known. Williams and Carbonetti (1986) earlier demonstrated that iron supplied to iron-stressed bacteria by aerobactin, unlike that delivered by enterochelin, does not confer sensitivity to the iron-dependent antibiotic streptonigrin. They suggested that aerobactin-derived iron exists in an intracellular complexed form rather than being released into a cytoplasmic pool. Thus, one possibility suggested by their data and by the results presented here is that aerobactin may have a role in intracellular storage and mobilization of ferric iron within the cytoplasm. Other possible explanations are concidered in Chapter 6.

Chapter 4.

Sensitivity to Cloacin DF13: The Role of IutA and the major outer membrane protein OmpF.

4.1. INTRODUCTION.

Outer membrane proteins of gram negative bacteria may participate in the binding and uptake of at least three biologically distinct types of molecule. In addition to their physiological functions such as the uptake of nutrients including iron complexes (Neilands, 1982), vitamin B_{12} (DiGirolamo and Bradbeer, 1971) and nucleosides (Hantke, 1976), they may be exploited by other agents. Outer membrane proteins may serve as receptors for the binding of specific bacteriophages (Schwartz, 1980). In addition, bacteriocins, narrow spectrum antimicrobial compounds produced by related bacteria, utilize outer membrane proteins as specific receptors (Konisky, 1982). While bacteriocins have various modes of action, such as degradation of ribosomes (Konisky and Nomura, 1967) or depolarization of membrane potential (Gould and Cramer, 1977), they share several characteristics: they are all protein species which are synthesized with cognate immunity proteins that protect the producing strain from their lethal action, they all display very narrow spectra of target organisms, which are closely related to their producing strain, and they all exibit single hit kinetics of killing.

The colicins, produced by strains of *Escherichia coli*, are the best studied of the bacteriocins. On the basis cross-resistance patterns of a large number of colicin-tolerant mutants have been divided into the group A and group B colicins (Davies and Reeves, 1975a, 1975b). Colicins of group B

require the inner membrane-associated protein TonB and its functionally associated proteins ExbB and ExbD (Davies and Reeves, 1975b; Eick-Helmerich and Braun, 1989; Gutermann, 1973). Colicins of group A require the activity of two inner membrane proteins, TolQ and TolR (Sun and Webster, 1987), the genes for which have recently been cloned and sequenced, and shown to be highly homologous with *exbB* and *exbD* respectively. This suggests some similarity in the transport mechanisms of the group A and B colicins, and presumably a common evolutionary origin of both the colicins themselves and the transport machinery required for their lethal action.

Cloacin DF13 is a bacteriocin produced by strains of *Enterobacter cloacae* that harbor plasmid CloDF13 (Stouthamer and Teize, 1966). Killing of sensitive enteric bacteria by cloacin DF13 involves three distinct stages (Krone *et al.*, 1986; van Tiel-Menkveld *et al.*, 1982), binding to a specific surface receptor protein, transport of an active fragment of the bacteriocin molecule through the cell envelope, and endoribonucleolytic cleavage of 16S rRNA, resulting in defective protein synthesis. The aerobactin receptor in several enterobacterial species acts as a receptor for cloacin DF13 (Bindereif *et al.*, 1982; Krone *et al.*, 1985a; van Tiel-Menkveld *et al.*, 1982). Thus, strains of *E. coli* that utilize aerobactin as a source of iron are sensitive to cloacin DF13 because they express the common aerobactin/cloacin DF13 outer membrane receptor protein IutA (Bindereif *et al.*, 1982). Killing of *E. coli* by cloacin DF13 was originally reported to be a TonB-dependent process like the group B colicins (Pugsley, 1984). It was later noted, however, that cloacin DF13 did not contain the consensus pentapeptide found in all TonB-dependent receptors and colicins so far studied, and killing was subsequently shown to be independent of TonB activity (Schramm *et al.*, 1987). In addition, cloacin DF13 appears to have extensive similarities with the E colicins which belong to the TonB-independent group A colicins (Konisky, 1982).

The aerobactin receptor is the only envelope-associated protein in *E. coli* reported to date to be required for cloacin DF13 activity. In this work the major outer membrane protein OmpF, and possibly an outer membrane protein of 31 kDa, are also shown to be essential for the lethal action of cloacin DF13.

4.2. MATERIALS AND METHODS

4.2.1. Bacteria plasmids and culture conditions. The bacterial strains used in this study are described in table 4.1. Plasmid pLG141 comprises a 6.5 kb *Bam*HI fragment of plasmid ColV-K30 that includes the aerobactin receptor gene *iutA* (Carbonetti and Williams, 1984), cloned in the vector plasmid pACYC184 (Chang and Cohen, 1978); *iutA* in pLG141 is expressed constitutively (Carbonetti and Williams, 1984). Plasmid pJPN73 is pCloDF13 tagged with an ampicillin resistance marker (Cooper and James, 1985). Plasmid DNA was introduced as required into bacterial strains by transformation as described in section 5.2.6. Bacteria were routinely grown with aeration at 37°C in nutrient broth (Oxoid No. 2) supplemented where necessary with chloramphenicol (20 $\mu g ml^{-1}$) to select for the presence of pLG141 or ampicillin (100 $\mu g ml^{-1}$) to select for the presence of pJPN73. Solid media contained 1.5% agar (Difco).

4.2.2. Outer membrane protein preparation and analysis. Cells from 10 ml cultures grown overnight in nutrient broth containing chloramphenicol were harvested by centrifugation at 5,000 x *g* for 10 min at 4°C and resuspended in 1 ml of 100 mM Tris-HCl (pH7.8) containing 10 mM $MgCl_2$. Cells were disrupted by sonication and after removal of cell debris by centrifugation at 10,000 x *g* for 5 min at room temperature the supernatant fraction was subjected to ultracentrifugation at 100,000 x *g* for 10 min at 4°C. Pellets containing total membranes were suspended in the same volume of Tris-$MgCl_2$ buffer containing 2% (v/v) Triton X-100 to dissolve inner membrane proteins, and again subjected to

ultracentrifugation. Pellet containing outer membranes were resuspended as before, incubated at room temperature for 30 min, recovered by ultracentrifugation and the final pellet was resuspended in 200 µl of Tris-$MgCl_2$-Triton X-100 buffer. Outer membrane proteins were separated by electrophoresis in 11% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) by the method of Laemmli (1970), or 9% gels containing 8 M urea as described by Pugsley and Schnaitman (1978). Gels were stained with Coomassie brilliant blue.

4.2.3. Preparation of cloacin DF13. Cloacin DF13 was prepared essentially as described by de Graaf *et al.* (1969). A 10 ml culture of strain F205 (pJPN73) grown overnight at 37°C in nutrient broth containing ampicillin was used to inoculate 500 ml of nutrient broth containing ampicillin and pre-warmed to 37°C. After 20 min incubation at 37°C with vigorous aggitation, mitomycin-C (Sigma) was added to a final concentration of 25 $ngml^{-1}$ 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 was 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 was disolved in the solution (to raise the ammonium sulphate saturation to 60%) and the solution was again stired overnight at 4°C. The solution was again subjected to centrifugation as described above and the supernatant was discarded. The pellet was redissolved in 10 ml 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 µm filter (Millipore). The solution was titrated by spotting 10 µl aliquots onto a lawn of M5al cells freshly seeded onto a nutrient agar plate and incubating overnight. The solution was found to produce zones of clearing in the bacterial lawn at dilutions of 10^4 or lower.

4.2.4. Cloacin sensitivity assay and inhibition by membranes. Cloacin DF13 inhibits growth of cultures of sensitive bacteria, but as cloacin preparations are diluted inhibitory activity is lost (Roberts *et al.*, 1989). Sensitivity of bacterial strains to cloacin DF13 was assayed by adding 0.5 ml of cell suspensions at an optical density at 620 nm (OD_{620}) of 0.5 to serial doubling dilutions of a cloacin DF13 preparation in 2 ml of nutrient broth, incubating with shaking at 37°C for 4 h, and again determining OD_{620}. To quantify cloacin DF13-binding to membranes, cloacin dilution series were preincubated with outer membranes preparations before titration against the cloacin DF13-sensitive *Klebsiella pneumoniae* indicator strain M5a1 (Cooper and James, 1985; Roberts *et al.*, 1989).

4.2.5. Aerobactin uptake and inhibition by cloacin DF13. ^{14}C-labeled aerobactin was prepared as described in section 3.2.3. The ability of mutants to take up exogenously supplied [^{14}C]aerobactin, and inhibition of uptake by preincubation of cells with cloacin DF13, were tested as described in section 2.2.6.

Table 4.1. Bacterial strains

Strain	Characteristics	Source, Reference
***E. coli* K-12**		
CC118	*araD139* Δ*(ara, leu)7697* Δ*lacX74* *phoA*Δ*20 galE galK thi rpsE rpoB* *argE*$_{am}$ *recA* OmpC$^+$ OmpF$^+$	(Manoil & Beckwith, 1985)
LG1700	CC118 carrying pLG141 (Cm^r IutA$^+$)	This study
LG1701	LG1700, cloacin DF13 resistant (*iutA*$^-$)	This study
LG1702	LG1700, cloacin DF13 tolerant (OmpC$^-$OmpF$^-$)	This study
LG1703	LG1700, cloacin DF13 tolerant (lacking a 31 kDa outer membrane protein)	This study
MC4100	*araD139 lacU169 rpsL relA* *thiA fibB* (OmpC$^+$ OmpF$^+$)	(Taylor *et al.*, 1983)
MH760	MC4100, *ompR*472 (OmpC$^-$ OmpF$^+$)	(Taylor *et al.*, 1983)
MH1160	MC4100, *ompR*101 (OmpC$^-$ OmpF$^-$)	(Taylor *et al.*, 1983)
MH1460	MC4100, *envZ*11 (OmpC$^+$ OmpF$^-$)	(Taylor *et al.*, 1983)
UT4400	*ompT*	(Grodberg *et al.*, 1988)
Klebsiella pneumoniae		
M5a1	cloacin DF13 sensitive	(Cooper and James, 1985)

4.3. RESULTS

4.3.1. Isolation of mutants. Spontaneous cloacin DF13- insensitive mutants of strain LG1700 were isolated by spotting partially purified cloacin DF13 onto bacterial lawns (approximately 10^7 cells per plate) on nutrient agar containing chloramphenicol. Fourteen independent colonies were picked from zones of inhibition after overnight incubation at 37°C. SDS polacrylamide gel electrophoresis of outer membrane protein preparations of these isolates revealed three classes of mutant (Fig. 4.1). The most numerous class (8 of the 14 mutants, represented by strain LG1701, lane B) were true cloacin DF13-resistant mutants which, compared with protein profiles of the parent strain LG1700 (lane A), had lost the 74 kDa IutA protein. Plasmid DNA isolated from each strain of this class was introduced by transformation into virgin CC118 cells; all transformants were cloacin DF13 resistant, confirming that the relevant mutations were plasmid borne (i.e. *iutA*).

Mutants of class 2 (5 isolates, represented by strain LG1702, lane C) lacked proteins corresponding to the major outer membrane proteins species OmpF and OmpC (apparent molecular weight 36,000). Class 3, represented by the single mutant LG1703 (lane D), had lost a protein of approximately 31 kDa; in addition, the 74 kDa IutA band was replaced by a novel protein of 71 kDa and there were differences in intensity of several other bands compared with wild-type. Transformation of strain CC118 with plasmid DNA isolated from mutants of classes 2 or 3 yielded transformants that were sensitive to cloacin DF13, indicating that the plasmids in these mutants were still *iutA*$^+$, and that the mutations causing cloacin insensitivity were chromosomal.

4.3.2. Cloacin DF13 binding. Fig. 4.2 shows a representative experiment in which approximately 10 doubling dilutions of a cloacin DF13 preparation (incubated with membranes prepared from the IutA$^-$ strain

CC118) were required for 50% inhibition of growth of the indicator strain *K. pneumoniae* M5a1. Addition of membranes prepared from the IutA$^+$ strain LG1700, however, significantly increased the level of cloacin DF13 required for 50% inhibition of M5a1 (to approximately 5-6 doubling dilutions) by binding, and so inactivating, bacteriocin molecules at each dilution. Interestingly, outer membranes prepared from the cloacin DF13 insensitive mutants LG1702 and LG1703 also titrated cloacin DF13 activity in the dilution series with the same efficiency. As would be expected of an *iutA*$^-$ strain, however, membranes of mutant strain LG1701 did not bind cloacin DF13, and therefore did not alter the effective bactericidal concentration of the cloacin preparation used (Fig. 4.2).

4.3.3. Aerobactin uptake. Similarly, strain LG1701 *(iutA)* did not bind exogenously supplied [^{14}C]aerobactin (Table 4.2), while mutant strains LG1702 and LG1703 (IutA$^+$) took up aerobactin at levels comparable to the cloacin DF13 sensitive parent strain LG1700. Moreover, preincubation of LG1702 and LG1703 cells with partially purified cloacin DF13 caused greater than 80% inhibition of aerobactin uptake, similar to the level of inhibition observed with the parental strain (Table 4.2). Mutants of classes 2 and 3 have normal receptor activity (despite the fact that the latter appears to express a slightly smaller protein than wild type IutA, see Fig. 4.1), but are presumably defective in uptake of active bacteriocin; they are therefore more accurately termed cloacin DF13-tolerant.

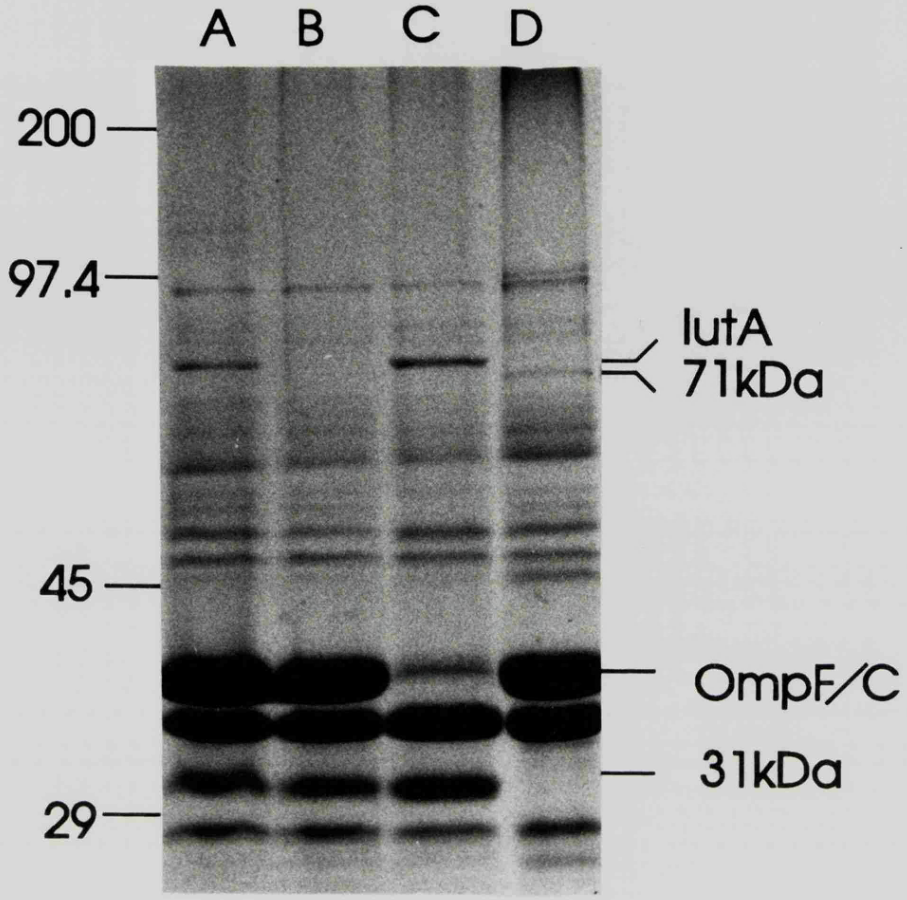
4.3.4. Involvement of OmpF in cloacin DF13 sensitivity. To determine whether the apparent loss of porin proteins from the outer membranes of class 2 mutants was responsible for cloacin DF13 insensitivity, strains were examined which carried well defined mutations at the *ompB* locus resulting in particular OmpC and OmpF phenotypes. Strains were transformed with plasmid pLG141 as described in 5.2.6, and tested for cloacin DF13 sensitivity as decribed in Materials and Methods. As

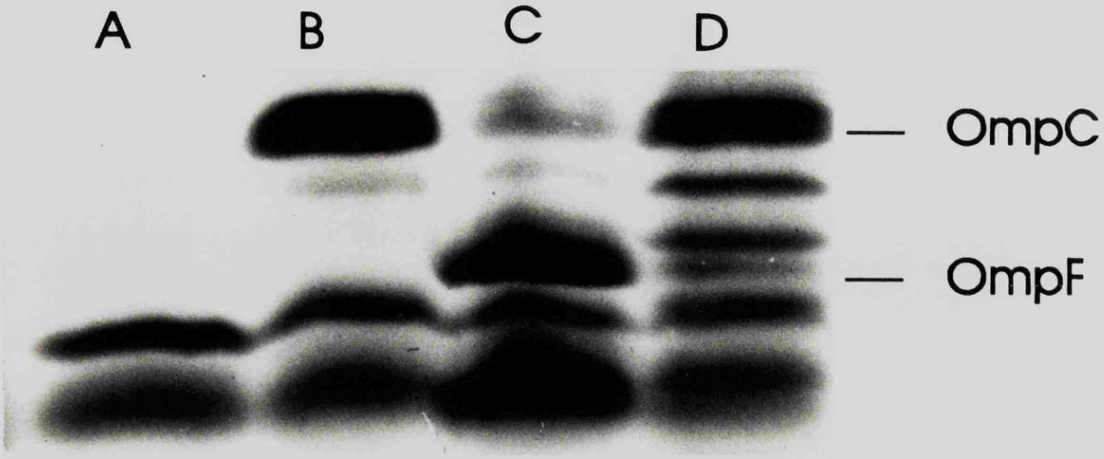
shown in Fig. 4.3a, strains MH1460 ($OmpC^+OmpF^-$) and MH1160 ($OmpC^-OmpF^-$) were insensitive to cloacin DF13; on the other hand strain MH760 ($OmpC^-OmpF^+$) showed markedly greater susceptibility to cloacin DF13 even than the parent strain MC4100. Using a gel system that resolves OmpF and OmpC proteins (Pugsley and Schnaitman, 1978), it was observed that strain MH760 expresses significantly higher levels of OmpF (in the absence of OmpC) than wild-type (Fig. 4.4).

4.3.5. OmpT as a candidate for the 31 kDa protein absent from outer membranes of mutant class 3. The mature form of the outer membrane protease OmpT has approximately the same molecular mass, calculated from nucleotide sequence data, as the protein band absent from the class 3 mutant (Grodberg et al., 1988). The possibility arose, therefore, that OmpT may be responsible for proteolytic cleavage of cloacin DF13 as it passes through the membranes of susceptible *E. coli* cells. To test this, the cloacin sensitivity of MC4100(pLG141) was determined in the presence and absence of 0.5 mM $ZnCl_2$, which completely inhibits OmpT activity in vitro (Sugimura and Higashi, 1988) and in vivo (Baneyx and Georgiou, 1990). Cloacin susceptibility was unaffected by the presence of $ZnCl_2$ (Fig. 4.3b), suggesting that OmpT is not involved in the lethal action of cloacin DF13. This was confirmed by the observation that the defined *ompT* mutant UT4400 (Grodberg *et al.*, 1988) containing plasmid pLG141 was sensitive to killing by cloacin DF13 (Fig. 4.3b). Therefore the nature of cloacin-insensitivity in the class 3 mutant remains unresolved.

Fig. 4.1. SDS polyacrylamide gel elecrophoresis of outer membrane proteins of strain LG1700 (lane A) and representative cloacin DF13-insensitive mutants LG1701 (lane B), LG1702 (lane C), and LG1703 (lane D). Molecular weight standards indicated on the left were myosin heavy chain (200,000), phosphorylase b (97,000), bovine serum albumin (68,000), ovalbumin (43,000) and carbonic anhydrase (29,000). The positions of the IutA protein, a 71 kDa protein expressed by LG1703, and OmpC and OmpF proteins are indicated on the right.

Fig. 4.4. Urea polyacrylamide gel electrophoresis to resolve major outer membrane proteins OmpF and OmpC. Strains tested were MH1160 (OmpC-OmpF-, lane A), MH1460 (OmpC+OmpF-,lane B), MH760 (OmpC-OmpF+, lane C), and MC4100 (OmpC+OmpF+, lane D). The positions of OmpF and OmpC are indicated on the right.


A
B
C
D
200
97.4
45
29
lutA
71kDa
OmpF/C
31kDa


A
B
C
D
OmpC
OmpF

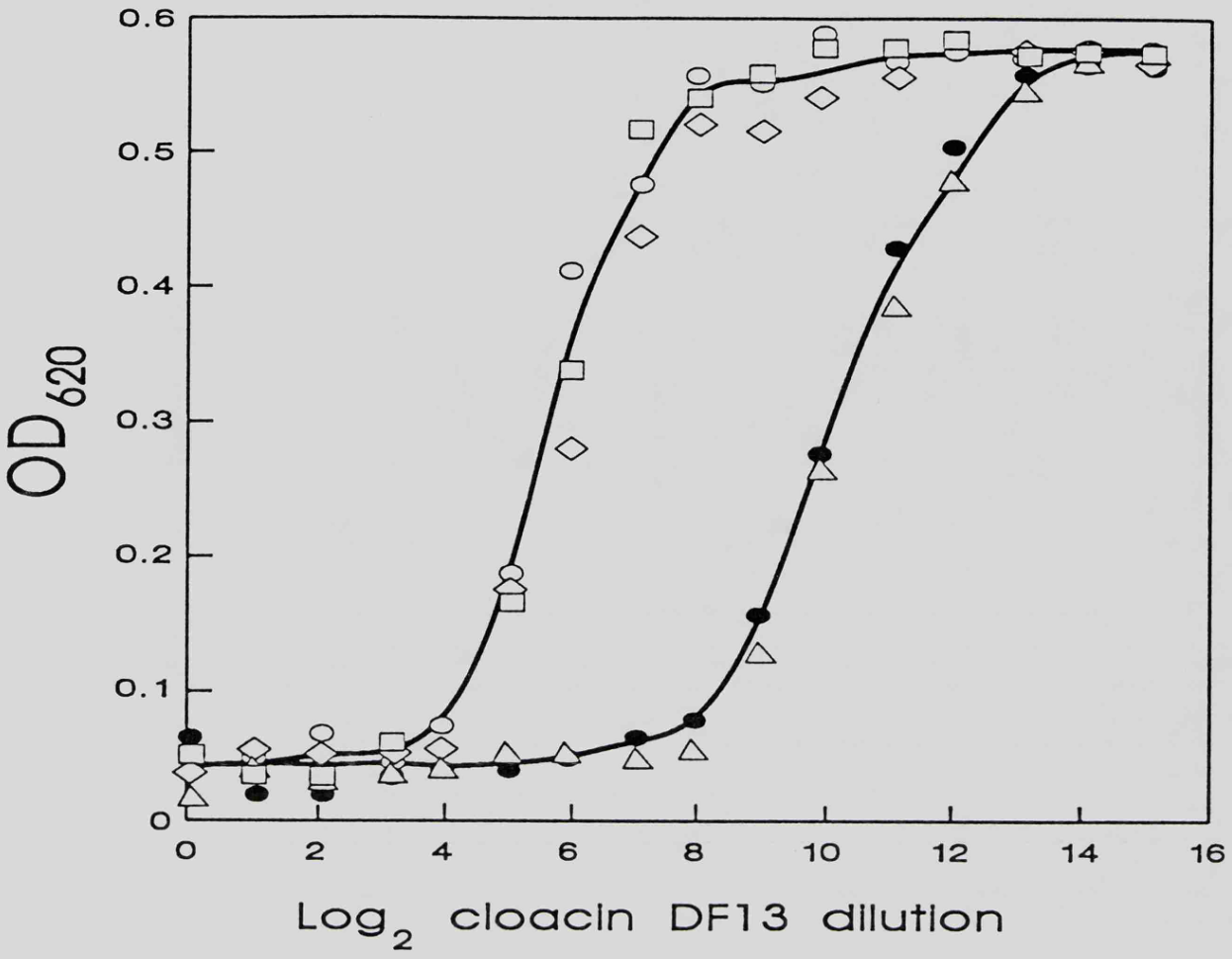


Fig. 4.2. Cloacin binding to membranes of cloacin DF13-insensitive derivatives of strain LG1700. Growth of the indicator strain M5a1 was monitored (culture OD_{620}) after addition to the indicated dilutions of a cloacin DF13 preparation treated with membranes prepared from strains LG1700 (IutA$^+$,○) and CC118 (IutA$^-$,●), and from cloacin DF13-insensitive mutants LG1701 (△), LG1702 (□), and LG1703 (◇).

Table 4.2. Uptake of radioactive aerobactin by cloacin DF13 insensitive mutants of strain LG1700

Strain	aerobactin (cpm) uptake[a] by:	
	untreated cells	cloacin DF13-treated cells[b]
LG1700	1219	194
LG1701	82	71
LG1702	1315	205
LG1703	1054	75
CC118	77	-[c]

[a] [^{14}C]aerobactin associated with to cells after incubation at 37^{o}C for 90 min.

[b] Cells were incubated with cloacin DF13 for 30 min at 37^{o}C before addition of [^{14}C]aerobactin. Untreated samples contained equivalent concentrations of bovine serum albumin.

[c] -, not done.

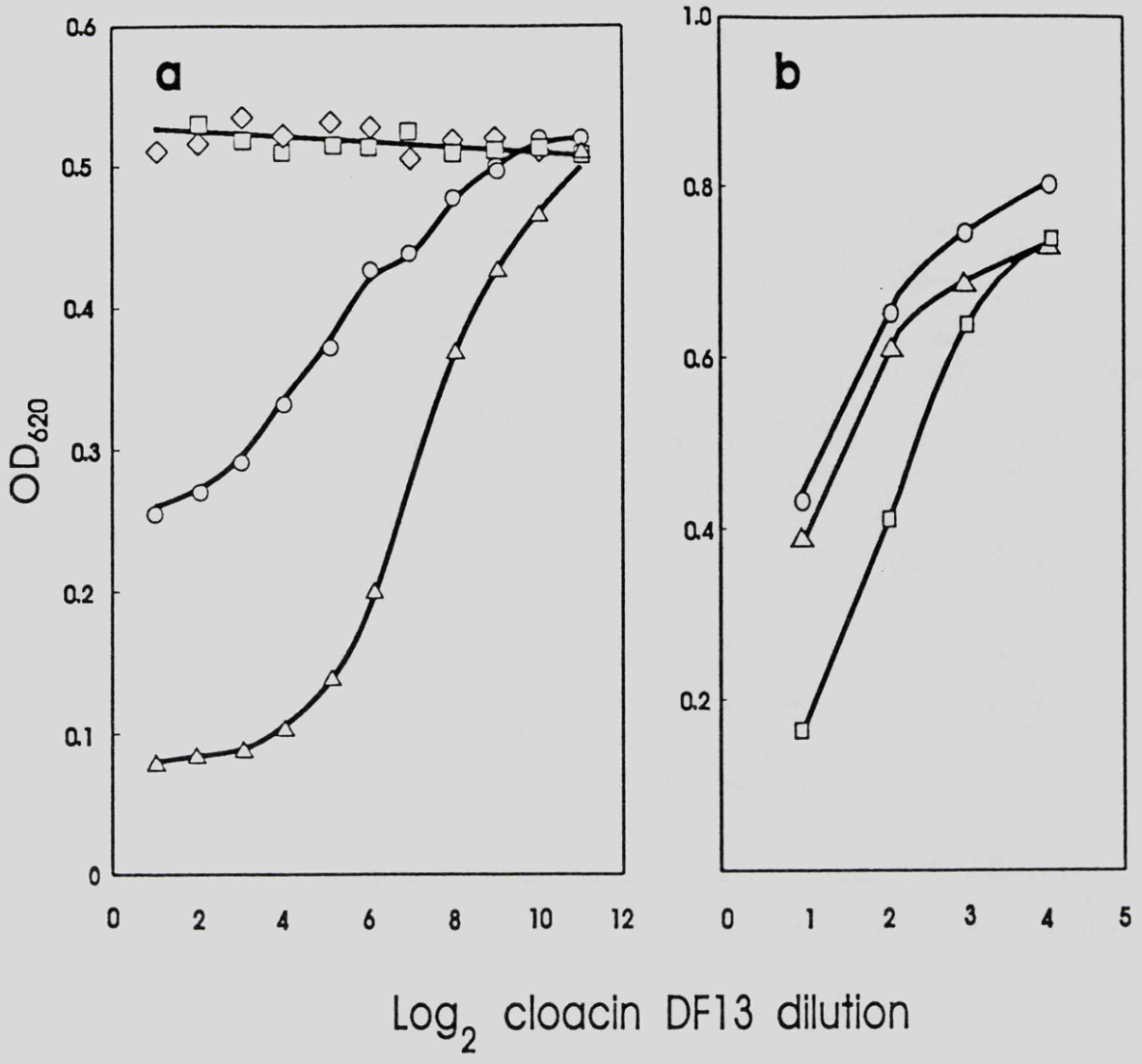


Fig. 4.3. Sensitivity to cloacin DF13 of *E. coli* strains altered in the activity of major outer membrane proteins. Bacterial growth was monitored (culture OD_{620}) after addition to the indicated dilutions of a cloacin DF13 preparation. (a) Strains tested were MC4100(pLG141) ($OmpC^+OmpF^+$, ○), MH760(pLG141) ($OmpC^-OmpF^+$, △), MH1160(pLG141) ($OmpC^-OmpF^-$, □), and MH1460(pLG141) ($OmpC^+OmpF^-$, ◇). (b) Sensitivity of strain MC4100(pLG141) was assayed in the presence (○) and absence (△) of $ZnCl_2$ to inhibit OmpT protease activity, and compared to that of strain UT4400(pLG141) (*ompt*$^-$, □)

4.4. DISCUSSION

The observation that cloacin DF13 resistant mutants of susceptible strains of *E. coli* were also unable to take up aerobactin provided the first evidence that aerobactin and cloacin DF13 share a common receptor (Bindereif *et al.*, 1982). Among fourteen spontaneous cloacin DF13 insensitive mutants examined in this study, the *iutA* type of mutation was indeed the most common, in all cases resulting in total absence of IutA protein from the outer membrane. Two additional classes of mutants were also identified on the basis of outer membrane protein pofiles. Binding of both aerobactin and cloacin DF13 were unaffected in these two classes (which are therefore most accurately designated cloacin DF13-tolerant mutants), emphasizing that, although the two very different ligands share a common receptor, their subsequent translocation across the outer membrane occurs by different processes. It has been known for some time that uptake of aerobactin (Williams, 1979), but not of cloacin DF13 (Schramm *et al.*, 1987), is dependent on the inner membrane protein TonB which is thought to provide energy for translocation of the siderophore across the outer membrane (Hancock and Braun, 1976).

The evidence provided here suggests roles for OmpF and for a 31 kDa outer membrane protein in the lethal action of cloacin DF13 for susceptible strains of *E. coli*. These proteins are not involved in the initial binding of cloacin DF13, nor presumably in its enzymic activity. Rather, they appear to be involved in the fragmentation and/or translocation of active cloacin DF13 across the cell envelope and into the cytoplasm of sensitive cells. The 31 kDa protein absent from mutant LG1703 has not yet been identified, but it is not the protease OmpT. Indeed, other alterations in the protein profile of LG1703, including conversion of the IutA protein from 74 kDa to about 71 kDa, might indicate increased protease activity in this mutant. Moreover, a further possibility is that the 71 kDa form of the receptor may have lost

translocation functions while retaining binding activity.

Class 2 mutants, represented by strain LG1702, have simultaneously lost the ability to express the two similar sized porin proteins OmpF and OmpC. Synthesis of these proteins in *E. coli* is regulated by the *ompB* (*ompRenvZ*) locus, which encodes the cytoplasmic protein OmpR and the inner membrane-associated EnvZ protein. EnvZ is thought to act as an environmental sensor that responds to changes in osmolarity of the surrounding milieu by switching OmpR between two functionally active states which separately act as inducers of the *ompC* and *ompF* gene promoters (Hall and Silhavy, 1981). Thus mutants of class 2 appear to be like strain MH1160 (*ompR101*), lacking active OmpR protein so that neither *ompC* nor *ompF* are expressed (Taylor *et al.*, 1983). Mutants of the MH1460 type (*envZ*11), in which the OmpR protein acts only on the *ompC* promoter (Taylor *et al.*, 1983), might be predicted, as indeed would mutants harbouring mutations within the OmpF structural gene, but such mutants were not observed in this study.

Membranes of wild type *ompB*$^+$ (*ompR*$^+$*envZ*$^+$) *E. coli* contain both OmpC and OmpF; in circumstances where one of these proteins is synthesized at sub-maximal levels, however, there may be compensatory expression of the other to maintain the total porin content of the outer membrane (Hall and Silhavy, 1981). For example, expression of OmpC is preferred over that of OmpF in conditions of high osmolarity (van Alphen and Lugtenberg, 1977), such as in brain-heart infusion broth. Therefore the observation that cells grown in brain-heart infusion broth were less sensitive to cloacin DF13 than cells grown in nutrient broth, despite the fact that they both expressed the same number of receptor molecules per cell (Krone *et al.*, 1986), may now be explained in terms of the relative lack of OmpF in the membranes of the former. Support for this idea is provided by the observation that the OmpC$^-$ strain MH760(pLG141) is significantly

more sensitive than wild-type to killing by cloacin DF13. MH760(pLG141) expresses compensatory high levels of OmpF, as has previously been demonstrated for this class of mutants (Hall and Silhavy, 1981). Thus sensitivity to cloacin DF13 is shown here to correlate with expression of OmpF.

The involvement of more than one outer membrane component in the sensitivity of *E. coli* to cloacin DF13 is reminiscent of the multi-component outer membrane receptor for colicin A described by Chai *et al.* (1982). They demonstrate the absolute requirement for OmpF and LPS, and enhanced activity in the presence of the *btuB* gene product in the killing action of colicin A. However, *ompF*$^+$*btuB* cells adsorbed colicin A as efficiently as *ompF*$^+$*btuB*$^+$ cells suggesting that OmpF is the protein to which colicin A initially binds and that the *btuB* gene product may be involved in a subsequent step. This is analagous to the binding of cloacin DF13 to IutA, with OmpF, in this case, playing the secondary role. Interestingly, however, the presence of the *btuB* gene product in cell free preparations was shown to enhance the ability of OmpF to inacivate colicin A. This is in contrast to the results described here for the inactivation of cloacin DF13 *in vitro* by similar membrane preparations of *iutA*$^+$ bacteria. The presence of OmpF in this case did not affect the ability of such preparations to inactivate the bacteriocin. Benedetti *et al.* (1989) investigated the role of OmpF in the sensitivity of cells to the BtuB-group colicins; colicins A and E1-9, grouped on the basis of their reduced activity against *E. coli* mutants lacking the outer membrane protein BtuB (Mock and Pugsley, 1982). They found that colicin A required BtuB and OmpF for initial binding, and OmpF but not BtuB for subsequent translocation. Colicin E1 required BtuB for both initial binding and subsequent translocation, but had no requirement for OmpF. Colicins E2 and E3 appeared to have a requirement for OmpF intermediate between colicin A and colicin E1 in that BtuB was required for both binding and translocation while OmpF was not involved in binding but did have a

role in subsequent translocation. In this respect, cloacin DF13 appears to behave like colicins E2 and E3 in that it does not require OmpF for binding but does require this porin for subsequent translocation.

Chai *et al.* (1982) suggested that OmpF and BtuB may form a specific pore across the outer membrane which facilitates the translocation of colicin A. Furthermore, they infered that such a pore may be involved in uptake of the physiological ligand of BtuB, vitamin B_{12}. They did not, however, investigate uptake of vitamin B_{12}. The work described in this chapter demonstrates that while OmpF is required for sensitivity of *E. coli* to cloacin DF13, it plays no role in the physiological function of the aerobactin receptor, the uptake of aerobactin. This would tend to argue against the possibility that OmpF serves to stabilize a given functional conformation of the IutA protein. It may be that OmpF stabilizes the IutA-cloacin DF13 complex, or that OmpF and IutA interact to form a specific pore across the outer membrane through which cloacin DF13, or a fragment thereof, can pass. Such a pore would, however, have no role in the uptake of aerobactin.

Chapter 5

Functional Analysis of the Aerobactin Receptor Protein by Studies on Mutant Proteins.

5.1. INTRODUCTION.

The complete nucleotide sequence of the aerobactin/cloacin DF13 receptor gene has been determined (Krone *et al.*, 1985b) and found to encode a precursor protein of 725 amino acids. Analysis of the primary structure of the first 25 amino acids revealed a peptide with typical characteristics of a signal sequence (Osborn and Wu, 1980) with a positively charged amino terminus, a hydrophobic core region and a conserved cleavage site. The predicted mature protein was thus 700 amino acids in length with a calculated molecular weight of 77,345 Da.

In common with other outer membrane proteins, the mature protein was rather hydrophilic in comparison with proteins found in most biological membranes, and was rich in β-sheet structure (as predicted by Chou-Fasman rules, Chou and Fasman, 1978; Lenstra *et al.*, 1977) at the expense of α-helix, although some α-helix was predicted in the amino terminal region. In comparing the deduced amino acid sequence with consensus sequences within the *E. coli* major outer membrane proteins identified by Nikaido and Wu (1984), the authors identified a region starting at amino acid 184 with the sequence Gly-Asn-Gly-Asp-X-Thr which was homologous to a conserved region located in the amino terminal part of the porins. This has been suggested to be involved in export of the protein and to act as a recognition signal for its final location in the outer membrane (Benson and Silhavy, 1983). In addition, the deduced amino acid sequence of the aerobactin/cloacin DF13 receptor contains a consensus pentapeptide ("TonB-

box") that has been identified in all TonB-dependent outer membrane receptor proteins and colicins (Fischer *et al.*, 1989; Postle, 1990a; 1990b; Schramm *et al.*, 1987). Genetic evidence suggests that the TonB boxes of the vitamin B_{12} receptor BtuB and the ferrichrome receptor FhuA interact directly with the cytoplasmic membrane protein TonB (Heller *et al.*, 1988; Schoffler and Braun, 1989).

No other information correlating the primary structure of the protein to its known biological activities is presently known. In this chapter I will describe a number of approaches used in attempts to correlate primary amino acid sequence with outer membrane localization, binding of cloacin DF13, and binding and translocation of aerobactin.

5.2. MATERIALS AND METHODS.

5.2.1. Bacterial strains and plasmids. Strain LG1700, a pLG141 transformant of strain CC118 (*araD139* Δ[*ara leu*]*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE*[Am] *recA1*), was used to screen for in-frame fusions of IutA and PhoA; it is deleted for the alkaline phosphatase gene and therefore does not express native alkaline phosphatase activity (Manoil and Beckwith, 1985). Strain L336 is strain CC118 containing an F' plasmid which has a temperature sensitive origin of replication and contains the composite transposon Tn*phoA* (Manoil and Beckwith, 1985). Strain BZB1022 (*cir gyrA*) was used to visualize IutA in outer membrane preparations as it lacks the similarly sized outer membrane protein Cir (Pugsley, 1985). Strain DH5α (*supE*44 δ*lacU*169[ϕ80 *lacZ*δM15] *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) was used to detect recombinant clones in vector plasmid pUC19 as it produces functional β-galactosidase, detectable as blue colonies on media containing 5-bromo-4-chloro-3-indolyl–β-D-galactoside (X-Gal) (Horwitz *et al.*, 1964), when provided with the α-

complementation (expression of the α-subunit of β-galactosidase) provided by intact pUC19 plasmids, but not recombinant plasmids derived from pUC19 containing DNA sequences cloned into the multiple cloning site of this plasmid (Hanahan, 1983). Plasmid pLG141 contains a 6.5 kb *Bam*HI fragment of pABN1, which expresses the aerobactin receptor protein (IutA) constitutively (Carbonetti and Williams, 1984), cloned into vector plasmid pACYC184 (Chang and Cohen, 1978). Plasmid pUC19 (Messing, 1983) was used for cloning *SalI-BamHI* fragments from plasmids generated by hexameric linker insertion mutagenesis of plasmid pLG1000 for double stranded sequencing. Plasmid pUC4K (Oka et al., 1981) was used as a source of the kanamycin resistance cassette. Plasmid pLG1000 was constructed by digesting plasmid pLG141 with *BamHI*, filling in the sticky ends with Klenow in the presence of all four deoxynucleotides and isolating both fragments independently by elution onto a dialysis membrane. The fragments were blunt-end ligated in a 10:1 insert to vector ratio before digestion with *BamHI* to select against any molecules which retained *BamHI* sites. The products were then used to transform competent BZB1022 cells. Chloramphenicol resistant colonies were tested for the presence of insert DNA by testing the transformants for sensitivity to cloacin DF13 in a plate assay. Of 60 transformant clones isolated, two were found to be cloacin DF13 sensitive. Plasmid DNA was isolated from the two chloramphenicol-resistant cloacin DF13-sensitive transformant clones and from one chloramphenicol-resistant cloacin DF13-resistant clone. The former contained plasmids similar to pLG141 except that the two *BamHI* sites had been destroyed and, in both cases, the insert was in the reverse orientation with respect to pLG141. One of these plasmids was called pLG1000 and used subsequently to produce hexameric linker insertion mutants in which a unique *BamHI* site could be introduced. The latter contained a plasmid similar to pACYC184, but in which the *BamHI* site had been destroyed.

5.2.2. Media and growth conditions. Bacteria were routinely grown in nutrient broth (Oxoid N°.2). Chloramphenicol (20 μgml^{-1}) was included to select for the presence of pLG141 or its derivatives, amicillin (100 μgml^{-1}) was included to select for pUC19 or its derivatives, and kanamycin was used to select for the presence of the pUC4K-derived kanamycin resistance cassette or Tn*phoA* in low copy number (50 $mgml^{-1}$), or to select for Tn*phoA* on high copy number pLG141-derivatives (300 μgml^{-1}). 5-bromo-4-chloro-3-indolyl phosphate (XP, 40 μgml^{-1}) was included in media to induce and detect alkaline phosphatase activity. Isopropylthio-β-D-galactoside (IPTG, 40 μgml^{-1}) and 5-bromo-4-chloro-3-indiolyl-β–D-galactoside (X-Gal, 40 $mgml^{-1}$) were included in media to detect β-galactosidase activity. Solid media contained 1.5% agar (Difco).

5.2.3. Standard DNA manipulations. Ligations and restriction digestions were carried out using Bethesda Research Laboratories enzymes and buffers according to the recommendations of the manufacturers. Reaction products were analysed by agarose gel electrophoresis in 0.8% agarose gels containing 500 $ngml^{-1}$ ethidium bromide in 0.04 M Tris-acetate, 1 mM EDTA. Bands were visualized by ultraviolet illumination and photography.

5.2.4. Standard DNA preparation. DNA for transformation or restriction analysis 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* and resuspended in 200 µl of 50 mM glucose, 25 mM Tris-HCl (pH8.0), 10 mM EDTA and incubated at room temperature for 5 min. 400 µl of 0.2 M NaOH, 1% SDS were added, and the mixture was incubated on ice for 5 min. 300 µl of 3M sodium acetate (pH 5.0) were added and the mixture was incubated for a further10 min on ice. The precipitate was pelleted by centrifugation at 10,000 x *g* for 10 min and the supernatant was removed to a fresh tube. 500

µl of propan-2-ol were added, and the mixture was incubated at room temperature for 10 min before harvesting the precipitate by centrifugation at 10,000 x *g* for 10 min. The supernatant was aspirated, and the pellet dried and redissolved in 100 µl of water to which 200 µl of 4.4 M LiCl were added, and the mixture was incubated for 10 min on ice. The precipitate was removed by centrifugation at 10,000 x *g* for 10 min, the supernatant removed and DNA precipitated by addition of 0.1 volumes of 3M sodium acetate (pH5.0), 0.7 volumes of propan-2-ol; the precipitate was harvested by centrifugation at 10,000 x *g* for 10 min. The final pellet was resuspended in 50 µl of sterile distilled water containing 0.1 $mgml^{-1}$ RNAse A (Sigma). For most other purposes large scale DNA preparation was essentially a scaled up version of the above method except that, after RNAse A-treatment for 10 min at 37°C, the solution was extracted twice with phenol and once with chloroform before ethanol precipitation, washing in 70% ethanol, vacuum-drying and resuspension in 200 µl of 10 mM Tris-HCl (pH8.0), 1mM EDTA (TE).

5.2.5. DNA preparation for double stranded DNA sequencing. High-purity plasmid DNA for sequencing was prepared using Quiogen™ kits and protocols. Cells from a 100 ml overnight culture containing appropriate antibiotics were harvested by centrifugation at 10,000 x *g* for 10 min and resuspended in 10 ml 50 mM Tris-HCl (pH8.0), 10 mM EDTA. 10 ml of 0.2 M NaOH were added and after gentle mixing the solution was incubated at room temperatute for 5 min. 10 ml of 2.55 M potasium acetate (pH4.5) were added. The solution was mixed immediately but gently, centrifuged at 20,000 x *g* for 30 min at 4°C, and the cleared lysate was removed. A Quiogen™ tip-500 was equilibrated with 10 ml of 750 mM NaCl, 50 mM 3-(N-morpholino)propane-sulphonic acid (MOPS) (pH7.0), 15% ethanol, 0.15% Triton X-100 and allowed to empty by gravity flow. The cleared lysate was applied to the equilibrated Quiogen™ tip and allowed to

enter the resin by gravity flow. The tip was washed three times with 10 ml of 1 M NaCl, 50 mM MOPS (pH7.0), 15% ethanol and the DNA was eluted with 15 ml of 1.25 M NaCl, 50 mm MOPS (pH7.0), 15% ethanol. The DNA was precipitated by addition of 0.7 volumes of propan-2-ol and centrifugation at 20,000 x *g* for 30 min. The pellet was washed in 70% ethanol, vacuum-dried and redisolved in 200 µl of TE.

5.2.6. 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 were then chilled on ice. 1 ml aliquots were dispenced into 1.5 ml Eppendorf™ tubes and cells were harvested by centrifugation at 10,000 x *g* for 2 min. Cell pellets were resuspended in 1 ml of 10 mM MOPS (pH7.0), 10 mM RbCl and harvested by centrifugation as above. Cell pellets were then resuspended in 1 ml of 100 mM MOPS (pH6.5), 50 mM $CaCl_2$, 10 mM RbCl and incubated on ice for 15 min. Cells were harvested as above and resuspended in 200 µl of 100 mm MOPS (pH6.5), 50 mM $CaCl_2$, 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 seconds and returned to ice. After addition of 1 ml of nutrient broth the cells were incubated at 37°C for 90 min before plating out dilutions on selective media. Colonies appearing after incubation at 37°C overnight were selected.

5.2.7. Conjugation and selection for in frame fusions of *iutA* and *phoA*. Cultures of strains LG1700 and L336 were grown at 30°C with aeration to an optical density of 0.5, and cells from 1 ml of each culture were sequentially harvested in the same 1.5 ml Eppendorf™ tube. Cells were resuspended in 100 µl of nutrient broth and spread over the surface of a 2.5 cm filter disk with a pore size of 0.4 µm (Millipore) which had been placed on the surface of a nutrient agar plate containing no antibiotics. The plate

was incubated overnight at 30°C and the cells were harvested from the filter by placing the filter in an Eppendorf™ tube and vortexing in 1 ml nutrient broth. The cell suspension was diluted 10 and 100-fold, plated onto nutrient agar containing 20 μgml^{-1} chloramphenicol, 300 μgml^{-1} kanamycin and 40 μgml^{-1} XP and incubated overnight at 42°C. Plasmid DNA was isolated from blue colonies and used to transform virgin cells of strain CC118. Transformants which produced blue colonies on medium containing XP were mapped by analysis of *BamH*1 restriction fragment sizes and precise map positions of Tn*phoA* insertions of selected mutants were obtained by double stranded DNA sequencing as described in section 5.2.11. All mutants were analysed for alkaline phosphatase activity and inner and outer membrane protein profiles were obtained as described in section 5.2.12.

5.2.8. *In vitro* hydroxylamine-induced mutagenesis. Hydroxylamine mutagenesis was carried out using the method of Eichenlaub (1979). 2 µg of purified pLG141 DNA were reacted with 40 µmol of hydroxylamine in 100 ml 50 mM Na-phosphate (pH6.0) at 75°C for 30 min. The DNA was dialized overnight against 4 l of 10 mM Tris-HCl (pH8.0), 100 mM NaCl, 1 mM EDTA to remove hydroxylamine and used to transform cells of strain BZB1022. Transformant colonies were screened for sensitivity to cloacin DF13.

5.2.9. Hexameric linker insertion mutagenesis. Insertion of 6-base oligonucleotides into *Hpa*II and *Hha*I sites of plasmid pLG1000 was carried out using the methods described by Barany (1985a, 1985b). pLG1000 DNA was prepared as descried in section 5.2.4. For insertion into *Hpa*II sites 20 µg of DNA were digested with 7.5 U of *Hpa*II in 300 µl of 20 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$ containing 25 μgml^{-1} ethidium bromide. For insertion into *Hha*I sites 20 µg of DNA were digested with 15 U of *Hha*I in 300 µl of 50 mM Tris-HCl (pH8.0), 10 mM $MgCl_2$, 50 mM NaCl

containing 25 μgml^{-1} ethidium bromide. In both cases incubation was at 37°C for for 60 min. Digestions were stopped by addition of 30 µl of 50 mM EDTA (pH8.0) and incubation at 65°C for 10 min. Full length linear DNA was isolated by preparative agarose gel electrophoresis and elution onto a dialysis membrane, using pLG1000 digested to completion with *Sal*I (which cuts pLG1000 once only) as a size marker for full length linear molecules. Single stranded hexameric oligonucleotides were synthesized by Debra Langton, Department of Biochemistry, University of Leicester.

Linkers for insertion into *Hpa*II sites were $^{5'}$OH-CGGATC-OH$^{3'}$. 10 µg of linker DNA was 5'-phosphorylated in 10 µl of 50 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM spermidine, 1 mM EDTA containing 1 mM ATP and 5 U of T4 polynucleotide kinase for 1 h at 37°C. The kinase was inactivated by incubation at 65°C for 10 min. 2 µg of the phosphorylated linkers were then ligated to 0.5 µg of the *Hpa*II-linearized pLG1000 in 10 µl of 20 mM Tris-HCl (pH7.6), 5 mM $MgCl_2$, 5 mM DTT containing 1 mM ATP and 1 U of T4 DNA ligase for 16 h at 4°C. After additon of a further 1 U of T4 DNA ligase the ligation reaction was continued at 16°C for 1 h. The ligation mix was heated to 65°C for 10 min to inactivate the ligase and, after addition of 5 ml of 500 mM Tris-HCl (pH8.0), 100 mM $MgCl_2$, 1 M NaCl, and dilution to a final volume of 50 µl, 20 U of *BamH*I were added and the DNA was digested at 37°C for 2 h to remove excess linkers. The DNA was purified by extraction with phenol:chloroform and recovered from the aqueous phase by precipitation with 0.1 volumes of sodium acetate (pH5.0) and 3 volumes of ethanol. After incubating on ice for 10 min the DNA was pelleted by centrifugation at 10,000 x *g* for 30 min. After washing in 70% ethanol the pellet was vacuum-dried.

Linkers for insertion into *Hha*I sites were $^{5'}$OH-GATCCG-OH$^{3'}$. In this case, unphosphorylated linker DNA was ligated to the *Hha*I-linearized pLG1000 as described above for the ligation of phosphorylated linkers into

*Hpa*II-generated ends. After inactivation of T4 ligase by heating to 65°C for 10 min, the DNA molecules were phosphorylated by addition of 2 µl of 0.5 M Tris-HCl (pH7.6), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA, 2 µl of 10 mM ATP, 6 µl of water and 10 U of T4 polynucleotide kinase and incubation for 30 min at 37°C. The kinase was then inactivated by heating to 65°C for 15 min. The DNA was purified as described for the *Hpa*II-linearized ligation products.

Both types of construction were then ligated to the kanamycin resistance cassette of pUC4K by redissolving in 20 mM Tris-HCl (pH7.6), 5 mM $MgCl_2$, 5 mM DTT containing 0.5 µg of a purified fragment of DNA including the kanamycin resistance gene (prepared by digesting pUC4K DNA with *BamH*I and isolating the fragment by preparative agarose gel electrophoresis and electroelution onto a dialysis membrane) and 5 Weiss units of T4 DNA ligase and incubating overnight at 16°C. The products of ligations were used to transform cells of strain BZB1022 as described in section 5.2.6 and chloramphenicol resistant, kanamycin resistant colonies were isolated. To select for insertions into the *iutA* gene individual clones were tested for sensitivity to cloacin DF13. Plasmid DNA was isolated from cloacin DF13 resistant clones and digested with *BamH*I. After religation the DNA was used to transform cells of strain BZB1022 to chloramphenicol resistance, and transformant colonies were tested for sensitivity to kanamycin. Kanamycin sensitive transformant clones were selected from each transformation and plasmid DNA was isolated, digested with *BamH*I and analysed by agarose gel electrophoresis to determine the size of the molecule. Clones which produced full length linear molecules after digestion with *BamH*I were selected for mapping and phenotypic analysis.

5.2.10. Mapping the position of hexameric linker insertions. Plasmid DNA isolated from hexameric linker insertion mutants generated as described above was digested with *Sal*I and *BamH*I and analysed by

agarose gel electrophoresis. Unique clones were selected for precise mapping by double stranded sequencing. *Sal*I-*BamH*I fragments were isolated by preparative agarose gel electrophoresis and elution onto a dialysis membrane and ligated into pUC19 DNA cut with the same two enzymes. The ligation products were used to transform cells of strain DH5α to ampicillin resistance. White colonies appearing on plates containing ampicillin, IPTG and X-gal were isolated and DNA was prepared from them as described in section 5.2.5. The DNA was then used as a template for double stranded sequencing as described in section 5.2.11.

5.2.11. Double stranded DNA sequencing. Double stranded DNA sequencing was carried out by the chain termination method of Sanger *et al.* (1977) using a Sequenase^{TM} 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 prepared as described in section 5.2.5 were denatured in 0.2 M NaOH, 0.2 mM EDTA for 30 min at 37°C. The mixture was neutralized by adding 0.1 volumes 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. After the pelleted DNA was washed with 70% ethanol it was redissolved in 7 μl of distilled water, and 2 μl of 200 mM Tris-HCl (pH7.5), 100 mM $MgCl_2$, 250 mM NaCl (5 x Sequenase^{TM} reaction buffer) and 1 μl of primer. M13 forward primer supplied in the Sequenase kit at 0.5 pmolμl^{-1} was used to map *BamH*I sites created by hexameric linker insertion mutagenesis; 5'-CTGAGCAGCCCGGTT-3', designed by Julian Ketley, Department of Genetics, University of Leicester and synthesized by Debra Langton, Department of Biochemistry, University of Leicester at 10 ngμl^{-1} was used to sequence *iutA*::*phoA* gene fusions. The latter is comlementary to a sequence between base position 63 and 77 in the Tn*phoA* sequence. The tube was warmed to 65°C and allowed to cool to room temperature over a period of about 30 min. The tube was placed on ice

and the following components were added in order: 1 μl of 0.1 M DTT, 1 μl of labelling mix (7.5 μM each of dGTP, dCTP and dTTP) diluted 1:14 in water, 0.5 μl of α[^{35}S]dATP (approximately 10 Ciμl^{-1} and 10 μM), 2 μl of Sequenase^{TM} Version 2.0 (diluted 1:8 in 10 mM Tris-HCl(pH7.5), 5mM DTT, 0.5 mgml^{-1} BSA). After mixing, the solution was incubated for 3 min at room temperature. Into tubes labelled A, C, G or T, 2.5 μl of termination mixes (80 μM each of dATP, dCTP, dGTP and dTTP, 50 mM NaCl and 8 μM of the appropriate ddNTP) was added. After prewarming the termination mixes to 37^{o}C, 3.5 μl of the labelling mix was added to each termination mix and incubated at 37^{o}C for 5 min. 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each tube. The products of the sequencing reactions were heated to 75^{o}C for 3 min before analysis by denaturing gel electrophoresis using wedge gels composed of 6% acrylamide (19:1 acrylamide: N-methyl-bis-acrylamide) and 8.3 M urea in 45 mM Tris, 45 mM boric acid (pH8.3), 1.25 mM EDTA (0.5 x TBE). Running buffer was TBE, and electrophoresis was at 60 W for approximately 3 h. Gels were fixed in 10% acetic acid, 12% methanol for 2 x 15 min, dried and exposed to Fuji RX-100 X-ray film overnight before the film was developed.

5.2.12. Phenotypic analysis of mutant proteins. Outer membrane proteins were prepared as described in section 4.2.2 except that, in some cases, the total membrane fraction was initially resuspended in 200 μl of Tris-$MgCl_2$-Triton X-100 buffer and, after incubation at room temperature for 30 min, the suspention was subjected to ultracentrifugation and the supernatant fraction containing the cytoplasmic membrane proteins was also recovered. The pellet was then treated as described in 4.2.2 to obtain outer membrane fractions. Both outer membrane and cytoplasmic membrane preparations were analysed by SDS-PAGE as described in section 4.2.2. The protein content of outer membrane preparations was

determined by the method of Bradford (1976). To 4 µl of outer membrane protein preparation were added 16 µl of 100 mM Tris-HCl (pH7.8), 10 mM $MgCl_2$, 189 mM NaCl and 1 ml of staining solution (100 mg Coomassie Brilliant Blue G250 dissolved in 50 ml of 95% ethanol to which 100 ml of 85% phosphoric acid and 850 ml of water were added before filtering through Watman N°1 filter). After 10 min at room temperature the absorbance at 580 nm was measured. Protein concentration was determined as BSA-equivalents from a standard curve using 0-1 $mgml^{-1}$ BSA dissolved in 100 mM Tris-HCl (pH7.8), 150 mM NaCl, 10 mm $MgCl_2$, 0.4% Triton X-100. 3.5 mg of protein was added to each lane.

Alkaline phosphatase activity of cells expressing IutA::PhoA protein fusions was determined by the method of Brickman and Beckwith (1975). Cells from 5 ml of an exponential phase culture (harvested at an optical density of 1.0) were resuspended in 0.5 ml of ice cold 100 mM Tris-HCl (pH7.8), 10 mM $MgCl_2$. Cells were disrupted by sonication and unbroken cells were removed by centrifugation at 10,000 x *g* for 5 min. Total membrane fractions were recovered by ultracentrifugation at 100,000 x *g* for 10 min at 4°C and the supernatant was recovered. The membrane pellet was resuspended in 0.5 ml of the Tris-$MgCl_2$ buffer and both fractions were assayed as follows. To 0.5 ml of 0.2 M Tris-HCl (pH10), 5 mM $MgCl_2$, 5 mM p-Nitrophenyl phosphate on ice were added 0.4 ml of water and 0.1 ml of sample. Tubes were incubated at 37°C for 5 min and the reaction was stopped by the addition of 0.2 ml of 1 M K_2HPO_4. Absorbance readings at 420 nm and 550 nm were taken and units of alkaline phosphatase were calculated using the formula: activity = (1000 x A_{420} - 1.75 x A_{550} x dilution factor) / time x A_{600} (culture) and expressed as µmol/min/OD of cells.

Cloacin DF13 binding by membranes was determined by preincubating cloacin DF13 preparations with membranes and titrating the

residual cloacin DF13 against the indicator strain M5a1 as described in section 4.2.4. The ability of strains to take up [^{14}C]aerobactin was determined as described in section 2.2.6.

5.2.13. Computer predictions of secondary structure and physical properties of mutant proteins. Regions of the IutA protein sequence spanning 20 amino acids either side of the position of each two amino acid insertion mutation and the equivalent regions of their mutant counterparts were analysed using a software package designed by John Devereux (1982) and run on a VAX/VMS version V5.3-1 mini computer. Secondary structure predictions were made using the algorithms devised by Chou and Fasman (1978) and Garnier *et al.* (1978). Hydrophilicity was predicted using the method of Hopp and Woods (1981), surface probability was predicted according to Emini *et al.* (1985), and chain flexibility was predicted using the rules devised by Karplus and Schulz (1985).

5.3. RESULTS.

5.3.1. Generation and analysis of IutA::PhoA fusion proteins. In an attempt to determine if signals for translocation to, and stable insertion into the outer membrane could be isolated within amino terminal portions of the aerobactin receptor, a bank of fusions of various amino terminal portions of the aerobactin receptor with the catalytic portion of *E. coli* alkaline phosphatase (PhoA) was prepared by the method of Manoil and Beckwith (1985) as described in Materials and Methods. In-frame fusions which encode polypeptides that are exported across the cytoplasmic membrane can be detected by expression in the PhoA$^-$ strain CC118 growing on media containing XP, a substrate which is cleaved by alkaline phosphatase to yield a blue pigment. Approximately 20% of derivatives of LG1700 F'$_{ts}$-Tn*phoA* exconjugants selected for resistance to high levels of kanamycin at 42^{0}C (and thus likely to harbour Tn*phoA* on a high copy

number pLG141-derived replicon) secreted proteins with alkaline phosphatase activity as evidenced by the blue colouration of the colonies on media containing XP. Plasmid DNA isolated from 17 of 42 of such clones, when used to transform virgin CC118 cells, produced clones that were able to hydrolyse XP and thus must have contained in frame fusions of *iutA* (the only gene on plasmid pLG141 encoding an exported protein) and *phoA*. The remaining 25 clones presumably had Tn*phoA* inserts at multiple locations (including at least one producing an in frame fusion within a chromosomal gene encoding an exported protein) since the high levels of kanamycin used to would allow growth only of strains carying Tn*phoA* in a multicopy state.

The positions of the Tn*phoA* insertions into pLG141 were analysed by *BamH*I-mapping of the plasmids and all inserts mapped, as expected, within the *iutA* gene as shown in Fig.5.1. Alkaline phosphatase activity of whole cells of a selection of the hybrid clones spanning the range obtained was assayed, and all showed similar activity (Table 5.1). After sonication, removal of unbroken cells, and separation of membranes from supernatant fractions by ultracentrifugation, significant alkaline phosphatase activity was observed only in the supernatant fractions; no significant activity was detected in the membrane fractions, suggesting that the activity was confined to the periplasm in all cases (Table 5.1). SDS-PAGE analysis of outer membrane proteins prepared from cells expressing any of the fusion genes revealed that the 74 kDa band present in LG1700 was absent and no new proteins were observed (SDS-PAGE profiles of outer and cytoplasmic membrane preparations of 3 representative mutants are compared to the wild type and are shown in Figure 5.2). Interestingly a new protein of approximately 150 kDa was observed in the Triton-soluble envelope fraction of all Tn*phoA* mutants irrespective of the map position of Tn*phoA* within *iutA*. The identity of this protein is unknown but it is presumably a Tn*phoA*-encoded polypeptide independent of the fusion protein.

5.3.2. Generation and analysis of hydroxylamine-induced cloacin DF13 resistant mutants. Spontaneous cloacin DF13 resistant *iutA* mutants isolated and described in chapter 4 were invariably the result of lesions causing failure of insertion and/or stable association of a mutant protein into the outer membrane. It may be that these mutations were deletions or more complex DNA rearrangements. Spontaneous mutations occuring in *fhuA* and *fepA* resulting in resistance to lethal agents which utilize the receptors FhuA and FepA respectively have been shown to be predominantly of this type (Hantke and Braun, 1978, Elish *et al.*, 1988). In an attempt to isolate *iutA* mutants which were insensitive to cloacin DF13 but which, nontheless, inserted into the outer membrane, *in vitro* hydroxylamine-induced mutagenesis was used, as this technique introduces point mutations (Eichenlaub, 1979) which are less likely to drastically affect the stability of mutant proteins than deletions or more complex DNA rearangements.

Plasmid DNA was reacted with hydroxylamine as described in Materials and Methods and used to transform cells of strain BZB1022. Chloramphenicol-resistant colonies were isolated and tested for sensitivity to cloacin DF13. Of approximately 500 clones tested, only 4 were resistant to cloacin DF13. Outer membrane preparations were analysed by SDS-PAGE and, in all four cases, the outer membranes were devoid of a 74 kDa band (data not shown). This suggests that, even in the case of point mutations, the most common type of mutation within *iutA* leading to total resistance to cloacin DF13 results in non-insertion into the outer membrane.

5.3.4. Generation and mapping of two amino acid insertion mutants within *iutA*. In order to produce defined mutations within the *iutA* gene in a quasi-ramdom manner the techniques described by Barany (1985a; 1985b) were used. By these methods, insertion of two codons within the reading frame of cloned genes at many sites is possible. This method has

two advantages over hydroxylamine mutagenesis. First, the mutations are relatively easily mapped, and second, mutations which do not affect a given phenotype, as well as those that do, can be isolated and studied.

By choosing restriction sites which occur commonly within the gene and designing suitable hexameric oligonucleotides to ligate onto the ends created by cutting at the chosen site, it is possible to create a new restriction site and introduce two new codons at that site within the gene. Covalently closed circular DNA is digested with the chosen enzyme in the presence of ethidium bromide which selectively inhibits all but the first cut. After the plasmid is linearized it binds more ethidium bromide and thus becomes more refractory to subsequent cutting. In this way partial digestion conditions can be devised which yield digestion products which are enriched for full length linear DNA molecules. After ligation of the linkers, a kanamycin resistance cassette can be inserted to provide a biological selection for linker insertions which can be removed at a later stage.

The restriction enzymes *Hpa*II and *Hha*I were chosen because sites for both enzymes occur frequently within the *iutA* coding region. Synthetic oligonucleotides were designed for the two recognition sites chosen which would produce a *BamH*I site at the point of insertion. As two *BamH*I sites existed within pLG141, and the technique requires the generation of a unique site, it was necessary to construct a pLG141 derivative which did not contain *BamH*I sites. This was done as described in Materials and Methods to produce plasmid pLG1000.

Of 119 insertion mutants generated at *Hpa*II sites containing the kanamycin resistance cassette, 17 were found to be resistant to cloacin DF13. After removal of the kanamycin resistance cassette, 5 of these plasmids were found to be full length molecules; the remainder had deletions of various lengths and were not further studied. *Sal*I-*BamH*I

mapping revealed that all five insertions in full length plasmids had occured at the same *Hpa*II site. Of 225 insertion mutants generated at *Hha*I sites containing the kanamycin resistance cassette, 51 were found to be resistant to cloacin DF13. After removal of the kanamycin resistance cassette, 12 of these plasmids were found to be full length molecules and *Sal*I-*BamH*I mapping revealed that these fell into 5 classes. Double stranded DNA sequencing was used to precisely map the positions of all six unique insertion sites (Table 5.2). Their relative positions with respect to the entire gene are shown in Figs. 5.3 and 5.3a.

5.3.5. Phenotypic analysis of hexameric linker insertion mutants. Outer membrane protein profiles of cells of strain BZB1022 harbouring wild type and mutant plasmids are shown in Fig. 5.4. No protein of the appropriate size was detectable in the outer membrane of cells harbouring pLG1004 (lane 2) while the amount of mutant protein in the outer membrane preparations of cells containing plasmids pLG1003, pLG1005 and pLG1006 (lanes 3, 4 and 7 respectively) was markedly reduced in comparison to the wild type protein present in the outer membrane preparation on cells harbouring the parent plasmid pLG1000 (lane 1). Mutant proteins present in the outer membrane preparations of cells harbouring plasmids pLG1001 and pLG1002 (lanes 5 and 6 respectively) appear to occur in the outer membrane at similar levels to the wild type protein.

Outer membranes prepared from cells harbouring pLG1001 or pLG1002 were shown to inactivate cloacin DF13 with comparable efficiency to those prepared from cells harbouring the non-mutant plasmid pLG1000 using a cloacin DF13 titration assay (Fig. 5.5). Outer membranes prepared from cells harbouring no plasmid or the mutant plasmids pLG1003, pLG1004, pLG1005 or pLG1006 were unable to titrate cloacin DF13 molecules in this assay (Fig. 5.5). Cells harbouring plasmids pLG1001,

pLG1002 or pLG1003 were sensitive to cloacin DF13 in a plate assay whereas cells harbouring plasmids pLG1004, pLG1005 or pLG1006 were not sensitive.

The ability of cells harbouring the mutant plasmids to incorporate [^{14}C]aerobactin was determined as shown in Table 5.3. Cells harbouring mutant plasmid pLG1001, a plasmid in which the mutation mapped to the signal peptide of the precursor protein, incorporated aerobactin with an efficiency of approximately 160% as compared to the wild type. Cells harbouring plasmid pLG1002 also took up aerobactin with a greater efficiency than the wild type cells in the assay conditions used, this time with an efficiency of almost 200% of the wild type activity. No significant uptake was observed in cells harbouring plasmid pLG1004 and reduced aerobactin translocation was observed in cells harbouring pLG1006, pLG1003 or pLG1005, with efficiencies of 15%, 27% and 76% respectively with respect to cells harbouring pLG1000.

5.3.6. Predictive analysis of secondary structure and physical properties of wild type and mutant proteins. The predicted secondary structure of the wild type signal peptide was compared to that of the mutant encoded by plasmid pLG1001 and is shown in Fig. 5.6a. According to Chou Fasman rules the effect of the mutation is to increase the length of a stretch of β-sheet structure which precedes the α-helical region in which the cleavage site resides. In addition two potential reverse turns are introduced immediately preceding the α-helix and the α-helix is shortened within the signal peptide but extends further into the mature protein. According to the Garnier-Osguthorp-Robson prediction the α-helix spanning the cleavage site is completely abolished and replaced by a shorter β-sheet structure. The effect of the insertion within the signal peptide on the overall hydrophilicity of this region is shown in Fig. 5.6b. A slight increase in hydrophilicity is observed around the position of the insertion and thus the central

hydrophobic region of the signal peptide is shortened slightly towards the C-terminus, but the overall hydrophilicity profile is not greatly affected. Similarly a very slight increase in surface probability close to the site of insertion is shown in Fig. 5.6c, but again the overall surface probability profiles of the wild type and mutant proteins in this region are very similar. In addition a small peak of increased chain flexibility is observed at the site of insertion with respect to the wild type protein (Fig. 5.6d).

The effect of the insertion of the amino acids Gly and Ser after Ser278 in the protein encoded by plasmid pLG1002 on the predicted secondary structure of the receptor protein between residues 259 and 298 is shown in Fig. 5.7a. An extra potential reverse turn at the inserted glycine residue is predicted by Chou Fasman rules and according to Garnier-Osguthorp-Robson rules a region of β-sheet structure preceeding the insertion is increased from 6 amino acids to seven and an extra potential reverse turn immediately after this β-sheet region is introduced. In addition a region of β-sheet predicted to occur after the insertion is reduced from 8 amino acids to 7. Fig. 5.7b shows that a predicted peak in hydrophilicity at the centre of this segment of the protein is slightly increased by the insertion after Ser278. Little change in surface probability is observed in this mutant (Fig. 5.7c). Fig. 5.7d shows the predicted chain flexibility of the mutant and wild type proteins between residues 259 and 298. A marked increase in chain flexibility is observed at the position of the insertion.

Fig. 5.8a shows the predicted secondary structure of the receptor protein between residues 342 and 381 and the respective stretch of amino acids in the mutant protein encoded by pLG1003 in which the amino acids Gly and Ser have been inserted after Ser363. Chou Fasman rules predict a shortening of an α-helical region immediately spanning the insertion site from 12 amino acids to 10 and the addition of 2 potential reverse turns. Similarly, Garnier-Osguthorp-Robson rules predict a shortening of an a-

helical region, although the region is shorter than that predicted by Chou Fasman (8 amino acids in the wild type, 6 in the mutant) and no potential turns are introduced. The changes in secondary structure predicted in the protein encoded by mutant plasmid pLG1004, in which the same two amino acids (Gly, Ser) are inserted two amino acids further downstream than in plasmid pLG1003, are similar to, but less pronounced than the changes predicted for the pLG1003 protein (Fig. 5.9a). Very small changes in predicted hydrophilicity (Figs. 5.8b and 5.9c), surface probability (Figs. 5.8d and 5.10d), or chain flexibility (Figs. 5.8d and 5.9d) were observed in proteins encoded by either pLG1003 or pLG1004.

Fig. 5.11a shows the predicted secondary structure of the receptor protein between residues 404 and 443 and the equivalent region of the protein encoded by mutant plasmid pLG1005. According to Chou Fasman rules the insertion results in the interruption of a 10-amino acid region of β-sheet. Only the last 4 amino acids of this region, however, are also predicted to form β-sheet by Ganier-Osguthorp-Robson rules and this region is unaffected by the insertion. The effect of the insertion in encoded by plasmid pLG1005 on predicted hydrophilicity, surface probability and chain flexibility is to increase all three parameters (Figs. 5.10b, c and d, respectively).

The predicted secondary structure of the receptor protein between amino acid residues 601 and 640 was compared to that of the protein encoded by mutant plasmid pLG1006, in which the amino acids Ile and Arg are inserted after Arg619 (Fig. 5.11a). Chou Fasman rules predict the creation of two potential reverse turns preceeding a region of β-sheet into which the new amino acids are inserted. Garnier-Osguthorp-Robson rules, however, predict the abolition of two potential reverse turns immediately after the position of the inserted amino acids and the creation of a 6-amino acid stretch of β-sheet. Very little change in the hydrophilicity (Fig. 5.12b),

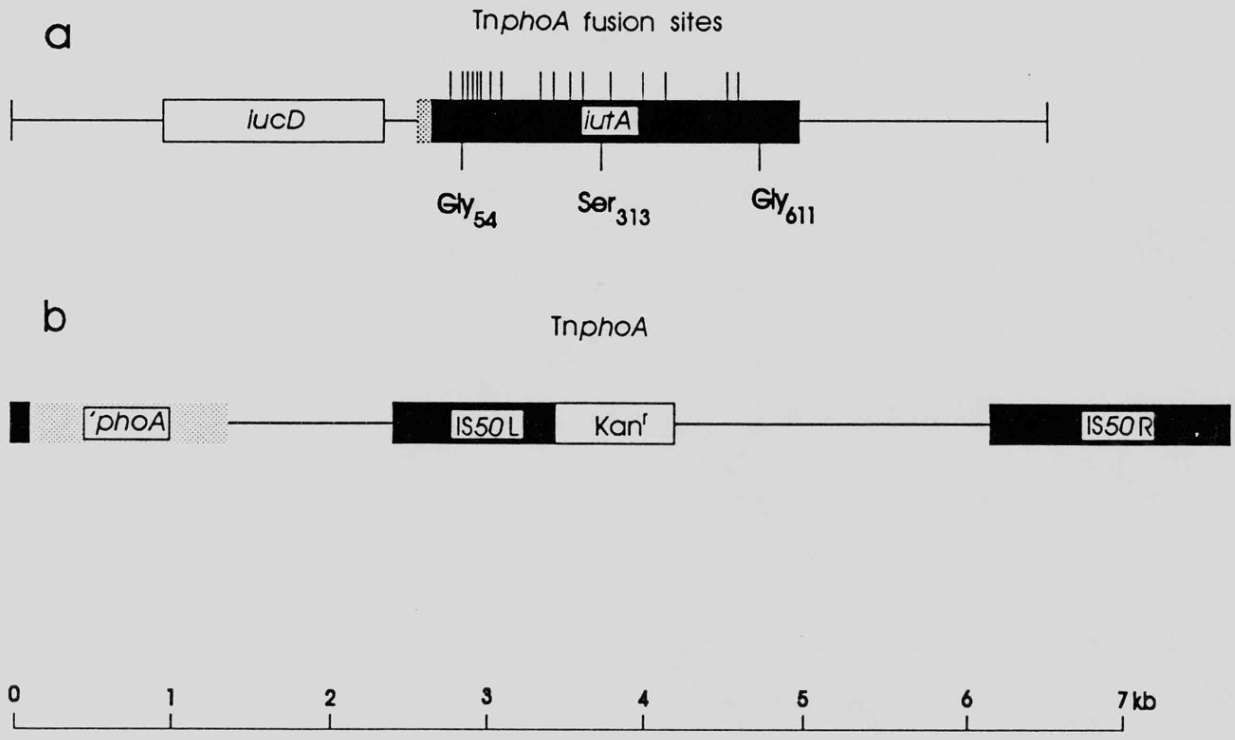


Figure 5.1. Positions of *iutA*::*phoA* fusions within the *iutA* gene. (a) The DNA depicted represents the 6.5 kb insert of pLG141. The filled box represents the sequence encoding the mature protein, the stippled box represents the sequence encoding the signal peptide and the empty box represents the *iucD* gene sequence. Bars above the line represent the approximate positions of the fusions numbered 1-17 from left to right determined by restriction mapping. Bars below the line represent the precice positions of fusions 1, 13 and 17 determined by sequencing which occur after Gly_{54}, Ser_{313} and Gly_{611} respectively. (b) The DNA depicted represents Tn*phoA*. The filled box on the left represents part of the IS*50*L sequence which continues towards the center of the transposon. IS*50*L is interupted by a DNA sequence containing most of the *E. coli* alkaline phosphatase gene but lacking a promotor and signal sequence (stippled box). The empty box represents the kanamycin resistance gene, and the filled box on the right represents IS*50*R (Manoil and Beckwith, 1985).

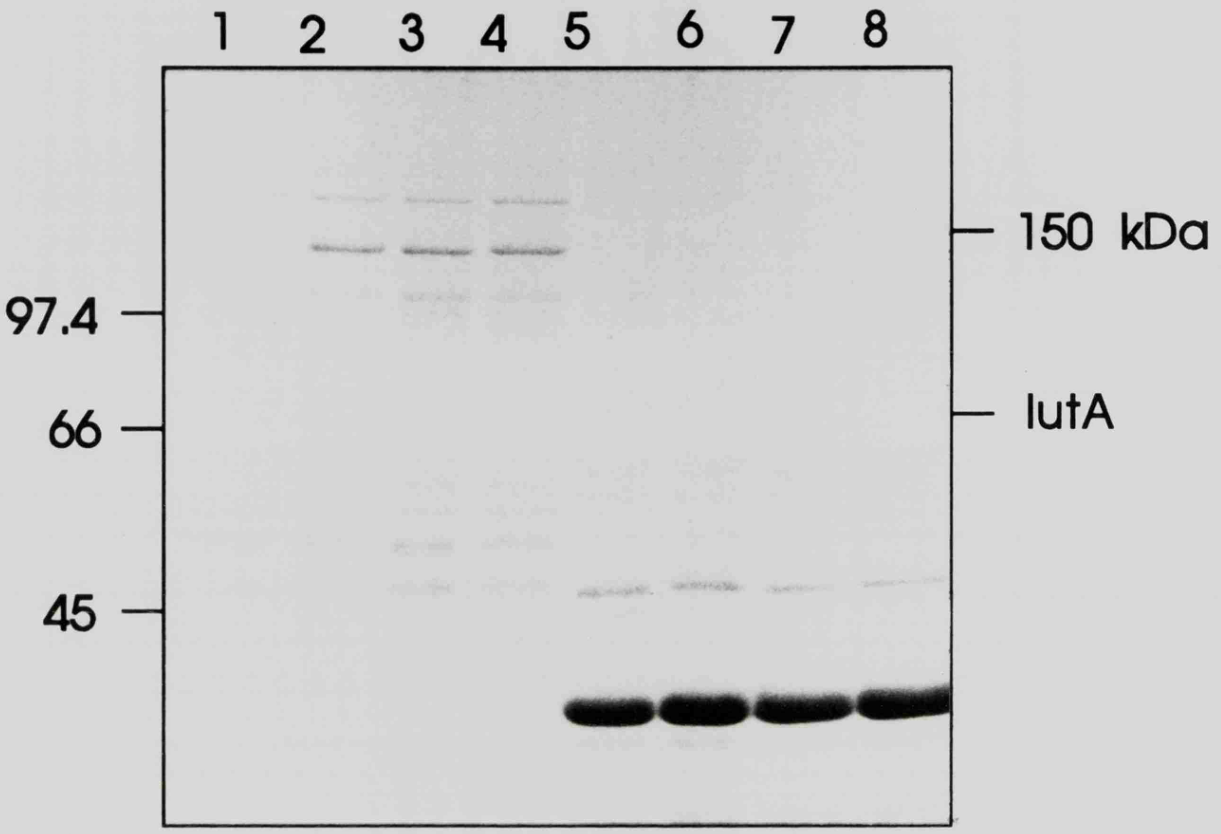


Figure 5.2. SDS polyacrylamide gel electrophoresis of cytoplasmic membrane proteins (lanes 1-4) and outer membrane proteins (lanes 5-8) of strain CC118 harbouring plasmids encoding IutA (lanes 1 and 5) or the catalytic portion of PhoA encoded by Tn*phoA* fused to N-terminal portions of IutA occuring after Gly_{54}[a] (lanes 2 and 6), Ser_{313}[a] (lanes 3 and 7) or Gly_{611}[a] (lanes 4 and 8). Molecular weight standards indicated on the left of the gel were phosphorylase B (97,400), bovine serum albumin (66,000) and ovalbumin (45,000). The position of IutA and of a 150 kDa cytoplasmic membrane protein expressed by strains containing Tn*phoA* are indicated on the right of the gel.

[a] Amino acids numbered from Gln1 of the mature IutA protein.

Table 5.1. Alkaline phosphatase activity of CC118 cells harbouring pLG141-derivatives encoding IutA::PhoA fusion proteins.

Fusion	Position of fusion[a]	Activity[b]		
		Whole cells	Sonnicates[c]	
			Membranes	Supernatants
1	Gly_{54}	32.9	1.9	47.3
9	nd[d]	29.3	2.0	40.7
13	Ser_{313}	34.5	2.3	52.3
15	nd[d]	35.2	1.8	45.9
17	Gly_{611}	30.3	2.4	42.4

[a] Last amino acid of the mature IutA protein encoded by the fusion.

[b] Alkaline phosphatase activity was determined by the method of Brickman and Beckwith (1975) as described in Materials and Methods.

[c] Cells were sonicated and, after removal of unbroken cells, membranes were recovered by ultracentrifugation and assayed separately from the supernatants.

[d] Not determined by sequencing. For approximate position see Figure 5.1.

Table 5.2. Two amino acid insertion mutations encoded by plasmids pLG1001-pLG1006.

Plasmid	Restriction site[a]	Amino acids inserted[b]
pLG1000	None	None
pLG1001	*Hha*I 231	Ala_{-4}-**Asp-Pro**-Pro_{-3}
pLG1002	*Hha*I 1078	Ser_{278}-**Gly-Ser**-Ala_{279}
pLG1003	*Hha*I 1333	Ser_{363}-**Gly-Ser**-Ala_{364}
pLG1004	*Hpa*II 1337	Ser_{365}-**Gly-Ser**-Gly_{366}
pLG1005	*Hha*I 1512	Ala_{422}-**Asp-Pro**-Gln_{423}
pLG1006	*Hha*I 2102	Arg_{619}-**Ile-Arg**-Val_{620}

[a] Nucleotide coordinates from the *iutA* sequence of Krone *et al.* (1985).

[b] Preexisting amino acids numbered from Gln_1 of the mature protein. Inserted amino acids are in bold.

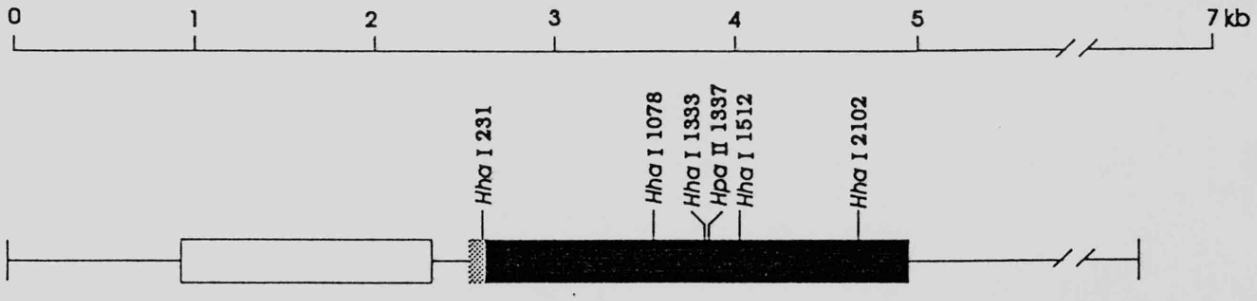


Figure 5.3. Positions of restriction sites used to create two codon insertion mutations within the *iutA* gene. The DNA depicted represents the complete insert within pLG1000. The filled box represents the sequence encoding the mature protein and the stippled box represents the sequence encoding the signal peptide. The empty box represents the *iucD* gene sequence.

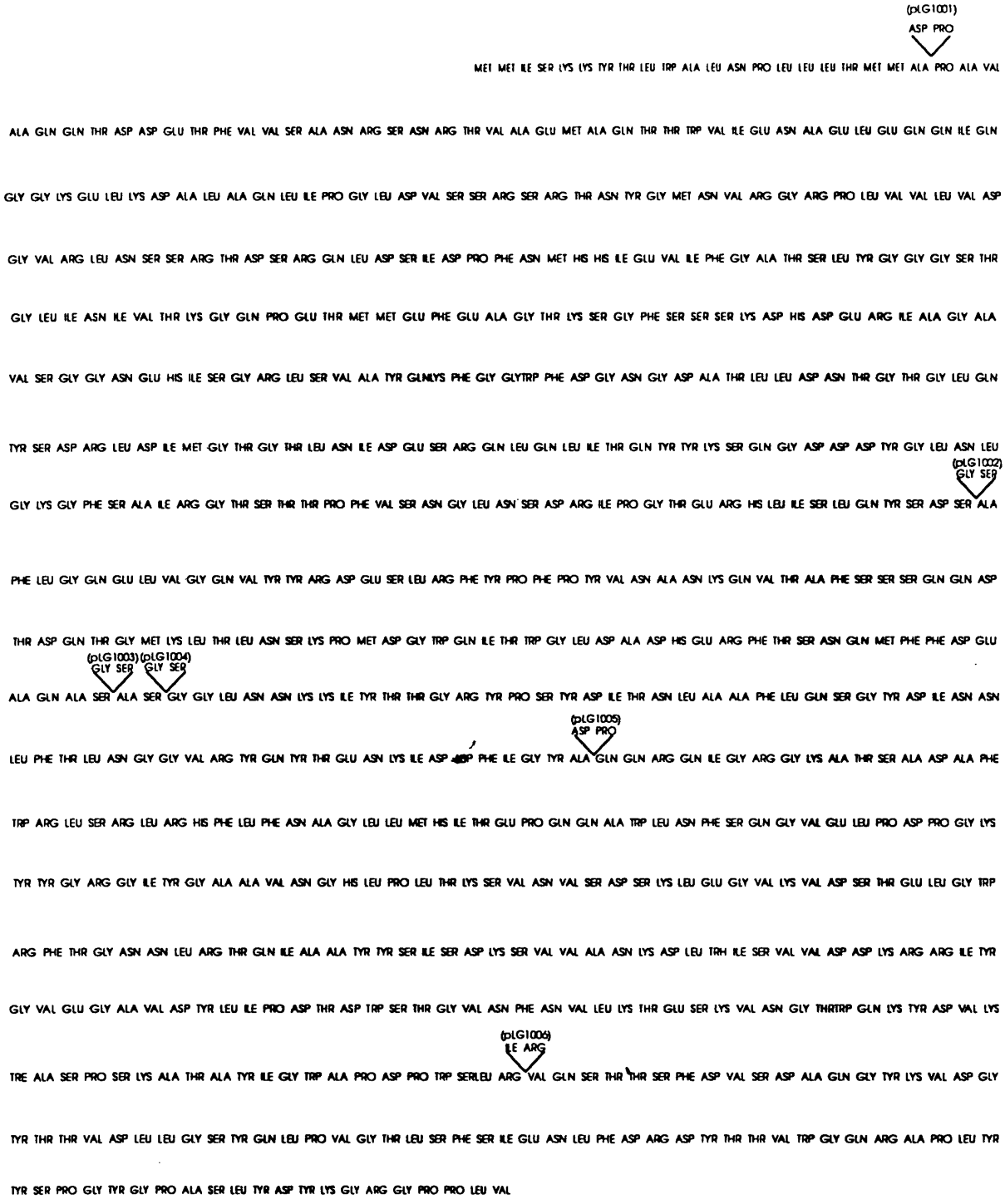


Figure 5.3a. Amino acid sequence of the IutA protein as determined from the nucleotide sequence and reproduced from Krone *et al.* (1985b). The amino acid insertions within mutant proteins encoded by plasmids pLG1001-pLG1006 are shown above the amino acid sequence and the plasmids encoding the insertions are included in parentheses above the inserted amino acids.

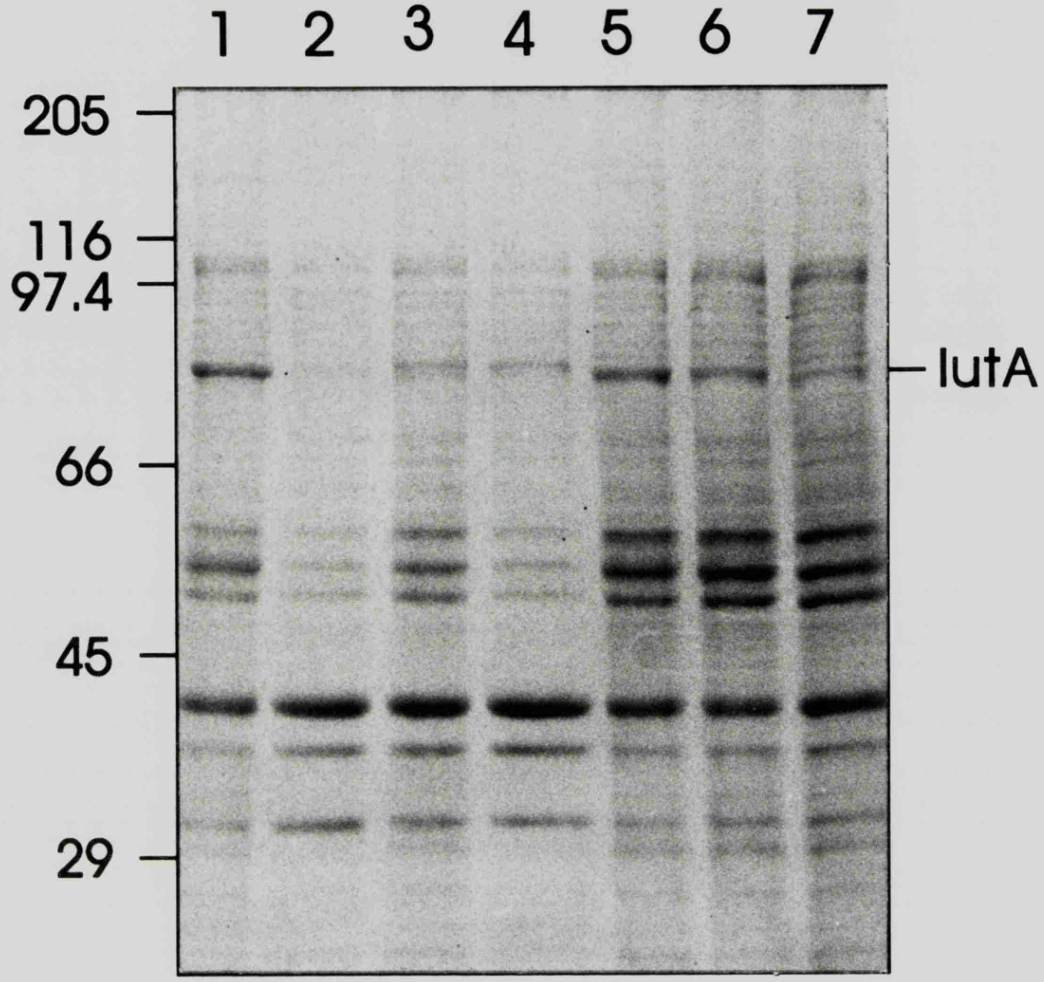


Figure 5.4. SDS polyacrylamide gel electrophoresis of outer membrane proteins of strain BZB1022 harbouring plasmids pLG1000 (lane 1), pLG1004 (lane 2), pLG1003 (lane 3), pLG1005 (lane 4), pLG1001 (lane 5), pLG1002 (lane 6) or pLG1006 (lane 7). Molecular weight standards indicated on the left of the gel were myosin heavy chain (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000). The position of the IutA protein is indicated on the right.

Table 5.3. [14C]aerobactin translocation by cells of strain BZB1022 harbouring plasmids encoding the IutA protein and two amino acid insertion mutant proteins.

Plasmid	Amino acids inserted[a]	Aerobactin translocation[b]
None	-	134±13[c]
pLG1000	None	2178±385
pLG1001	Ala_{-4}-**Asp-Pro-**Pro_{-3}	3510±332
pLG1002	Ser_{278}-**Gly-Ser-**Ala_{279}	4213±302
pLG1003	Ser_{363}-**Gly-Ser-**Ala_{364}	588±12
pLG1004	Ser_{365}-**Gly-Ser-**Gly_{366}	155±5
pLG1005	Ala_{422}-**Asp-Pro-**Gln_{423}	1643±127
pLG1006	Arg_{619}-**Ile-Arg-**Val_{620}	326±6

[a] Preexisting amino acids numbered from Gln_1 of the mature protein. Inserted amino acids are in bold.

[b] 14C emmissions were measured in a window of 0-156 keV.

[c] Variance is the standard deviation from 3 independent determinations.

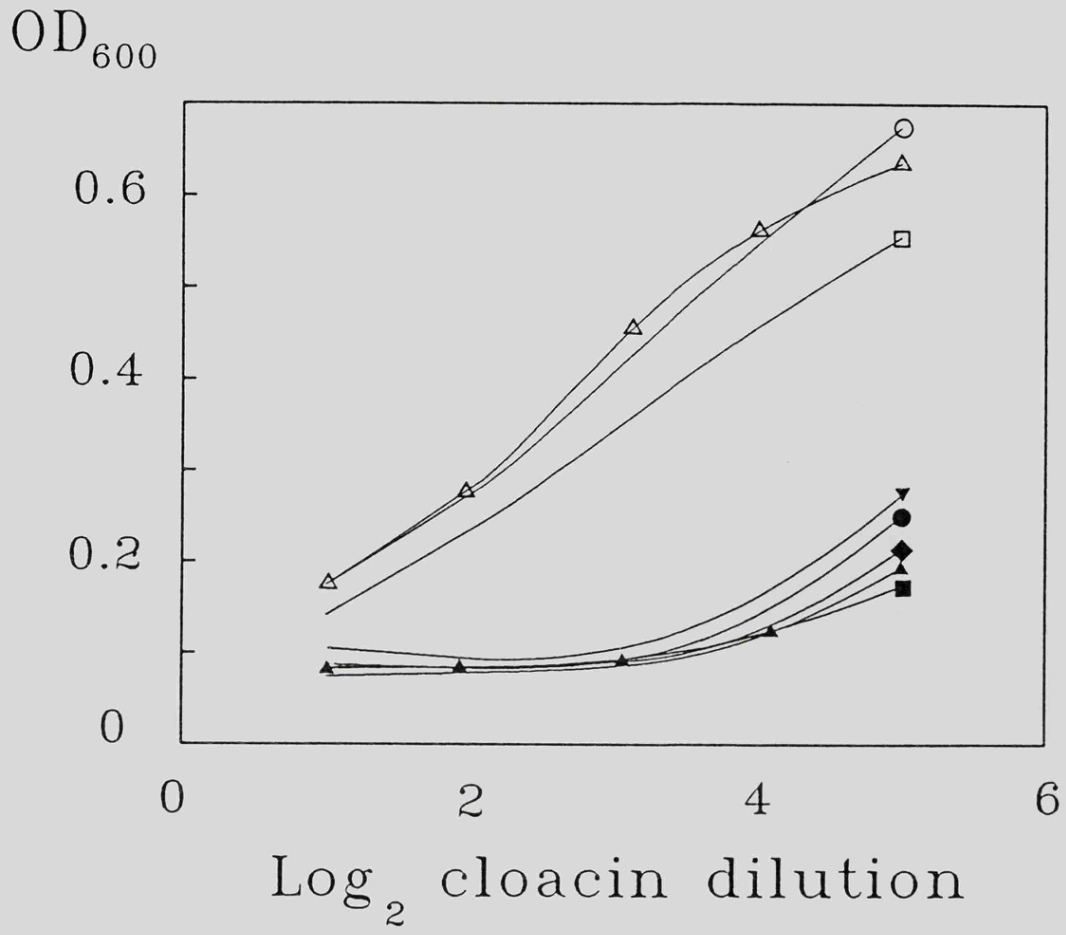


Figure 5.5. Cloacin binding to membranes prpared from cells of strain BZB1022, or BZB1022 harbouring plasmids encoding IutA or two-amino acid insertion mutant derivatives of IutA. Growth of the indicator strain M5a1 was monitored (culture OD_{600}) after addition to a cloacin DF13 dilution series pre-treated with membranes prepared from strains BZB1022 (▲), or BZB1022 harbouring plasmid pLG1000 (△), pLG1001, (○), pLG1002 (□), pLG1003 (●), pLG1004 (■), pLG1005 (◆) or pLG1006 (▼).

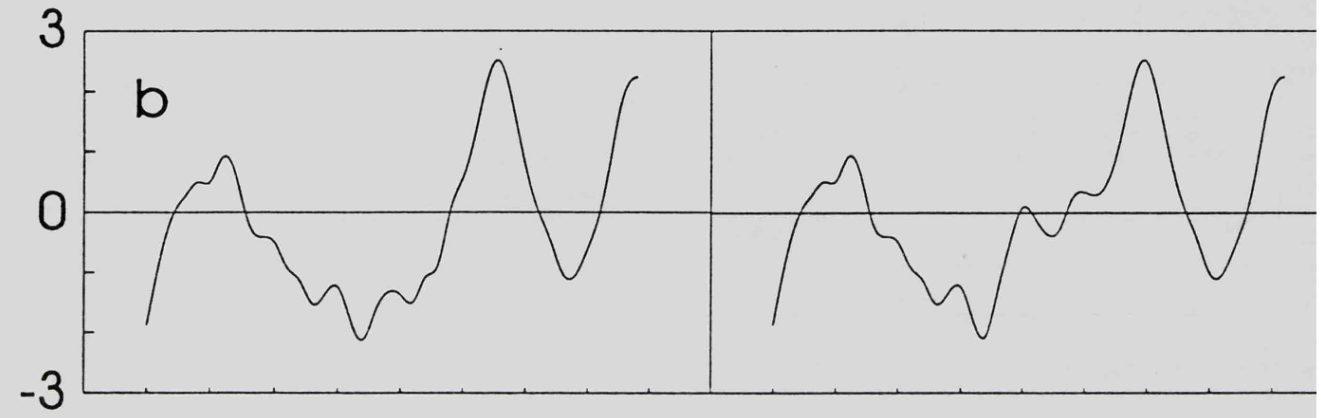
Figure 5.6. Predicted changes in secondary structure and physical properties of the IutA signal peptide caused by the insertion of aspartic acid and proline after Ala_{-4}. (a) The amino acid residues which comprise the signal peptide (underlined) as well as the first 15 amino acid residues of the mature protein of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1001, are depicted. The inserted amino acids are shown in bold. The charge distribution of this region of the proteins encoded by both plasmids is depicted below as is the predicted secondary structure according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR). 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

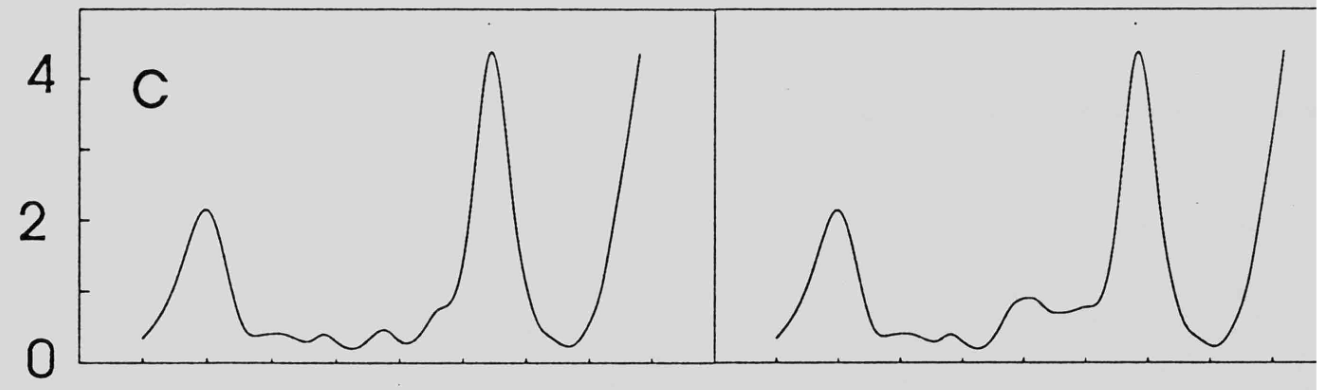
[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).

a

Residue N°.		-25 -1	
15			
Residue		MMISKKYTLWALNPLLLTMMADPPAVAQQTDDETFVVSANRS	
Charge	pLG1000	++ ---	+
	pLG1001	++ - ---	+
CF[a]	pLG1000	HHHHHHBBBBBBBBBBB HH--HHHHHHttttBBBBBBBTT	
	pLG1001	HHHHHHBBBBBBbbbbbbbbb ttHHHHHHHHHHBBBBBBBTT	
GOR[b]	pLG1000	BBBB BBBBB HHHHHHH--HHHHHH BBBBB	TT
	pLG1001	BBBB BBBBB BBBBB BBBBB	TT





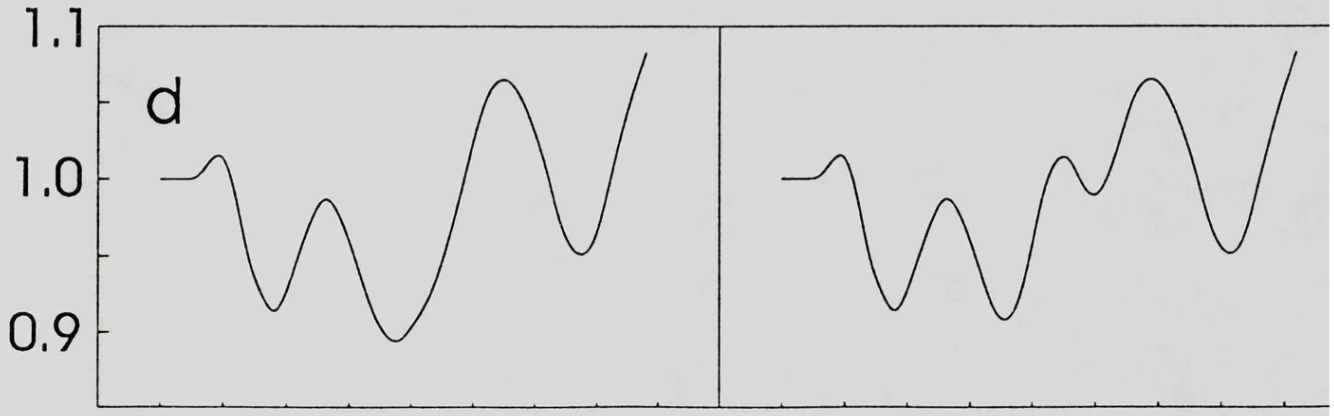


Figure 5.7. Predicted changes in secondary structure and physical properties of the IutA protein between residues 259 and 298 of the mature protein caused by the insertion of glycine and serine residues after Ser_{278} (a) The amino acid residues of this region of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1002, are depicted. The inserted amino acids are shown in bold. The predicted secondary structure of both wild type and mutant proteins according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR) are shown below. 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).

a

```
Residue N°.    259                                              298
Residue          NSDRIPGTERHLISLQYSDSGSAFLGQELVGQVYYRDESLRF

CF[a]  pLG1000     TT  TT   BBBBBBBTTt--HHHHHHBBBBBBBhhhhhhh
       pLG1002     TT  TT   BBBBBBBTTTThhhhhhhBBBBBBBhhhhhhh

GOR[b] pLG1000    TTT      BBBBBBTT   --      BBBBBBBBTTT TT
       pLG1002    TTT      BBBBBBBTTT          BBBBBBBTTT TT
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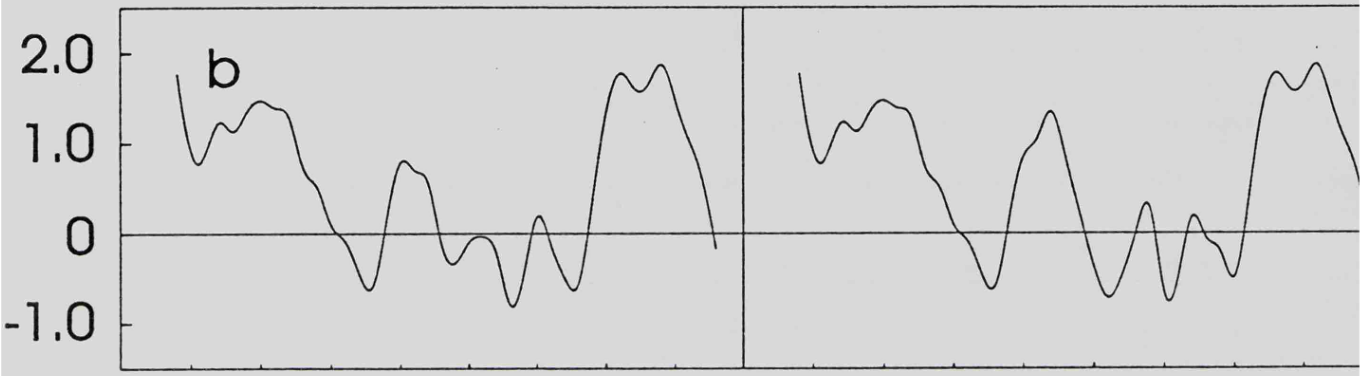

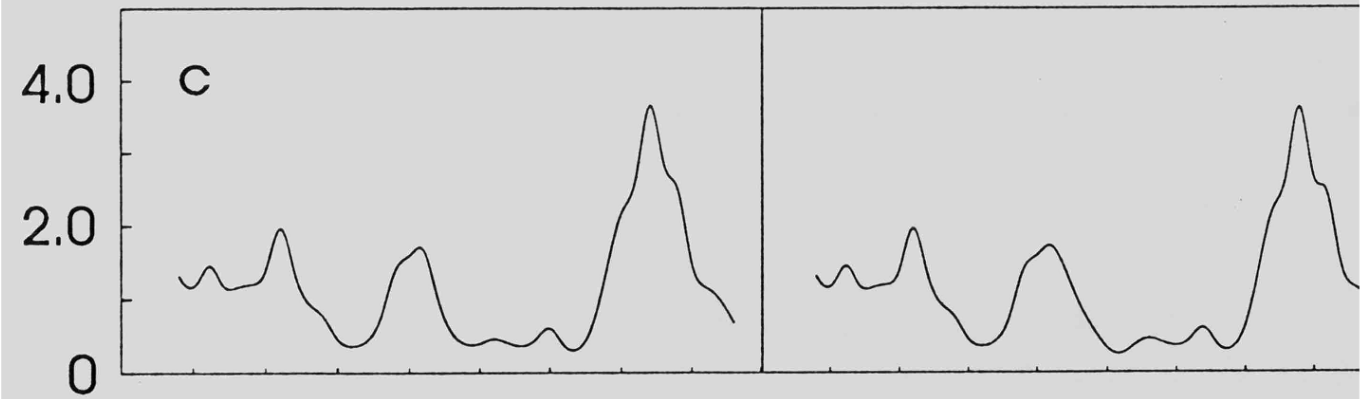

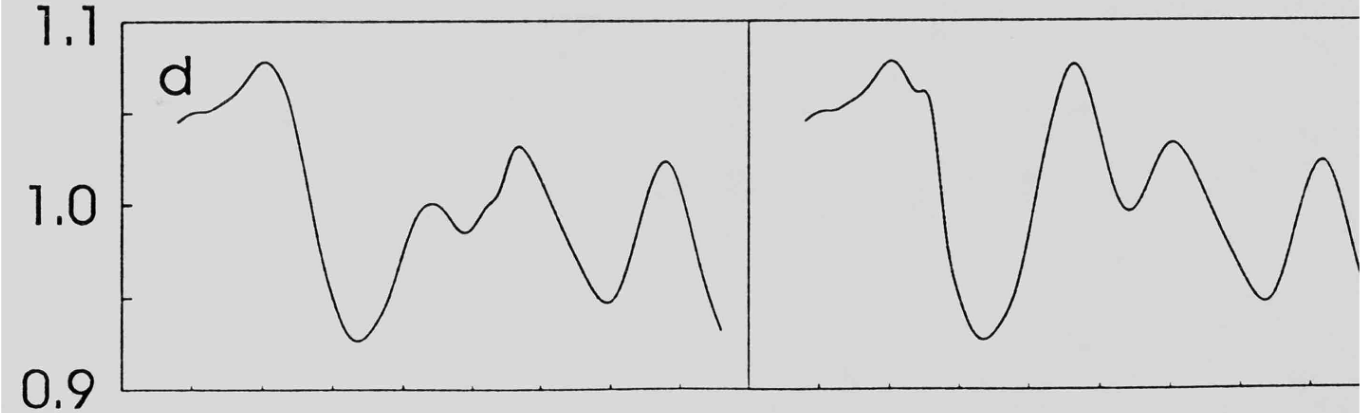

Figure 5.8. Predicted changes in secondary structure and physical properties of the IutA protein between residues 344 and 383 of the mature protein caused by the insertion of glycine and serine residues after Ser_{363} (a) The amino acid residues of this region of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1003, are depicted. The inserted amino acids are shown in bold. The predicted secondary structure of both wild type and mutant proteins according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR) are shown below. 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).

a

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Residue N°.      344                                          383
Residue          DADHERFTSNQMFFDLAQASGSASGGLNNKKIYTTGRYPSYD

CF[a]  pLG1000   hhhhhhhhTTHHHHHHHHHH--HHttbbbbbbbbbbttttTTt
       pLG1003   hhhhhhhhTTHHHHHHHHHH  TTTtbbbbbbbbbbttttTTt

GOR[b] pLG1000   HHHHHHH     HHHHHHHH--        BBBBBBTT TTTT
       pLG1003   HHHHHHH     HHHHHH            BBBBBBTT TTTT
```
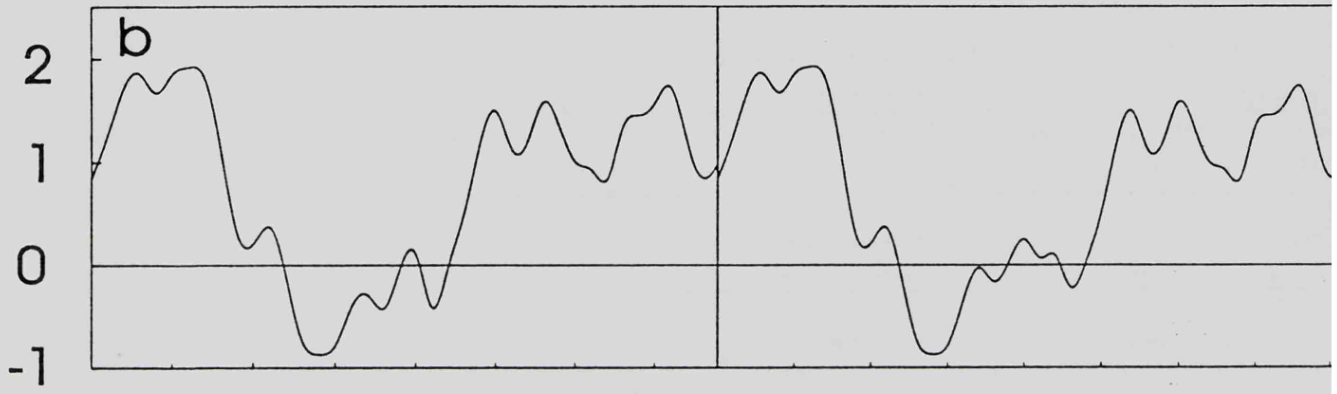
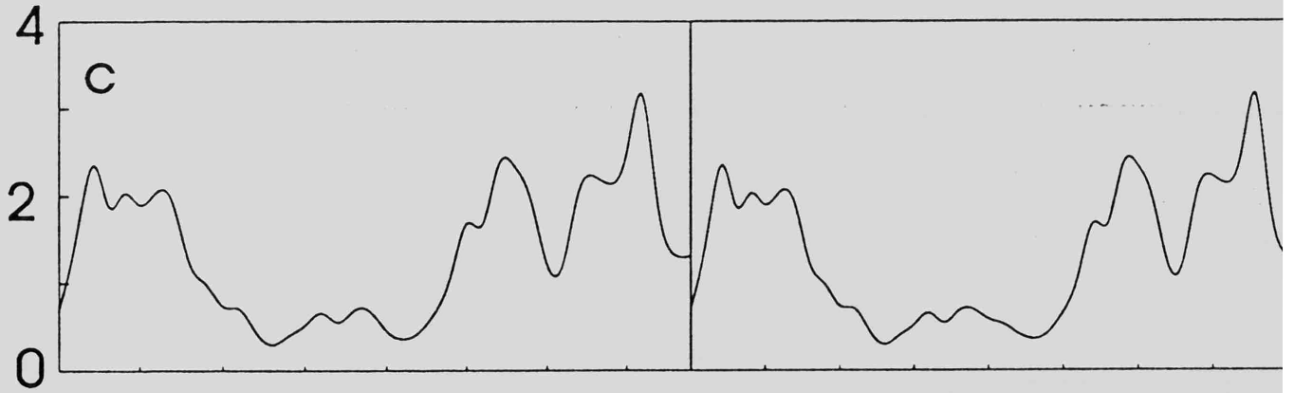





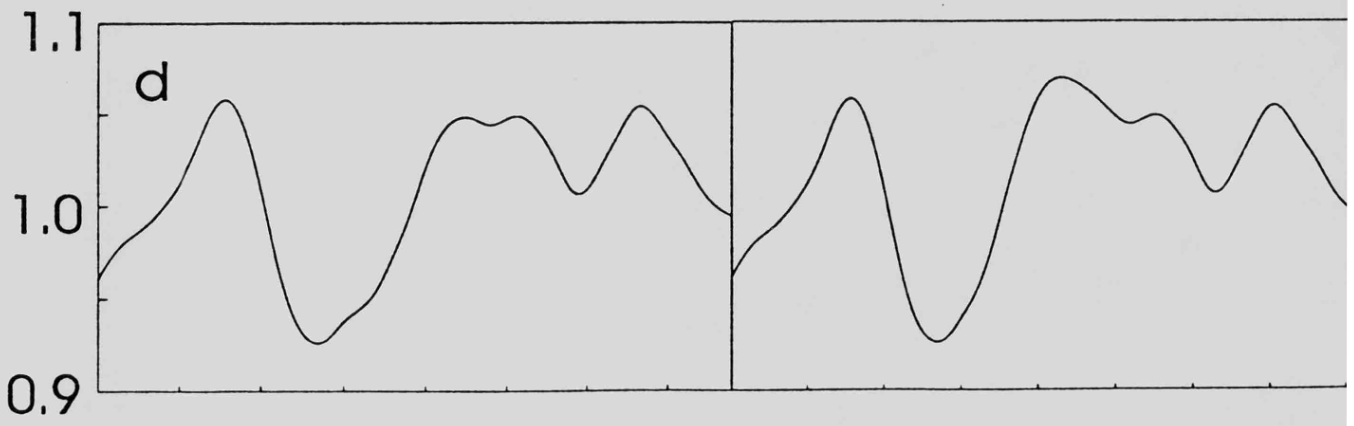


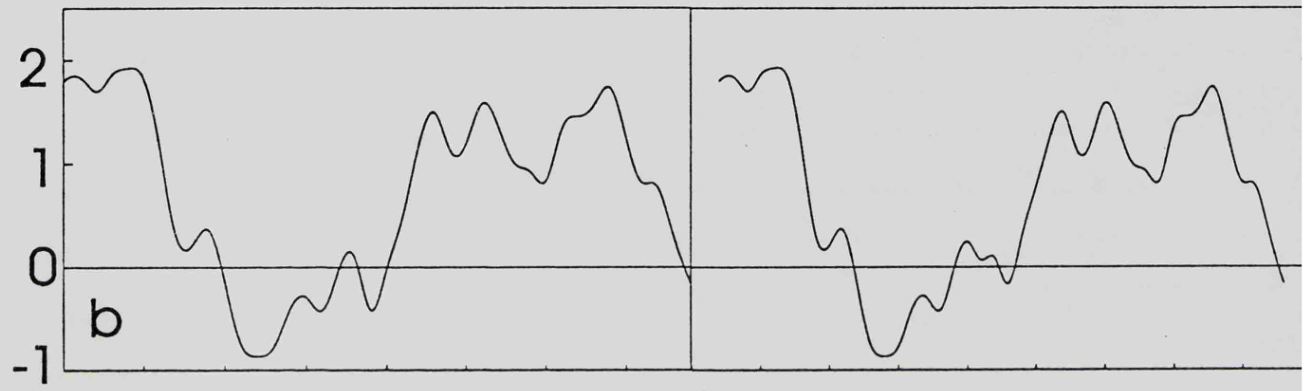
Figure 5.9. Predicted changes in secondary structure and physical properties of the IutA protein between residues 346 and 385 of the mature protein caused by the insertion of glycine and serine residues after Ser_{365} (a) The amino acid residues of this region of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1004, are depicted. The inserted amino acids are shown in bold. The predicted secondary structure of both wild type and mutant proteins according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR) are shown below. 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

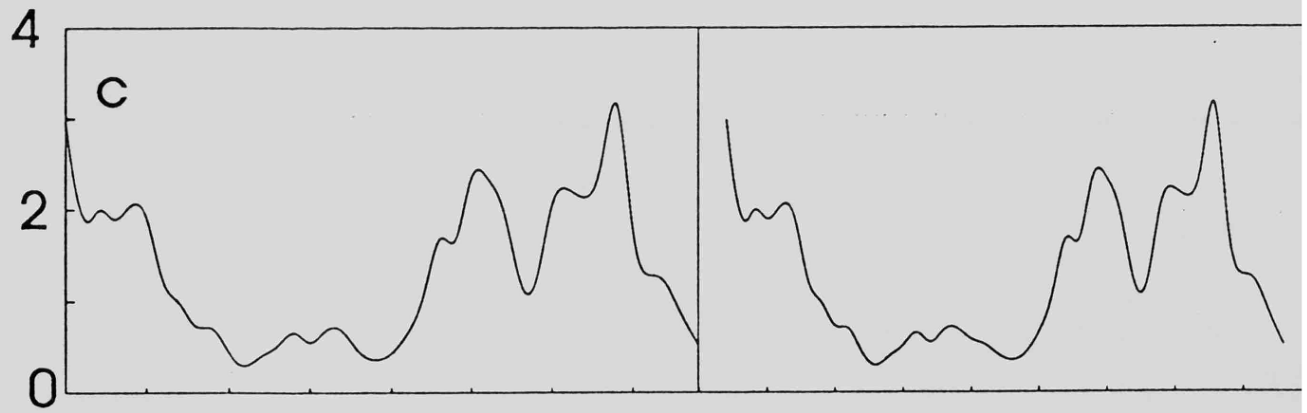
[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).

a

```
Residue N°.     346                                                 385
Residue         DHERFTSNQMFFDLAQASASGSGGLNNKKIYTTGRYPSYDIT

CFa  pLG1000          TTHHHHHHHHHHHH--ttbbbbbbbbbbttttTTtt
     pLG1004          TTHHHHHHHHHHHHHTTTtbbbbbbbbbbttttTTtt

GOR[b] pLG1000    TTT    HHHHHHHH  --     BBBBBBTT  TTTT
       pLG1004    TTT    HHHHHHH          BBBBBBTT  TTTT
```





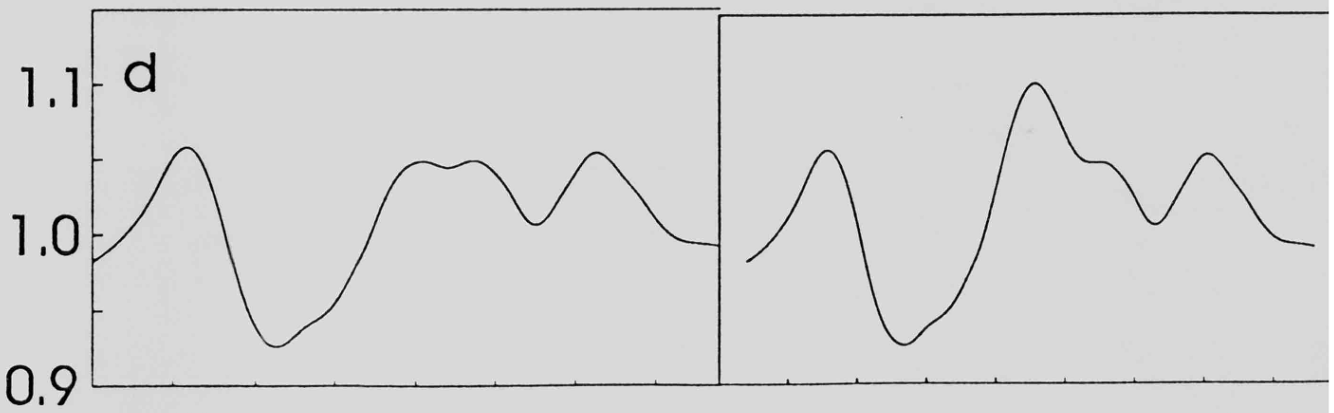


Figure 5.10. Predicted changes in secondary structure and physical properties of the IutA protein between residues 403 and 442 of the mature protein caused by the insertion of aspartic acid and proline residues after Ala_{422} (a) The amino acid residues of this region of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1005, are depicted. The inserted amino acids are shown in bold. The predicted secondary structure of both wild type and mutant proteins according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR) are shown below. 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).

a

```
Residue N°.      403                                          442
Residue            NGGVRYQYTENKIDDFIGYADPQQRQIGAGKATSADAFWRLS

CF[a]  pLG1000     tttBBBBB tt    BBBBB--BBBBB tthhhhhhhhhh
       pLG1005     tttBBBBB tt    BBBBB  BBBBB tthhhhhhhhhh

GOR[b] pLG1000     TTT BBBBTTTTTTTTTT -- BBBB       HHHHHHHH
       pLG1005     TTT BBBBTTTTTTTTTT    BBBB       HHHHHHHH
```

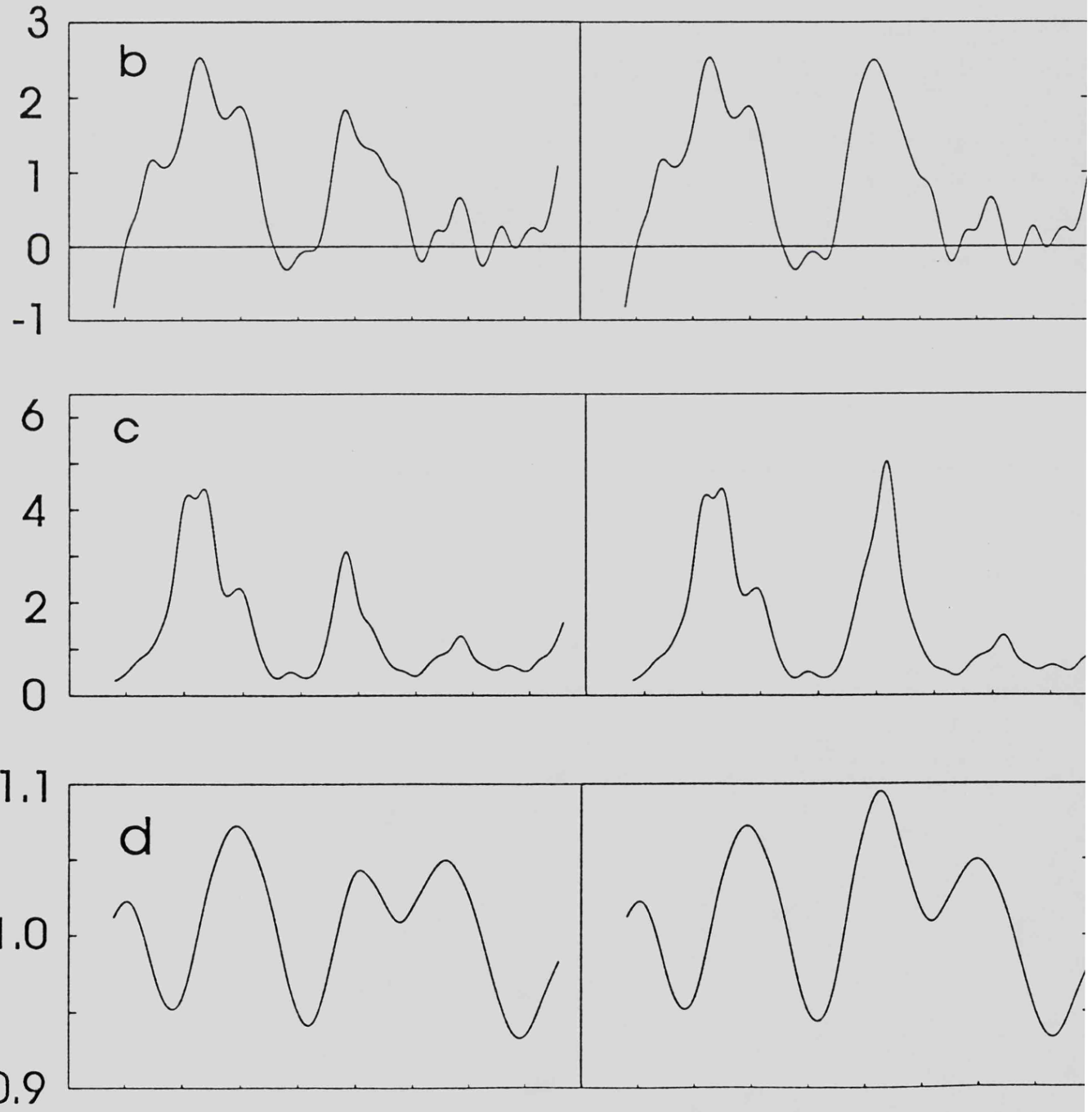
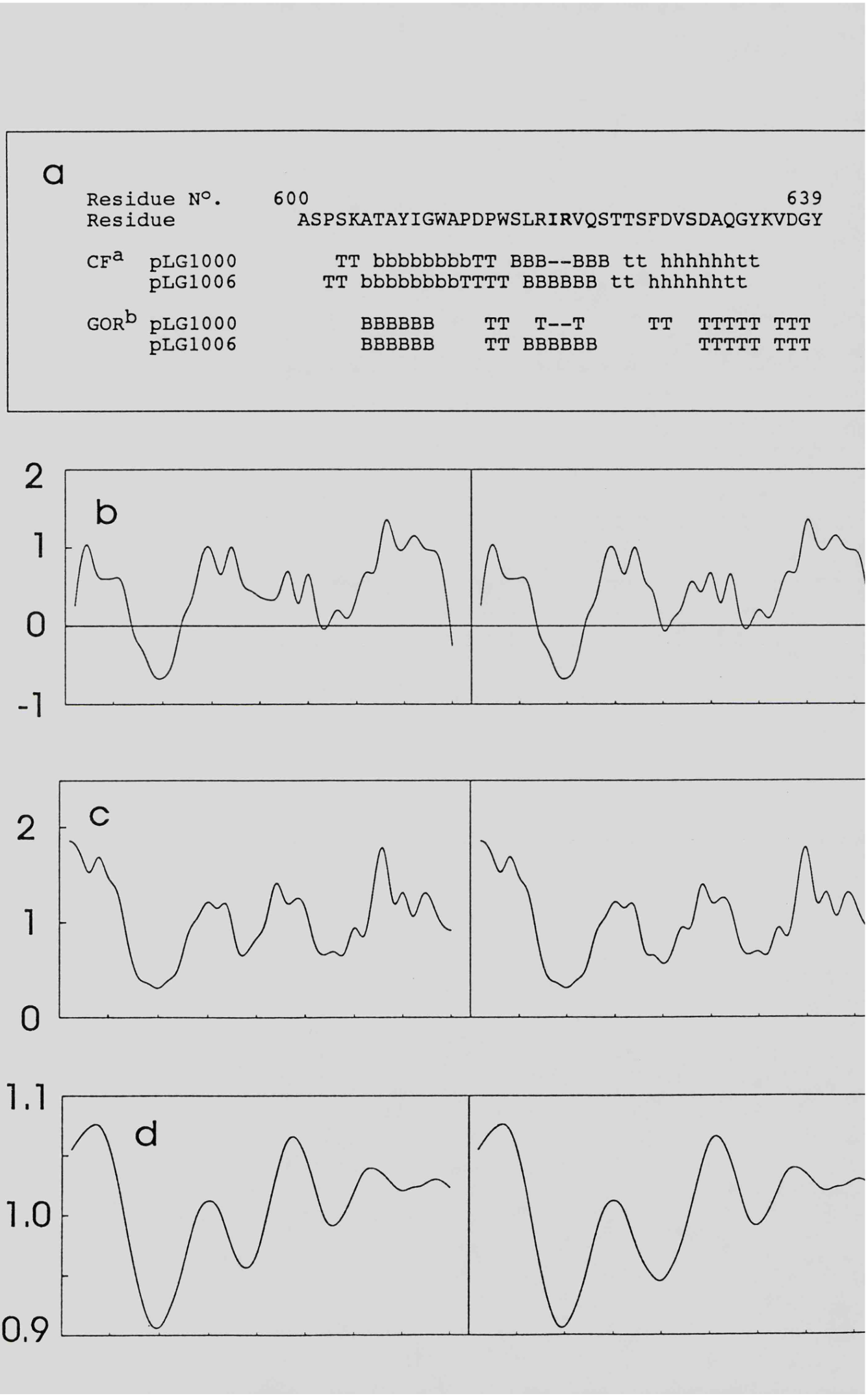


Figure 5.11. Predicted changes in secondary structure and physical properties of the IutA protein between residues 600 and 639 of the mature protein caused by the insertion of ispleucine and arginine residues after Arg_{619} (a) The amino acid residues of this region of the wild type protein, encoded by plasmid pLG1000, and the mutant protein, encoded by plasmid pLG1006, are depicted. The inserted amino acids are shown in bold. The predicted secondary structure of both wild type and mutant proteins according to the method of Chou and Fasman (1978) (CF) and Garnier *et al.* (1978) (GOR) are shown below. 'H' indicates α-helix, 'B' indicates β-strand and 'T' indicates a potential reverse turn. Lower case equivalents indicate the same structures but predicted with less certainty. (b) The hydrophilicity of this region of the wild type (left) and mutant (right) proteins as predicted by the algorithm of Hopp and Woods (1981) is shown. Positive values indicate hydrophilic regions. (c) The surface probabilities of wild type (left) and mutant (right) proteins in this region according to the method of Emini *et al.* (1985) are depicted. (d) The chain flexibility as predicted by Karplus and Schulz (1985) of wild type (left) and mutant (right) proteins in this region are depicted.

[a] Secondary structure prediction using the algorithm of Chou and Fasman (1978).

[b] Secondary structure prediction using the algorithm of Garnier *et al.* (1978).


a
Residue N°. 600 639
Residue ASPSKATAYIGWAPDPWSLRIRVQSTTSFDVSDAQGYKVDGY
CF[a] pLG1000 TT bbbbbbbbTT BBB--BBB tt hhhhhhtt
pLG1006 TT bbbbbbbbTTTT BBBBBB tt hhhhhhtt
GOR[b] pLG1000 BBBBBB TT T--T TT TTTTT TTT
pLG1006 BBBBBB TT BBBBBB TTTTT TTT
b
2
1
0
-1
c
2
1
0
d
1.1
1.0
0.9

surface probability (Fig. 5.11c) or chain flexibility (Fig. 5.11d) is predicted to result from the insertion of Ile and Arg in this mutant protein.

5.4. DISCUSSION.

Fusion proteins containing various N-terminal stretches of IutA and a catalytic C-terminal fragment of *E. coli* alkaline phosphatase were not localized to the outer membrane of *E. coli* cells harbouring plasmids encoding such gene fusions, even though they contained up to 87 % of the mature IutA protein. This evidence suggests that sequences within the mature IutA protein which are necessary for export to and/or stable association with the outer membrane are scattered throughout most of the length of the polypeptide chain. This is supported by the observation that all 4 hydroxylamine-induced (and thus probably point) mutants which had become resistant to the bacteriocin cloacin DF13 did not associate with the outer membrane. Morover, of 5 two-amino acid insertions within the mature protein sequence, 4 resulted in a significant reduction in the amount of receptor protein found in the Triton-insoluble envelope fraction.

This is consistent with a view that outer membrane proteins, unlike proteins associated with other biological membranes, may have assembled hydrophobic regions resulting from close apposition of regions containing polar or even charged amino acid residues, which are distant from one another in the primary amino acid sequence. These may be assembled together into the outer membrane as amphipathic structures, such as the β-barrel made up of eight or more antiparallel β-strands (Finer-Moore and Stroud, 1984; Kennedy, 1978). Such structures could present a hydrophobic exterior to the hydrophobic outer membrane microenvironment, while the hydrogen bonding requirements of the polar groups could be satisfied either by other polar groups within closely apposed regions or, in the case of

channel forming regions, by water molecules (Kennedy, 1978).

Indeed most outer membrane proteins are suprisingly polar and contain few, if any, stretches of hydrophobic residues in α-helical comformation characteristic of transmembrane segments of proteins which associate with other biological membranes. It has been suggested that the unusual nature of the membrane-spanning regions of outer membrane proteins may have evolved to prevent insertion of outer membrane proteins into the cytoplasmic membrane, where they would be highly deleterious, particularly those forming large aqueous channels (Kadner, 1990). The ability of fusion proteins containing N-terminal fragments of outer membrane proteins and truncated outer membrane proteins to assemble into the outer membrane has been studied by a number of groups working on several different proteins but no clear cut pattern has emerged. Early work by Benson and Silhavy *et al.* (1983) and Benson et al. (1984) using *lamB::lacZ* gene fusions indicated that if 49 or more residues of the mature LamB protein were present the fusion protein would co-fractionate with the outer membrane. More recently, however, Tommassen *et al.* (1985) studied the cellular localization of fusions between the outer membrane protein PhoE and β-galactosidase and found that, although some of the fusions co-fractionated with the outer membrane during centrifugation through sucrose gradients, they were found to be exclusively located in the cytoplasm when immunocytochemical labelling of ultrathin cryosection and electronmicroscopy were used. When these workers analysed the LamB-LacZ hybrids of Benson *et al.* (1984), the hybrids were again found by electronmicroscopy to be exlusively in the cytoplasm (Tommassen *et al.*, 1985). Similar results have since been shown for hybrids of FhuA with β-galactosidase (Coulton *et al.*, 1988). Voorhort *et al.* (1988) have shown that induction of LamB-LacZ hybrid proteins results in the appearance of membrane-like structures in the cytoplasm and this may explain these

discrepancies. Such results suggest that outer membrane proteins fused to β-galactosidase are not appropriate for studies of the localization of wild type proteins.

More promising results, however, have been obtained using fusions to the periplasmic protein PhoA which have been used in a number of studies examining the topology of both inner and outer membrane proteins (Boyd *et al.*, 1987; Coulton *et al.*, 1988; Herrero *et al.*, 1988, Manoil and Beckwith, 1986; Murphy and Klebba, 1989). Coulton *et al.* (1988) reported that FhuA-PhoA fusion proteins were exported to the periplasm and that, while fusions containing up to 88 amino acids were released along with periplasmic proteins, those containing 180 or more amino acids of the mature FhuA protein were associated with the outer membrane. This evidence suggests that information between $FhuA_{88}$ and $FhuA_{180}$ confers stable association with the outer membrane. These results are, however in contrast to those of Jackson *et al.*, 1986) who found that a series of truncated FhuA polypeptides, in which the C-terminus had been truncated by the insertion of translational stop signals within the reading frame, were found exlusively in the Sarkosyl-soluble (cytoplasmic membrane) fraction even when less than 5% of the polypeptide was missing. This suggests that C-terminal residues are required for assembly into and/or stable association with the outer membrane. The discrepancy may possibly be explained, however, if the truncated proteins were in fact localized to the outer membrane but, as the authors suggest, in a Triton-soluble "assembly intermediate" form (Jackson *et al.*, 1986).

Analysis of the subcellular location of fusions of the outer membrane protein FepA and PhoA has implicated a region between amino acids $FepA_{178}$ and $FepA_{227}$ of the mature FepA protein as being necessary and sufficient for final assembly into and stable association with the outer membrane (Murphy and Klebba, 1989). In contrast, a truncated FhuE

protein lacking only 24 amino acids (and containing an additional 5 amino acids encoded by vector pACYC184) at the C-terminus did not assemble into the outer membrane despite the fact that it was shown to be as stable as the wild type protein (Sauer *et al.*, 1990). In conclusion it appears that some outer membrane proteins such as FhuA and FepA contain information within the N-terminus that is sufficient for stable association with the outer membrane. Others, like FhuE, however, contain necessary structural information necessary for stable association with the outer membrane located throughout most of the length of the polypeptide. Evidence provided here suggests that, in this respect, IutA is like FhuE.

Regions of proteins within membranes are thought to be rarely, if ever, in a non-regular conformation because such regions are unable to form intramolecular hydrogen bonds and the hydrophobic interior of the membrane would not supply a source of hydrogen bonds (Kennedy, 1978). Mutations that drastically affect the stable association of a protein in the membrane might, therefore, be expected to perturb regular structures within the protein. Secondary structure predictions of regions of the IutA protein surrounding the insertion mutations encoded by the mutant plasmids pLG1003, pLG1004, pLG1005 and pLG1006 were made using the algorithms of Chou and Fasman (1978) and Garnier *et al.* (1978) in an attempt to correlate the effect of the mutations on protein structure with their phenotypic effect (i.e. reduction in the amount of receptor protein recovered in the Triton-insoluble outer membrane fraction of cells harbouring the mutant plasmids). The predictive programs must obviously only be taken as being suggestive of various structures existing at particular regions within a protein as they do not always correlate particularly well even with each other. Both programs were originally devised to predict the secondary structure of soluble globular proteins and thus their applicability to integral membrane proteins is questionable. On the other hand, it has

been suggested that such structure predictions are in fact more reliable for membrane proteins than for soluble proteins (Jahnig, 1990). Of the algorithms used, that of Garnier *et al.* (1978) has been suggested as being most suitable for proteins of the outer membrane (Nau and Konisky, 1989); for example, it predicted that the porins had essentially no α–helix, which was in accordance with what has been experimentally determined (Rosenbusch, 1974).

The mutation encoded by plasmid pLG1003 is predicted to have an α-helical region immediately adjacent to (Garnier-Osguthorp-Robson, GOR) or spanning (Chou-Fasman, CF) the position of insertion. Such an α-helical region might be expected to be a membrane spanning domain, although it is not long enough according to either prediction to entirely span the membrane. In both cases, the α-helix is shortened by 2 amino acid residues by the insertion. Paradoxically, the insertion mutation in pLG1004, which is just 2 codons downstream of that in pLG1003, appears to have a less drastic effect on the α-helical domain predicted by GOR, reducing its length by only a single residue despite the fact that the phenotypic lesion associated with pLG1004 is more pronounced than that of the protein encoded by plasmid pLG1003.

Secondary structure predictions of the protein encoded by plasmid pLG1005 predict essentially no change (GOR) or the interruption of a region of β-strand (CF). It is unlikely that a region of non-regular structure such as that predicted using GOR could exist within the membrane; it is therefore more likely that the CF prediction of a region of β-strand is correct. Thus the mutation in this protein might result in instability due to the disruption of this predicted structure. Predictions using the two methods are again at variance when used to predict the secondary structure of the region that is mutated in plasmid pLG1006. In this case a regular β-strand is actually created by the mutation according to the GOR prediction, whereas the CF

method predicts the introduction of two extra potential reverse turns preceding a β-sheet structure. As such no conclusions can be drawn as to the structural lesion caused by this mutation.

Plasmid pLG1001 encodes a protein with a two amino acid insertion within the signal peptide. Signal sequences of both prokaryotic and eukaryotic origin, while exibiting a low degree of sequence homology, do exibit an overall similarity in terms of their physical properties (von Heijne, 1983). The signal peptide can be sub-divided into a positively charged N-terminal region (n-region), a central hydrophobic region (h-region), and a more polar C-terminal region (c-region). The c-region in bacteria is usually 6 residues long and contains small hydrophobic residues at positions -1 and -3. The signal sequence of the IutA protein conforms to this typical pattern, with alanine occupying both of these positions. The residue at position -2 is usually a large charged residue, and the c-region usually has a net negative charge. The c-region of the IutA protein, by contrast, has a valine residue at the -2 position and has no net charge. The central h-region is usually in α-helical conformation (Stader *et al.*, 1986) yet both GOR and CF methods predict β-sheet in this region of the IutA signal sequence. There is a predicted α-helix spanning the cleavage site according to both algorithms, which, in pLG1001 is shifted further into the mature protein according to the CF algorithm, and totally abolished according to the GOR algorithm. Presumably this is not important in processing, since the protein encoded by plasmid pLG1001 appears to be processed at least as efficiently as the wild type protein. Indeed, the mutant protein may be processed more efficiently than the wild type protein because the rate of aerobactin translocation in cells harbouring pLG1001 is greater than that of the wild type. This may be because, with the addition of an aspartic acid residue, the mutant actually conforms more closely to the consensus pattern of signal peptides in that, unlike the wild type, it has a net negative charge of 1 on the c-region.

The reduction in the rate of aerobactin translocation by cells harbouring plasmids pLG1003, pLG1004 and pLG1006 in comparison to that of cells expressing the wild type protein can probably be explained in terms of the amount of protein present in the outer membrane, as evidenced by the intensity of the bands on the SDS-PAGE profile of Triton-insoluble membrane proteins. Cells harbouring plasmid pLG1005, however, translocate aerobactin at levels almost comparable to wild type, even though the amount of protein present in the outer membrane preparation is very much less than in the membranes of cells expressing the wild type protein. This may possibly reflect insertion of the mutant protein into the membrane in an active form which is nontheless less stable (and less resistant to extraction with Triton X-100) than the wild type protein. As such levels of the mutant protein in live cells may be greater than levels reflected in the profile of the Triton-insoluble membrane protein preparation. Jackson *et al.* (1986) found that, although C-terminally truncated mutants of the FhuA polypeptide were isolated in the total envelope fraction, they were nontheless sarcosyl-soluble; these authors concluded that such proteins constituted "assembly or maturation" mutants which were localized to the outer membrane but in a sarkosyl-soluble form. Similarly, Halegoua and Inouye (1979) reported sarkosyl-soluble precursors of the outer membrane protein OmpA which were, nontheless, translocated to the outer membrane. As cells harbouring plasmids pLG1001-3 were sensitive to cloacin DF13 presumably the regions that have been mutated in these proteins are not directly involved in cloacin DF13 binding and translocation. The insensitivity of cells harbouring plasmids pLG1004 or pLG1006 may simply relect the low levels of the mutant proteins encoded by these plasmids that are stably assembled into the outer membrane. The protein encoded by plasmid pLG1005, however, as judged by the aerobactin translocation activity probably exists in the outer membrane of live cells at levels approaching those of the wild type protein. Thus, the insensitivity of cells

harbouring plasmid pLG1005 to cloacin DF13 suggests that the region mutated in this protein is involved in the binding and/or translocation of the bacteriocin. This is in agreement with data reported by Marolda *et al.* (1991) who correlated reduced sensitivity to cloacin DF13 confered by a cloned 76 kDa aerobactin receptor from an enteroinvasive *E. coli* with respect to the pColV-K30 encoded receptor with the C-terminal portion of the protein. Fusion proteins between the two homologous proteins showed sensitivities to cloacin DF13 very similar to the wild type protein from which their C-terminus was derived (Marolda *et al.*, 1991). Cloacin DF13 titration assays using outer membranes prepared from cells harbouring the mutant plasmids were generally in agreement with the results from the plate assays of cloacin sensitivity of cells harbouring the mutant proteins. Cells harbouring plasmid pLG1003, however, were sensitive to cloacin DF13 in a plate assay and yet no cloacin DF13 binding activity could be deteceted in membrane preparations prepared from these cells. Again this might reflect the presence of the mutant protein encoded by this plasmid in the outer membrane of living cells in a form which is not resistant to extraction with Triton X-100.

Cells harbouring plasmid pLG1002 translocate aerobactin, in the conditions of the assay, with an efficiency of almost 200% with respect to cells expressing the wild type protein. There does not, however, appear to be significantly more protein in the outer membrane protein profile, and the ability of membranes containing this mutant protein to titrate cloacin DF13 is comparable to that of membranes containing the wild type protein. Nau and Konisky (1989) have compared the primary amino acid sequences of 5 TonB-dependent outer membrane transport proteins (Cir, FepA, BtuB, FhuA and IutA) and aligned them according to the shuffling program of Lipman and Pearson (1985). It is notable that the position of the insertion within the protein encoded by pLG1002 occurs within a hexapeptide for which there is no equivalent region in the amino acid sequences of the other

four TonB-dependent proteins compared. In a study of the FhuA protein using two-codon insertion mutagenesis, Carmel *et al.* (1990) isolated an insertion mutation that introduced Ser-Ser after $FhuA_{321}$ which abolished ferrichrome uptake (as evidenced by the inability of cells expressing the mutant protein to grow on ferrichrome as a sole iron sourse) but did not affect any of the other phenotypes (outer membrane localization, binding of phages T5, T1, ϕ80, UC-1 or sensitivity to colicin M). This insersion occurs at a site within the FhuA amino acid sequence which is very close in the alignment of the 2 proteins by Nau and Konisky to the insertion site which occurs after $IutA_{278}$. It is tempting to speculate that this hexapeptide within IutA might thus constitute part of the aerobactin binding site of the IutA receptor and that the region identified by the insertion of ser-ser after $FhuA_{321}$ is similarly part of the ferrichrome binding site. Predictive analysis suggests that the insertion has little effect on the secondary structure, hydrophilicity or surface probability. However, a very marked local increase in predicted chain flexibility of this region is observed which may account for the increased rate of aerobactin translocation by this protein. Interestingly, the equivalent region of a highly homologous chromosomally-encoded aerobactin receptor protein of a *Shigella flexneri* serotype 6 strain contains a non-conservative replacement of aspartic acid for Ala_{279} within this region as deduced from the nucleotide sequence (Susi Iravati Kuswandi, personal comunication). This replacement causes a predicted increase in chain flexibility similar to that seen in the insertion mutation. Cells containing a pUC19-derived plasmid containing a 7.0 kb *BamHI-Hind*III fragment containing this gene translocate aerobactin with almost 400% efficiency with respect to cells harbouring a similar construction encoding the aerobactin receptor of plasmid pColV-K30. Although this is further evidence suggestive of an important role for this region in aerobactin translocation, differences in promotor strength, efficiency of ribosome binding, translation or processing of the two proteins,

or functional differences within other regions of the *Shigella* protein can not be ruled out. To determine with any certainty the role of this region in binding and/or translocation of aerobactin it will be necessary to study the activity of a panel of mutant proteins containing single amino acid replacements within this region, which could be generated using oligonucleotide-directed mutagenesis techniques.

Chapter 6

General Discussion

The possession of the aerobactin-mediated iron uptake system is an important virulence determinant for many enteric bacteria. It allows bacteria to obtain sufficient iron to meet their requirements *in vivo* where the availability of this essential trace element is restricted by the host iron-binding proteins. The aim of this project was to further investigate the mechanisms involved in the uptake of aerobactin by *E. coli*. Furthermore, the prospect of utilizing the aerobactin receptor as a protective antigen was investigated.

The enteric bacteria, in common with other Gram-negative species, are surrounded by a highly assymetric outer membrane (OM) in addition to the cytoplasmic membrane (Nikaido and Vaara, 1985; Sukupolvi and Vaara, 1989). The OM serves as a barrier to hydrolytic enzymes, bile salts and other noxious agents that may be present in the intestinal tract, so protecting the cytoplasmic membrane from the action of lipases, bile salts and detergents, the peptidoglycan from the action of lysozyme, and proteins of the cytoplasmic membrane and periplasm from protease activity. The enteric bacteria are also very resistant to certain antibiotics, especially hydrophobic compounds, because of the impermeability of the OM (Nikaido and Vaara, 1985; Sukupolvi and Vaara, 1989). In addition, bacterial components which are contained within the OM are not accessible to antibodies.

As with other biological membranes the basic matrix of the OM is a lipid bilayer in which a number of proteins are embedded. However, the bilayer of the OM is highly unusual in that, although the inner leaflet is composed mainly of phospholipid as it is in other membranes, the outer leaflet contains predominantly lipopolysaccharide (LPS) molecules which

are not found in other membranes (Nikaido and Nakae, 1979). In some Gram-negative bacteria, such as *Neisseria* species, patches of phospholipid in the outer leaflet of the OM are thought to form a hydrophobic pathway through which hydrophobic compounds can diffuse (Lugtenberg and van Alphen, 1983). In the enteric bacteria, on the other hand, LPS molecules are thought to occupy the outer leaflet of the OM in normal conditions to the total exclusion of phospholipid (Lugtenberg and van Alphen, 1983). This has important implications in terms of the permeability of the OM towards hydrophobic compounds. LPS is extremely refractory to such molecules, so that hydrophobic compounds are largly excluded from enteric bacteria. This explains the high resistance of these bacteria to hydrophobic antibiotics (Sukupolvi and Vaara, 1989). The low permeability of LPS to hydrophobic compounds is thought to be due to the relatively low degree of lateral mobility of LPS molecules in the bilayer. Negatively charged groups on LPS molecules are neutralized by divalent cations such as Ca^{2+} or Mg^{2+} which produce salt bidges between adjacent molecules and thus restrict lateral movement (Sukupolvi and Vaara, 1989).

The proteins of the OM are rather limited in number and the protein profile is dominated by very large amounts of a small number of protein species (Osborn and Wu, 1980). In *E. coli* these major OM proteins are murein lipoprotein and OmpA, structural proteins which are thought to have a role in anchoring the OM to the underlying peptidoglycan layer (Sukupolvi and Vaara, 1989), and the proteins OmpF and OmpC, which serve as small water filled pores (Benz, 1988). In addition to these major proteins a number of minor proteins of the OM exist, some of which may be expressed to levels approaching those of the major OM proteins under certain conditions (Konisky, 1979; Neilands, 1982). The family of proteins which serve as receptors for aerobactin in certain enteric bacteria are such proteins, present at low levels in conditions of sufficient iron, but

derepressed to much higher levels in conditions of iron limitation (Bindereif *et al.*, 1982; Grewal *et al.*, 1982).

Aerobactin receptor proteins from different species of enteric bacteria may show polymorphisms and a range of different sizes as determined by SDS-PAGE. Proteins with an apparent molecular weight of 85 kDa, for example have been reported in strains of *Enterobacter cloacae* (Krone *et al.*, 1985b), while proteins with an apparent molecular weight of 76 kDa have been reported in strains of *Klebsiella edwardsii* (Krone *et al.*, 1985b), *Klebsiella pneumoniae* (Williams et al., 1989), *Shigella flexneri* (Derbyshire *et al.*, 1989) and enteroinvasive *E. coli* (Marolda et al., 1991). In most cases the reason for the size heterogeneity has not been studied at the molecular level. Marolda *et al.* (1991) showed by restiction mapping that the differences were confined to the C-terminal half of the protein encoded by the chromosome of an enteroinvasive *E. coli* strain. A chromosomally encoded aerobactin receptor gene cloned from a strain of *Shigella flexneri* serotype 6 has recently been sequenced and shown to be virtually identical to the ColV-K30 receptor gene sequence (Suzi Iravati Kuswandi, personal comunication). Interestingly, however, two suppressed frame shifts occur within this gene resulting in two short streaches of altered amino acids before the reading frame is restored. In one case a single nucleotide insertion is supressed by a single nucleotide deletion further downstream and, in the other case, a single nucleotide insertion is supressed by an insertion of two nucleotides further downstream. This results in a mature protein which is one amino acid longer and with a calculated molecular weight some 500 Da larger than the ColV-K30 encoded protein. Although this does not account for the full difference in apparent molecular weight as determined by SDS-PAGE analysis between the two proteins this is not perhaps surprising since both proteins migrate anomalously fast, possible reflecting resistance to complete denaturation by boiling in SDS.

Unlike proteins of other membranes, proteins of the OM do not generally have stretches of hydrophobic amino acids in α-helical conformation which may be expected to form membrane spanning domains. Structural information for correct insertion and stable association with the OM is in some cases contained in residues which may be located throughout most of the length of the polypeptide chain. Work described in chapter 5 has shown this to be the case for the aerobactin receptor. N-terminal fusions of the aerobactin receptor to alkaline phosphatase containing as much as 87 % of the wild type residues are not assembled into the OM, and 4 two-amino acid insertion mutations at various positions within the polypeptide chain caused reduced association of the mutant proteins with the OM compared to the wild type protein. This technique also identified regions of the polypeptide chain that are important for binding and translocation of the ligands which utilize this receptor.

The possession of an OM is clearly an advantage to bacteria in that it protects them from a variety of harmful compounds. However it does present them with a problem in that they have to obtain nutrients from the external milieu across a highly impermeable lipid bilayer. Proteins present in the OM allow these bacteria to overcome this problem in a number of ways depending on the type of nutrient.

Most small hydrophilic compounds cross the OM by simple diffusion through non-specific pores produced by trimeric proteins called porins (Benz, 1988). In *E. coli* the two major porins are OmpF and OmpC which are inversely regulated in response to changes in osmolarity (see chapter 4). These pores are relatively non-selective and any hydrophilic molecule with a molecular weight of less than about 600 are able to pass relatively freely (Decad and Nikaido, 1976; Payne and Gilvarg, 1968), although the charge, hydrophobicity and shape of a molecule will also effect on its ability to pass through the porin channels (Nikaido and Vaara, 1985). Diffusion through

the OM via the porins is entirely concentration-dependent and therefore requires a relatively high concentration of the nutrient in the external milieu.

Some larger molecules are able to cross the OM via specific channels which facilitate their diffusion. An example of this is the channel formed by the *E. coli* LamB protein which provides a specific route for the transport of maltose and maltodextrins (Wandersman *et al.*, 1979). Nucleosides are thought to be similarly transported by the Tsx protein in *E. coli* (Hantke, 1976; McKeown *et al.*, 1976). As with non-specific diffusion through porins, facilitated diffusion across the OM is driven only by a concentration gradient across the OM, thus requiring a relatively high concentration of the nutrients to be transported in the external milieu.

Some essential nutrients exceed the maximum molecular weight for transport via the porins, and in addition, are usually encountered only at low concentrations in the environment. The siderophore iron chelates and vitamin B12 come into this category. Ferric dicitrate and aerobactin are just within the molecular weight range which would allow transport through the porins, but as the molecular weight of a compound approaches maximum the rate of diffusion is greatly reduced (Nikaido and Rosenberg, 1981; 1983). Thus, because aerobactin and ferric dicitrate are both likely to be present at very low concentrations, the rate of diffusion would probably not be sufficient to meet the needs of the cell.

The enteric bacteria have solved this problem by evolving active transport machineries for such compounds mediated by specific OM receptor proteins with a high affinity for the ligands to be transported (Braun and Hantke, 1981; Neilands, 1982). The OM, however, is separated from cytoplasmic ATP, and the only electrical potential that can exist across the OM is the Donnan potential, as the presence of the porins will allow free

diffusion of protons and other small ions. The OM does not, therefore, have obvious access to the usual sources of energy to drive active transport processes. This problem has been overcome by coupling the membrane potential of the cytoplasmic membrane to the OM transporter proteins via the cytoplasmic membrane protein TonB and functionally related proteins (Postle, 1990b). Active transport across the inner membrane is also an active process, in this case requiring ATP (Reynolds *et al.*, 1980; Matzanke *et al.*, 1986). Work described in chapter 3 shows that the active uptake of aerobactin across the two membranes occurs by processes that are independent of one another. Furthermore, unlike transport of ferric dicitrate, aerobactin may be translocated into the cytoplasm as an intact chelate. In the presence of high levels of endogenous aerobactin, however, the intact chelate may only need to penetrate the OM, the iron being released (by an unknown mechanism) within the envelope and transported across the cytoplasmic membrane, and the apo-aerobactin remaining in the periplasm from where it can presumably be rapidly recycled.

Although the OM protects enteric bacteria from many harmful external molecules, some agents take advantage of the OM receptors elaborated by the bacteria to gain entry to the periplasm and beyond. Many bacteriocins, for example, translocate an active portion across the OM after first binding to a specific receptor protein (Konisky, 1982). The mechanism of translocation is not, however, the same as that of the physiological ligand. Aerobactin, for example, is translocated across the OM via the IutA protein in a TonB-dependent process. Sensitivity to cloacin DF13, a bacteriocin which exploits the aerobactin receptor, does not, however, require TonB-related functions (Schramm et al., 1987). Work described in chapter 4 demonstrates that penetration of the OM by cloacin DF13 requires the porin protein OmpF, as do a number of colicins of the BtuB group (Benedetti *et al.*, 1989). OmpF acts at a stage subsequent to initial binding of cloacin

DF13 to IutA, and is thus assumed to be involved in translocation of the bacteriocin across the OM. Aerobactin translocation, on the other hand, is independent of the presence of OmpF.

In addition to providing binding sites for bacteriocins, proteins of the OM also provide targets for specific antibodies. As the OM proteins have very specific functions, such as siderophore binding, their structures are expected to be constrained by functional requirements. As expected, therefore, functionally equivalent OM proteins of different strains and species of enteric bacteria show a considerable degree of homology (Chart and Griffiths, 1985; Henrikson and Maeland, 1987; Hofstra and Dankert, 1979). A consequence of this is that antibodies are likely to be present in the blood and secretions of animals that have been exposed to one strain of enteric bacteria which will cross-react with OM proteins of other enteric bacteria. Indeed normal human serum has been shown to contain antibodies to the enterochelin receptor and to OmpA (Griffiths *et al.*, 1985), both of which are highly conserved between enteric species (Chart and Griffiths, 1985; Hofstra and Dankert, 1979). Work described in chapter 2 shows that wild type strains of *E. coli* are able to shield the aerobactin receptor, as well as other OM proteins, with the long oligosaccharide side chains of their LPS molecules. While these molecules are themselves antigenic, they are not highly conserved; at least 160 O-serogroups of *E. coli* exist (Orskov *et al.*, 1977). Thus, only antibodies against a homologous O-serotype will recognize these outermost molecules of the OM, and the highly conserved OM receptor proteins will be protected from interaction with specific immunoglobulin.

To summarize, the OM acts as a selective barrier preventing access of deleterious agents to the cell surface at two levels. First hydrophobic compounds and hydrophilic compounds above a critical molecular weight are prevented from penetrating the OM and gaining access to the periplasm, cytoplasmic membrane and cytoplasm. Certain specific molecules required

by the cell, however, are allowed to bypass this generally impermeable membrane via active transport processes involving specific OM protein receptors. Second the OM proteins themselves are protected from immunoglobulins, bacteriocins, etc. by the oligosaccharide side chains present on the LPS molecules. The uptake of aerobactin by *E. coli* exemplifies the selective permeability of the OM. It is a hydrophilic molcule which is likely to be present in low concentrations *in vivo*. As such its diffusion through the non-specific porin chanels would be at most negligible. It therefore requires active transport across the OM via a specific protein receptor. This receptor protein is protected from specific immunoglobulin and cloacin DF13 by a second permeability barrier. Oligosaccharide side chains of smooth LPS found in wild type *E. coli* isolates sterically hinder access to the receptor.

Future work is needed to further relate the primary structure of the aerobactin receptor to its biological activities. Regions identified as being important in aerobactin binding and translocation, cloacin DF13 binding and translocation, and stable association with the outer membrane could be further investigated by studying the properties of mutant proteins created by oligonucleotide-directed mutagenesis. This technique could be used, for example, to create specific mutations within the region identified by hexameric linker insertion mutagenesis as being involved in the binding and/or translocation of aerobactin. Cloning and sequencing of homologous proteins from other enteric species, particularly those which are more distantly related to the ColV-K30-encoded receptor, might also be useful in identifying highly conserved motifs which might be suggestive of functionally imposed constraints on divergence. Such regions, if found, could then be subjected to *in vitro* mutagenesis to establish their functional role.

Uptake of exogenous aerobactin, but not the iron that it carried, was shown to be apparently inhibited by the presence of endogenous aerobactin.

However it may be that these results were influenced by the use of α,α'-dipyridyl as an inducer of iron restriction. It is known that this chelator of ferrous iron is toxic to *E. coli* (Neilands, 1982) and can effect the differential expression of iron regulated membrane proteins (Chart *et al.*, 1986). In addition iron responsive regulation of TonB has been demonstrated using ferric iron chelators to restrict the availability of of iron but not when dipyridyl is used (Postle, 1990b). This possibility could be investigated by using ferric iron chelators such as transferrin or desferral.

The molecular basis of the differential uptake of aerobactin-derived iron and aerobactin across the cytoplasmic membrane of aerobactin producing cells also needs to be investigated. This phenomenon may have important implications for the design of antibacterial drugs based on the structure of aerobactin. Catechol-substituted antibiotic compounds have been shown to "hijack" TonB-dependent iron transport pathways (Curtis *et al.*, 1988; Nikaido and Rosenberg, 1990). If such a rationale was adopted to utilize the aerobactin transport system to deliver bacteriocidal aerobactin analogues, the site of action within the bacterium would have to be taken into consideration since little uptake beyond the periplasm might be expected in cells producing aerobactin.

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