Investigations on virulence determinants in *Escherichia coli* and *Neisseria* species

PhD Thesis

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Summary

Escherichia coli is the most common cause of extraintestinal infections such as uroinfection (cystitis, pyelonephritis), and neonatal meningitis. Paradoxically it is also the predominant facultative member of the normal intestinal flora. Pathogenic *E. coli* and commensal *E. coli* typically differ from each other with respect to virulence attributes and they also belong to distinct clones. The definition of virulent clones is traditionally based on O:K:H serogroups, the possession of specific virulence factors, including for example pili that mediate adherence to the host cells, resistance to the bactericidal activity of human serum, production of haemolysin and cytotoxic necrotising factor, presence of chromosomal aerobactin and increasing amounts of K capsular antigen.

In this thesis in chapter 3, we proved that different type (type I, P, S) fimbriae, which play role in colonisation, bind to subepithelial connective tissue protein fibronectin, type I collagen and laminin (called extracellular matrix protein). The fimbriae might contribute to tissue invasion of bacteria. Fimbriated strains were more virulent in nephropathogenicity assay than non-fimbriated mutant strains.

The cytotoxic alpha-haemolysin is able to increase virulence of bacteria causing damage to RBC and PMNLs. In chapter 4, we proved that the host infected with haemolytic *E. coli* produced antibodies against haemolysin. The effect of alpha-haemolysin was inhibited by these antibodies. These sera also protected the mice from lung toxic effect of haemolytic *E. coli* and haemolytic *Proteus* strains. We found only one haemolytic *E. coli* strain (origin from mouse and haemolysin encoded on plasmid) which failed to evoke lung toxic effect. Serum produced against this strain did not protect from the lung toxicity of other haemolytic *E. coli* and *Proteus* strains.

Some genes encoding tRNAs represent the target structure for certain phages or plasmids, which integrate into the chromosome. The "pathogenicity islands (Pais)" are also associated with tRNA specific loci as flanking sequences.

Genes encoding tRNAs may mediate "global regulators". In chapter 5, we demonstrated the key role of genes encoding *leuX* and *selC* specific tRNA in virulence. We also proved that in uropathogenic *E. coli* 536 strain these genes, which are associated to Pais, influence production of type 1 fimbriae, flagella, enterobactin and serum resistance, which are necessary for full *in vivo* virulence.

The RecA protein plays role in the so-called SOS response of bacteria of DNA damage and induces also the conversion from lysogenic to lytic phage replication. It has previously been shown that the RecA protein has an influence on virulence of *Salmonella, Shigella*, and enteroinvasive *E. coli* (EIEC). In chapter 6, we demonstrated the effect of the deletion of *recA* gene from enterohaemorrhagic *E. coli* (EHEC) on the *in vivo* virulence. The *recA* mutants of EHEC strains 933 and 8624 have lost their virulence potencial in contrast to UPEC strain 536 in which *recA* mutation did not affect the virulence in intravenous lethality and lung toxicity assay.

Commensal *Neisseria* specieses also belong to normal flora. *Neisseria meningitidis* that is a pathogenic member of *Neisseriae* may also occur in normal flora without causing infection and disease. However, *N. meningitidis* sometimes causes fatal disease, in contrast to commensal *Neisseria,* which is rare in serious diseases. In chapter 7, we demonstrated different virulence factors (pili, porA - class 1 outer membrane protein, and IgA protease) in commensal *Neisseria* strains. We proved that there is a relationship between meningococcal pili and pilus types produced by commensal *Neisseria*. These data suggest that interspecies transfer of genes occurs and sequences are acquired through transformation of *Neisseria* from other *Neisseria* species.

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Abbreviations

ADP	adenosine diphosphate
Ag	antigen
AHCH	Alder Hey Children's Hospital
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHI	brain-heart infusion broth
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CAS	Chrome Asurol S
CFA	colonisation factor antigen
CFLP	Carworth Farm Lane Petter
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster ovary cells
Chr	chromosome
CLDT	cytolethal distending toxin
CNF	cytotoxic necrotising factor
Da	dalton
DAEC	diffusely adhering *E. coli*
2,3-DHBA	2,3-dihydroxybensoic acid
2,3-DHBS	2,3-dihydroxy-N-bensoylserine
DIG	digoxigenen
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
EAggEC	enteroaggregative *E. coli*
EAST1	enteroaggregative *E. coli* heat-stable enterotoxin 1
ECM	exracellular matrix
ED	effective dosis
EDTA	ethylenediamine tetra acetic acid
EHEC	enterohaemorrhagic *E. coli*
Ehly	enterohaemolysin
Ehx	EHEC toxin
EIEC	enteroinvasive *E. coli*
EIET	EIEC enterotoxin
EM	electron microscopy
EMB	eosin methylene blue agar
ENS	enteric nervous system
EPEC	enteropathogenic *E. coli*

ETEC	enterotoxigenic *E. coli*
Fab	antigen binding fragment
Fim	type 1 fimbriae
Gal	galactose
GalNAc	N-acetylgalactosamine
Gb_3	globotriaosylceramid Galα(1-4)Galβ(1-4) Glcβ1-1ceramide
Gb_4	globotetraosylceramide Gal NAcβ(1-3)Galα(1-4)Galβ(1-4) Glcβ1-1ceramide
GC-C	guanylate cyclase C
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1--1ceramide
GlcNac	N-acetylglucosamine
Glc	glucose
Gly	glycine
GM1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	haemagglutination
H and E	haematoxylin and eosin
HDTMA	hexadecyl-trimethyl-ammonium bromide
HEp-2	human epidermoid carcinoma
Hly	haemolysin
HRMEC	human renal microvascular endothelial cells
HUS	haemolytic uremic syndrome
IgA	Immunglobuline A
kDa	kilodalton
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LD	lethal dosis
LT	heat-labile enterotoxin
LPS	lipopolysaccharide
M9	Minimal 9
MDa	megadalton
MRHA	mannose-resistant haemagglutination
MSHA	mannose-sensitive haemagglutination
NA	nutrient agar
NAD	nicotinamide adenine dinucleotid
NB	nutrient broth
NBT	Nitro blue Tetrasolium
NeuNAc	N-acetylneuramic acid
PAA	particle agglutination assay
Pai	pathogenicity island
PAS	periodic acid Schiff's stain
PBS	phosphate-buffered saline
PBST	PBS Tween 20

PCR	polymerase chain reaction
PGE	prostaglandin E
PFGE	pulsed field gel electrophoresis
PHLS	Public Health Laboratory Services
Pil	pilin
Pl	plasmid
PMNL	polymorphonuclear leukocytes
PorA	porin A
Prf	P-related fimbriae
RBC	red blood cell
RecA	general recombination and DNA repair protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
RTX	repeat in toxin
SDS	sodium dodecyl sulphate
Sfa	S fimbrial adhesin
SLT	Shiga-like toxin
ShET2	Shigella enterotoxin
SPF	specific pathogen-free
s*s	conserved cysteine residues / regions
SSC	sodium chloride, trisodium citrate
ST	heat-stable enterotoxin
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin
Sv	semivariable
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA buffer
T-H	Tamm-Horsfall
TSA	tryptone soya agar
TSB	tryptone soya broth
Tris	Tris-(hydroxymethyl)-methylamine
UPEC	uropathogenic *E. coli*
UV	ultraviolet
VR	variable region
VT	Vero cell cytotoxin
VTEC	Vero cell cytotoxin producing *E. coli*

Contents

Chapter 1.
Introduction

1.1. General introduction of *Escherichia coli*

Theodor Escherich described first the organism as *Bacterium coli* now known *Escherichia coli* in the faeces of a normal infant in 1885 (Escherich T 1885, Escherich, T. 1889). In 1918 Aldo Castellani and Albert J. Chalmers classified first the microorganism as *Escherichia coli*, which fermented glucose and lactose producing acids and gas (Castellani, A. and A.J. Chalmers 1920).

E. coli is the predominant nonpathogenic facultative anaerobe member of the human intestinal flora. The organism typically colonises the infant gastrointestinal tract within hours of life and thereafter *E. coli* and the host derive mutual benefit (Drasar, B.S. and M.J. Hill 1974). *E. coli* becomes member of the commensal flora of human and animal intestine and usually remains harmlessly confined to the intestinal lumen, however, in the debilitated or immunsupressed host, or when gastrointestinal barriers are violated even normal "nonpathogenic" *E. coli* can cause infection. Some *E. coli* strains have developed the ability to cause disease of the gastrointestinal, urinary or central nervous system in even the most robust human hosts. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body.

Three general clinical syndromes result from infection with pathogenic *E. coli* strains: I. urinary tract infection; II. sepsis/meningitis; and III. enteric/diarrheal disease.

Diarrheagenic strains of *E. coli* can be divided into at least six different categories with corresponding distinct pathogenic schemes (Levine, M.M. 1987). There was no way of distinguishing avirulent strains from those capable of causing diarrhoea until 1933, when Goldschmidt used the "serological typing" to study the epidemiology of infantile gastroenteritis in institutions (Goldschmidt, R. 1933). Kauffmann laid the foundation of serological typing of *E. coli* in 1944 (Kauffmann, F. 1944, Kauffmann, F. 1947). His scheme for the serological classification of *E. coli* is still used in modified form today. *E. coli* strains were serotyped on the basis of their three types O (somatic), H (flagellar) and K (capsular) surface antigens, that were based solely on bacterial agglutination

results in absorbed and nonabsorbed rabbit antisera, and on the influence of heating of bacteria on agglutination and immunogenicity. Twenty-five O antigens, 55 K antigens, and 19 H antigens were included in the Kaufmann-Knipschildt-Vahlne antigenic scheme in 1966. These numbers have subsequently increased to 173 O antigens, 80 K antigens, and 56 H antigens (Ørskov, F. and I. Ørskov 1984, Ørskov, I. *et al.*, 1991). The serotyping techniques for determination of O and H antigens are still based on bacterial agglutination. The serological specificity of the O antigens is based on the complex structure of the polysaccharide side chain of the lipopolysaccharide (LPS). Many *E. coli* are motile and may be determined by their H antigens. The presence of K antigens was determined originally by means of bacterial agglutination tests: an *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture was heated was considered to have a K antigen. Several different molecular structures, including fimbriae belonged to K antigens. Now they include only acidic polysaccharides. Proteinaceous fimbrial antigens have been removed from the K series and have been given F designation. The antigens gave rise to the O:H system of classification, which has played an important role in the epidemiology and pathogenesis of *E. coli* infection.

Identification of pathogenic *E. coli* strains requires that these microorganisms be differentiated from non-pathogenic members of normal flora. Serotypic markers correlate, sometimes very closely, with specific categories of diarrheagenic *E. coli,* however, these markers are rarely sufficient in and of themselves to reliably identify a strain as diarrheagenic. An exception is the O157:H7 serotype that serves as a marker for virulent enterohaemorrhagic *E. coli* strains. Limited sensitivity and specificity of serotyping is tedious and expensive and is performed reliably only by a small number of reference laboratories. Thus, detection of diarrheagenic *E. coli* has focused increasingly on the identification of characteristics which themselves determine the virulence of these organisms (Nataro, J.P. and J.B. Kaper 1998). This may include *in vitro* phenotypic assays, which correlate with the presence of specific virulence traits.

Development of molecular diagnostic methods gave new most reliable techniques for differentiating diarrheagenic strains from non-pathogenic members of the stool flora and determining virulence of pathogenic *E. coli*.

1.2. *E. coli* virulence markers

E. coli, like most pathogens follow the steps of infection: 1. colonisation of a mucosal site, 2. evasion of host defences, 3. multiplication, and 4. host damage. The most conserved feature of pathogenic *E. coli* strains is their ability to colonise the mucosal surface. Certain surface adhesins play significant role in the colonisation of mucosal surfaces.

1.2.1. Adhesins and colonisation factors

The adhesive properties of *E. coli* were first recognised by Guyot in 1908. He observed that some strains possessed the ability to agglutinate red blood cells (RBC) from a number of animal species and humans (Guyot, G. 1908). In the classic studies of Duguid *et al.*, (Duguid, J.P. *et al.*, 1955) the haemagglutination (HA) was recognised as an adhesive property of *E. coli*, which in number of instances correlated with the presence of fimbriae observed under the electron microscope. The adhesive affinity of *E. coli* for many cell types other than RBC has since been recognised, indicating the potential role of adhesins as colonisation factors. The term adhesin is defined as a microbial surface component that mediates specific attachment to an eucaryotic cell membrane. In recent years many of them was recognised.

Most pathogenic *E. coli* strains produce fimbrial adhesins, which are easily identified by electron microscope. Their binding capacity can be determined by haemagglutination. However, certain adhesins are devoid of any detectable haemagglutination activity but mediate adherence to eucaryotic cells.

E. coli adhesins may be classified and identified according to morphology, function or antigenic properties. A large number of fimbrial antigens have been

characterised, although the fimbriae of some strains have yet to be identified. The fimbrial adhesins may form morphologically stable rod like structures up to 2 μm in length and 7 nm in diameter (Hacker, J. 1992). Furthermore, fimbriae with a more flexible morphology exist, which form fibrilla-like pattern (de Graaf, K. 1990). Some fimbriae (CFA/III and Longus) are flexible and frequently seen folded back on themselves in bundles (Knutton, S. *et al.*, 1989). Some *E. coli* strains express fibrous surface proteins called curli. Similar surface organelles designated thin aggregative fimbriae were also found in *Salmonella enteritidis* (Collison, S.K. *et al.*, 1992, Collison, S.K. *et al.*, 1991, Olsen, A. *et al.*, 1993). A prominent property of curli polymers is their ability to specifically interact with numerous human proteins such as the matrix proteins fibronectin and laminin and proteins of the fibrinolytic and contact-phase systems. This ability should facilitate the adaptation of curli-expressing bacteria to different niches in the infected host.

The pathogenic *E. coli* produce adhesive proteins which are able to bind to erythrocytes or other eucaryotic cells but are devoid of any fimbrial structures (Duguid, J.P. *et al.*, 1979). These adherence factors are termed non-fimbrial adhesins or A-fimbrial adhesins.

By bacterial adherence to host epithelial cells pathogenic *E. coli* strains avoid being swept along by the normal flow of body fluids (urine, intestinal contents, and blood) and eliminated. Attachment is considered a necessary first step in the colonisation of host mucosal surfaces and a precedent to invasive infection in many situations (Johnson, J.R. 1991). Once colonisation has been established, a separate range of virulence factors determines whether the organisms can invade the host and survive. Most of the evidence for the function of these factors is circumstantial but it is persuasive, particularly because it appears that they act additively (Johnson, J.R. 1997). The adhesins were summarised in Table 1.

Table.1.

Fimbrial adhesins	F type	MRHA	MSHA	Receptor	Morphology	Genetic location	*E. coli* subtype
Type I	F1A		+	α-D-mannozides laminin,fibronectin	stable rod-like	Chr.	Pathogenic and Non-pathogenic
CFA I	F2	+		sialic acid	stable rod-like	Pl.	ETEC human
CS1 (CFAII)		+		unknown	stable rod-like	Pl.	ETEC human
CS2 (CFAII)		+		Asialo GM	stable rod-like	Chr.	ETEC human
CS3 (CFAII)	F3	+		GalNac-β1-4Gal	flexible fibrillar	Pl.	ETEC human
CS4 (CFAIV)		+			stable rod-like	Pl.	ETEC human
CS5 (CFAIV)		+			stable rod-like	Pl.	ETEC human
CS6 (CFAIV)		-		unknown	non-fimbrial	Pl.	ETEC human
CS8 (CFAIII)					bundle forming rod-like		ETEC human
CS10 2230					non-fimbrial		ETEC human
CS11 PCFO148					flexible fibrillar		ETEC human
CS12 PCFO159					stable rod-like		ETEC human
CS13 PCFO9					flexible fibrillar		ETEC human
CS14 PCFO166					stable rod-like		ETEC human
CS15 8786					non-fimbrial		ETEC human
CS17					stable rod-like		ETEC human
CS18 PCFO20					stable rod-like		ETEC human
CS19					stable rod-like		ETEC human
CS20					stable rod-like		ETEC human
CS21 Longus					bundle forming		ETEC human
K88	F4			Contains Galα(1-3)Gal	flexible fibrillar	Pl.	ETEC porcine
K99	F5			Neu-5Glcα(2-3)Lac (NeuGlcα3Galβ4Glcβ)	semiflexible	Pl.	ETEC porcine ETEC bovine
987P	F6			unknown	stable rod-like	Pl.	ETEC porcine
F41				N-acetylgalactosamine	stable rod-like		ETEC bovine ETEC ovine ETEC porcine
F17				N-acetylDglucosamin	stable rod-like		ETEC bovine ETEC porcine
F1845				D^r blood group Ag	stable rod-like	Chr.	EPEC human
EAF					stable rod-like	Pl.	EPEC human
AF/RI				unknown	stable rod-like	Pl.	EPEC rabbit
EHEC fimbriae					stable rod-like	Pl.	EHEC human
Curli				fibronectin, laminin	thin coiled fibrils	Chr.	EAggEC human
P (Pap)	F7-F16	+		αDGal(1-4)βDGal (P Group Ag)	stable rod-like	Chr.	UPEC human
Prs	F13			Forssman glycolipid GalNAcα(1-3)βGalNAc	stable rod-like	Chr.	UPEC human UPEC dog
G				N-acetylDglucosamin (G blood group)	stable rod-like	Chr.	UPEC human
D^r O75X				D^r blood group Ag	stable rod-like	Chr.	UPEC human
S (SfaI SfaII)		+		α-sialyl 2-3 βGal	stable rod-like	Chr.	UPEC human MENEC human
Type 1C F1C	-	-		unknown	stable rod-like	Chr.	UPEC human
F 165				Forssman glycolipid	stable rod-like	Chr.	Sepsis pigs
CS31A				unknown	stable rod-like	Chr.	Sepsis calves ETEC

1.2.2. Capsular polysaccharide (K antigen) and serum resistance

The capsules are important virulence determinants, which enable the bacteria to evade or counteract the unspecific host defence during the early phase of infection. Smith first showed the relation between capsular polysaccharides and *E. coli* invasiveness in 1927 and later Kaufmann also demonstrated this in 1974. Smith, who investigated strains from white scours in calves, showed that spontaneous uncapsulated mutants could be obtained from certain "colibacteria", and that such mutants were less virulent when injected intraperitoneally in guinea pigs (Smith, T. and G. Bryant 1927, Smith, T. 1927, Kaufmann, F. 1974). Kétyi *et al.* inoculated several rough strains with or without the K1 antigen directly into the bladder through the abdominal wall of suckling mice. However these strains were not isogenic, the encapsulated strains were more virulent (Kétyi, I. *et al.,* 1983).

Capsular polysaccharides of which *E. coli* has more than 80 types, are linear polymers of repeating carbohydrate subunits that sometimes also include a prominent amino acid or lipid component. Among human strains, a greater proportion of urinary than faecal *E. coli* isolates is encapsulated and is typeable with standard anti-K sera. Certain K types, including K1, K2, K3, K5, K12, K13, K20, and K51 are overrepresented among isolates from patients with cystitis and especially pyelonephritis in comparison with faecal strains (Kaijser, B. *et al.,* 1977, Ørskov, F. and I. Ørskov 1985, 1992, Westerlund, B. *et al.,* 1988).

The K1 polysaccharide capsular antigen is most frequently found on *E. coli* strains isolated from adults and neonates with bacteraemia and urinary infection. The prevalence of faecal carriage of K1 strains increases with age, climbing from 22 % of premature infants to 45 % of adult women (Sarff, L.D. *et al.,* 1975). The best known association of the K1 capsule with human disease is in neonatal meningitis, in which 79 % of *E. coli* isolates are positive for K1 antigen (Sarff, L.D. *et al.*, 1975, Robbins, J. *et al.*, 1974, Czirók, É. *et al.*, 1986). The reason for this association may be myriad and include the neonatal immune system's incomplete ability to localise and fight infection. The studies of infants with meningitis and animal models of meningitis have shown that high level of

bacteremia is required for the development of *E. coli* meningitis. Previous investigations have determined that the encapsulated or $K1^+$ strains are phagocytosed less well by polymorphonuclear leukocytes (PMNLs) than the uncapsulated strains. The degree of impairment of phagocytosis is proportional to the amount of polysaccharide (Howard, C.J. and A.A. Glynn 1971). Phagocytosis of K1 strains increases after disruption of capsular polysaccharide by heating. Capsular polysaccharide blocks opsonisation by interfering with complement deposition in a dose dependent mode. The anticomplementary effect of capsular polysaccharides may occur in part because cell surface polysialic acids (such as K1 polysaccharide) increase the binding of the inhibitor B1H to $C3_b$, thereby preventing the formation of C3 convertase and blocking activation of the complement cascade (Leying, H.S. *et al.*, 1990). The anticomplementary activity of capsular polysaccharide probably contributes to the increased survival in serum of some encapsulated strains. Capsular polysaccharides from pathogenic *E. coli* strains are also poor immunogens in animals and humans. This may be because of molecular mimicry, with the host immune system possibly "blind" to the K1 polysaccharide because of this compound's similarity to host structures (Silver, R.P. 1988). The K1 polysaccharide is a homopolymer of NeuNAc (sialic acid) units linked 2-8 and randomly acetylated at C-7 and C-9, which is structurally identical to the capsular polysaccharide of *Neisseria meningitidis* group B and related to human trisialogangliosides containing a NeuNAc-(2-8)-NeuNAc moiety (Johnson, J.R. 1991). These may contribute to the development of high level bacteremia.

1.2.3. Iron-sequestering mechanism (bacterial siderophores: aerobactin, enterochelin)

All living cells need iron (Weinberg, E.D. 1978). Bacteria compete with both the host and with each other for a variety of essential nutrients once they have arrived at their preferred niche. Iron is a critical nutrient whose availability to bacteria is restricted by high-affinity host iron-storage proteins, such as hemin,

ferritin, transferrin, and lactoferrin. As a result, bacterial pathogens have developed sophisticated mechanisms for iron acquisition within the host. Bacterial strategies for iron acquisition include removal of iron from host proteins by secreted bacterial iron chelators known as siderophores with subsequent bacterial receptor-mediated uptake of iron-siderophore complexes and secretion of haemolysins that can release iron from heme. Clinical situations associated with increased iron availability, such as haemochromatosis, chronic liver disease, transfusion therapy are associated with an elevated risk of *Yersinia enterocolitica*, *Vibrio vulnificius* and *E. coli* bacteremia (Cantinieaux, B. *et al.*, 1988, Bullen, J.J. *et al.*, 1991, Starks, A.M. *et al.*, 2000). As with other critical virulence-associated attributes, bacteria rely on multiple redundant systems for essential nutrient acquisition; thus some but not all mutant strains with single defects in iron uptake are avirulent (Relman, D.A. 1999).

E. coli uses iron for oxygen transport and storage, DNA synthesis, electron transport, and metabolism of peroxides (Bagg, A. and J.B. Neilands, 1987). As almost all of the iron is complexed with host iron proteins, several iron chelation systems are employed for iron acquisition. In *E. coli*, the hydroxamate siderophore aerobactin is the most effective system. Aerobactin is a small molecule (Mw 616) formed from the condensation of two lysine molecules and one citrate (Neilands, J.B. *et al.*, 1985). Following secretion by *E. coli* cells, aerobactin extracts Fe^{3+} from host iron-binding proteins and is taken up through a 74 kDa outer membrane receptor protein. Strains with the aerobactin system have a growth advantage in low-iron conditions, including in serum and dilute urine (Braun, V. *et al.*, 1984, Montgomerie, J.Z. *et al.*, 1984, Williams, P.H. 1979). The aerobactin system has many advantages over other siderophores. *E. coli* can use but does not synthesise ferrichrome and cannot transport enough citrate to support growth in low-iron conditions in the absence of other siderophores. Although enterobactin (enterochelin), the other major specialised siderophore of *E. coli*, has a higher affinity constant for iron than does aerobactin when deprotonated (de Lorenzo, V. and J.L. Martinez 1988, Williams, P.H. and N.H. Carbonetti 1986), enterobactin's affinity constant at neutral pH is much lower (Bagg, A. and J.B. Neilands 1987).

Enterobactin deferrates transferrin more rapidly than does aerobactin in aqueous solutions, however, the reverse is true in serum or in the presence of albumin, presumably, because enterobactin (but not aerobactin) binds to and is inactivated by proteins. Enterobactin is less soluble and less stable than aerobactin (Bagg, A and J.B. Neilands 1987). Release of iron from enterobactin requires hydrolysis of the siderophore, whereas aerobactin is continuously recycled without hydrolysis. In contrast to enterobactin, which leaves iron free in the cytosol, aerobactin delivers iron directly to bacterial iron centres. Neither enterobactin nor aerobactin is large enough alone to stimulate antibody production, but enterobactin (a catechol) binds to serum proteins in a haptenlike fashion, giving rise to antienterobactin antibodies that probably limit its usefulness *in vivo* (Bindereif, A. and J.B. Neilands 1985). Finally, aerobactin production is stimulated by milder degrees of iron deprivation than are to stimulate enterobactin production. Aerobactin production is regulated by the intracellular iron concentration through the *fur* (ferric uptake regulation) gene product (Bagg, A. and J.B. Neilands 1987, Bindereif, A. and J.B. Neilands 1983, Braun, V. and R. Burkhardt 1982, Valvano, M.A. *et al.*, 1988).

In *E. coli* strains the aerobactin system is encoded by a five genes operon. Four genes encode the enzymes needed for aerobactin synthesis and a fifth gene encodes the outer membrane receptor protein (Carbonetti, N.H. and P.H. Williams 1984). In contrast to aerobactin, the enterobactin is thought to require seven genes, designated *entA-G* for biosynthesis. The *entC*, *entB*, and *entA* gene products convert chorismic acid to 2,3-dihydroxybensoic acid (2,3-DHBA), and *entD-G* gene products synthesise enterobactin from 2,3-DHBA and L-serin. These gene products act as multienzyme complex and are membrane associated (Nahlik, M.S. *et al.*, 1987, Earhart, C.F. and F. Hantash 1995).

1.2.4. Protein toxins

Bacterial toxins harm host cells by a variety of mechanisms. These toxins (1) help bacteria spread in tissues, (2) lyse host cells, (3) block protein synthesis

and (4) act pharmacologically by elevating or depressing normal cell function.

1.2.4.1. Haemolysins

An alternative method for pathogens to obtain iron is to haemolyse erythrocytes by means of a haemolysin to release haem. Kayser in 1903 reported that some *E. coli* cultures lysed erythrocytes and the culture supernatant retained haemolytic activity after being filtered through a filter (Kayser, H. 1903). Smith in 1963 described the difference between the cell-bound (β-haemolysin) and cell-free (α-haemolysin) haemolysin of *E. coli* (Smith, H. W. 1963). The α-haemolysin produces visible haemolysis on blood agar around colonies and is present in cell free filtrates, while β-haemolysin is not found in the filtrates or in the supernatant, but only can be measured from bacterial lysates. Cell-associated haemolysin was not neutralised by antiserum prepared against the cell-free haemolysin. Walton and Smith have shown that spontaneous nalidixic acid resistant mutants of a nonhaemolytic *E. coli* strain become haemolytic. These resistant mutant strains produced a haemolytic phenotype in the absence of nalidixic acid, but the intensity of the haemolysis produced was markedly enhanced by the presence of nalidixic acid in the medium. The haemolytic activity was different from that of the α or β haemolysin and was therefore designated γ-haemolysin (Walton, J.R. and D.H. Smith 1969).

E. coli α-haemolysin which is usually present in *E. coli* causing extraintestinal infections, is the best characterised toxin, which belongs to the RTX (for repeat in toxin) family. The RTX derives from common structural motif in these toxins, i.e. tandem arrays of a glycin-rich, nine-amino-acid sequence, L-X-G-G-X-G-(N/D)-D-X. The α-haemolysin causes cell lysis through production of pores in the target cell membrane. It affects both erythrocytes and leukocytes. Leukotrienes, histamine, and adenosine triphosphate (ATP) can be released by this toxin from human polymorphonuclear granulocytes. Haemolysin is toxic to a range of host cells in ways that probably contribute to inflammation, tissue injury, and impaired host defences. It stimulates superoxide anion and hydrogen peroxide

release from and oxygen consumption by renal tubular cells (Keane, W.F. *et al.*, 1987). The activity of α-haemolysin is neutralised by specific antiserum. The α-haemolysin is a 110 kDa protein encoded by the *hlyA* gene which is part of the *hlyCABD* operon. It is activated by an acylation event requiring HlyC and is secreted through a *sec*-independent pathway involving both HlyB and HlyD (Bauer, M.E. and R.A. Welch 1996). The HlyB and HlyD proteins that mediate export belong to the ABC superfamily of bacterial transporters. In some strains of *E. coli* (origin from animals) *hlyCABD* is carried by plasmid, but other strains (origin from human) *hlyCABD* is located on the chromosome (Müller, D. *et al.*, 1983).

Beutin *et al.*, have described a new type of *E. coli* haemolysin, called enterohaemolysin (Beutin, L. *et al.*, 1988). Enterohaemolysin (Ehly) causes lysis of washed but not unwashed erythrocytes. This type of haemolysin is found in some classical enteropathogenic *E. coli* (EPEC) (O26; O111) (Beutin, L. *et al.*, 1986, 1989) and enterohaemorrhagic *E. coli* (EHEC) strains. It is phenotypically, serologically, and genetically different from α-haemolysin. Enterohaemolysin is thought to be a 33 kDa protein with low activity when expressed in K-12 strains, but can form a dimer of 66 kDa with increased activity in wild type host (Stroeher, U.H. *et al.*, 1993). It is not secreted by bacteria into the culture medium, but associated with the outer membrane. A monoclonal antibody to α-haemolysin failed to react with Ehly, and DNA from Ehly-producing *E. coli* did not hybridise with an α-haemolysin-specific gene probe (Beutin, L. *et al.*, 1989). The *ehly* genes have been shown to be present on lamboid-like temperate phages, which have been isolated from VTEC and EPEC strains (Beutin, L. *et al.*, 1993). The other type of enterohaemolysin, which is genetically and serologically unrelated from previously described enterohaemolysin, is named EHEC toxin (Ehx). A 60 MDa plasmid, present in most EHEC strains, confers a haemolytic phenotype on washed sheep blood agar plates, although only very small zones of lysis are apparent (Schmidt, H. *et al.*, 1994). This haemolytic determinant was previously known as enterohaemolysin, but it shall be called EHEC toxin (Ehx) as proposed by Bauer and Welch (1996) in order to differentiate from the enterohaemolysin.

The Ehx determinant has been cloned and DNA sequence analysis has shown that two genes (*ehxA* and *ehxC*) are present, and share approximately 60 % homology to the *hlyA* and *hlyC* genes of the *E. coli* α-haemolysin operon (Schmidt, H. *et al.*, 1995). The fact that the *hlyB* and *hlyD* genes are lacking in the EHEC operon and thus Ehx may not be secreted may explain the small zones of lysis observed when EHEC are grown on blood agar and lack of haemolytic activity in culture supernatants. This hypothesis is supported by the fact when *hlyBD* genes are supplied in trans to either wild type EHEC or K-12 strains carrying plasmid encoded *ehxAC*, zones of haemolysis become much larger and haemolytic activity can be detected in culture supernatants (Bauer, M.E. and R.A. Welch 1996).

1.2.4.2. Cytotoxins

1.2.4.2.1. Cytotoxic necrotising factor (CNF)

Cytotoxic necrotising factor was recognised and described first in *E. coli* stains from humans with diarrhea by Caprioli in 1983 (Caprioli, A. *et al.*, 1983). CNF positive strains have also been frequently isolated from septicaemia, urinary tract infections in humans and enteritic piglets and calves (Blanco, J. *et al.*, 1990).

Two types of cytotoxic necrotising factors, CNF1 and CNF2 (first named Vir cytotoxin) (Oswald E. *et al.*, 1989), have been detected in extracts of *E. coli* strains (De Rycke, J. *et al.*, 1990). About 99 % of CNF1-positive *E. coli* strains are haemolytic and the majority of these strains was isolated from extraintestinal infection. CNF1 might impair migration and proliferation of bladder cells and potentially interfere with repair of the bladder epithelium damaged by haemolysin. Using bladder cell monolayers as an *in vitro* model, Island *et al.* found that repair of experimental wounds was inhibited by CNF1-containing bacterial extracts (Island, M.D. *et al.*, 1999).

The CNF1 and CNF2 are similar in size with apparent molecular masses of 110 to 115 kDa, respectively, and are immunologically related. They are lethal for mice (both toxins), chickens (CNF2) (De Rycke, J. *et al.*, 1987) and lambs (De

Rycke, J. and G. Plassiart 1990), and necrotic for rabbit skin (both toxins) or mouse footpads (CNF2 only). These toxins are Rho small GTPase modifying toxins that perturb F-actin assembly in host cells resulting in multinucleation due to a blockage in cytokinesis (Oswald, E. *et al.*, 1994). CNF1 can trigger the entry of non-invasive bacteria into HEp-2 (human epidermoid carcinoma) cells through phagosome-like organelles. This may permit non-invasive CNF-producing bacteria to be taken up by epithelial cells in the gut (Falzano, L. *et al.*, 1993). CNFs have been found only associated with bacterial cytosol and are not secreted by *E. coli* in culture. Certain virulence factors from Gram-negative bacteria, such as the invasion proteins from *Shigella* (Ipa proteins), *Yersinia* (Yop proteins, or *Salmonella* (inv proteins) are secreted by a mechanism triggered by cell contact named the type III secretion system. CNF1 could, in certain conditions, be massively released from the uropathogenic strain *E. coli* J96 by cell contact (Hofman, P. *et al.*, 1998), indicating that CNF1 is probably secreted by *E. coli* via a type III mechanism.

In addition CNF1 is chromosomally encoded by *cnf1* gene, whereas CNF2 is encoded by *cnf2* gene in an F-like plasmid that was initially designated as Vir plasmid (Oswald, E. *et al.*, 1989, Oswald, E. and J. De Rycke 1990). Interestingly, it has been shown that the *cnf1* gene is located within a group of virulence determinants named pathogenicity islands (PAIs). In the uropathogenic J96 *E. coli* strain, the *cnf1* gene lies within the PAI-II (Blum, G. *et al.*, 1995).

1.2.4.2.2. Cytolethal distending toxin (CLDT)

Some *E. coli* strains isolated from humans with diarrhea produce cytolethal distending toxin (CLDT) that causes progressive distension and ultimately death of Vero, HEp-2, HeLa, or CHO (Chinese hamster ovary) cells. In 1987 Johnson and Lior, using strains of *E. coli* O128 isolated from children with diarrhea, described a new type of activity in CHO cells and named the responsible factor cytolethal distending toxin. CLDT activity is destroyed by treatment with trypsin or by heating (70°C for 15 min) and is retained by a 30000 molecular weight filter.

Rabbit antiserum prepared to a CLDT$^+$ *E. coli* strain neutralises CLDT activity. CLDT activity is not neutralised by antibody to cholera toxin, *E. coli* LT, *E. coli* Shiga-like toxin, or *Clostridium difficile* cytotoxin. CLDT can be differentiated from Shiga-like toxin by its effect in CHO cells, in which Shiga-like toxins are inactive. Cytotoxic necrotising factor causes cellular distention similar to that by CLDT but with prominent multinucleation not seen with CLDT. Although CLDT induces transient CHO cell elongation, like that associated with heat-labile toxin (LT), the progression to cellular distension and eventual cell destruction is unique to CLDT. Y1 adrenal tumor cells are responsive to LT but not to CLDT (Johnson, W.M. and H. Lior 1988). Furthermore, *E. coli* CLDT is negative in the rabbit ligated ileal loop and suckling mouse assays.

Two groups of workers have cloned and sequenced the gene encoding CLDT from *E. coli*. Three genes, which are arranged in an apparent operon and designated *cdtA*, *cdtB*, and *cdtC* encode CLDT. These three genes specify polypeptides with predicted apparent molecular masses of approximately 25 to 35 kDa (CdtA), 28-30 kDa (CdtB), and 20 to 21 kDa (CdtC) (Scott, D.A and J.B. Kaper 1994, Pickett, C.L. *et al.*, 1994). Genetic and molecular cloning evidence suggests that all three polypeptides are required for toxicity, however, the nature of the CLDT holotoxin and its subunit arrangement are unknown. CLDT may inhibit cytokinesis without directly blocking DNA synthesis. CLDT causes an accumulation of actin stress fibers, coincident with cellular distension and cessation of cell division. CLDT may affect the function of RhoA. However, the dissimilarities between CLDT and CNF action, which include massive cellular distension, limited multinucleation, and slow progression to cell death mediated by CLDT but not CNF, suggest that these toxins possess different modes of action (Aragon, V. *et al.*, 1997).

1.2.4.2.3. Enteroinvasive *E. coli* (EIEC) cytotoxin

The possibility that a cytotoxin and an enterotoxin may play roles in the pathogenesis of EIEC-mediated diarrhea and dysentery respectively, was proposed

by Fasano and colleagues, who demonstrated the presence of such activities in culture filtrates and cell lysates of EIEC strains. The Vero cell cytotoxic activity of EIEC that was detected at low levels is distinct from enterotoxic activity and is not neutralised by anti-SLT-I or anti-SLT-II antibodies. The cytotoxin activity was detected in the <30 kDa fraction (Fasano, A. *et al.*, 1990). No experimental data on the function of this cytotoxin in EIEC disease have been reported.

1.2.4.2.4. Shiga-like toxin (SLT)

The Shiga toxins (Stxs) are a family of bacterial cytotoxins produced by *Shigella dysenteriae* type 1 and Shiga toxin producing *E. coli* (STEC). These toxins were formerly called Shiga-like toxins. Many isolates of classical enteropathogenic *E. coli* (EPEC) serotypes and enterohaemorrhagic *E. coli* (EHEC) O157 serotype, although able to induce diarrhoea in children and adult volunteers, neither produce *E. coli* heat labile or heat stable enterotoxins nor are they enteroinvasive. These strains produce Vero toxin that kills Vero cells (Konowalchuk, J. *et al.*, 1977, Johnson, W.M. *et al.*, 1983). In 1977 O'Brien *et al.* reported that extract of certain pathogenic strains of *E. coli* were cytotoxic for HeLa cells and that this cytotoxic activity could be neutralised by antitoxin prepared against crude *S. dysenteriae* 1 (Shiga) toxin. Bacterial extract and culture filtrates of Shiga-like toxin elaborating *E. coli* strains were enterotoxic for rabbit, mediating fluid accumulation in ligated ileal loops, paralytic and lethal for mice, and able to inhibit protein synthesis in HeLa cells (O'Brien, A.D. *et al.*, 1977, O'Brien, A.D. and R.K. Holmes 1987). The effects of the *E. coli* O157:H7 preparations and the purified toxins of H30 *E. coli* (serotype O26) and *S. dysenteriae* 1 strains were compared on HeLa and Vero cells. The results substantiated that the Vero cell cytotoxin (VT) and the *E. coli* Shiga-like toxin (SLT) were the same (O'Brien, A.D. *et al.*, 1983). Recently Shiga-like toxin designation is used.

Two immunologically non-cross-reactive groups of SLTs have been isolated from EHEC and a single EHEC strain can produce SLT-I, SLT-II, or both toxins. Scotland *et al.* demonstrated that SLT-I or VT-I cytotoxic activity is

neutralisable by antiserum against Shiga toxin produced by *S. dysenteriae* type 1 and SLT-II or VT-II is not neutralised by anti-Shiga toxin antibodies (Scotland, S.M. *et al.*, 1985). SLT-I predominates in cell lysates and SLT-II is the more active toxin in culture filtrate, when both toxins are produced by the same strain (Strockbine, N.A. *et al.*, 1986). SLTs are phage encoded (SLT-I and SLT-II) or chromosomally encoded (SLT-IIc, d, e, and Shiga toxin). SLT-I differs from Shiga toxin of *S. dysenteriae* type 1 by only one amino acid threonin at position 45 in the Shiga toxin A polypeptide and a serine at the corresponding position in SLT-I. SLT-II has only 50-60 % homology with SLT-I. The Shiga toxin and, presumably, SLT-I holotoxin are known to consist of a single A subunit noncovalently linked to five copies of the B subunit. The B polypeptide of Shiga toxin or SLT-I and SLT-II (~7.9 kDa per monomer) forms a pentamer that is responsible for binding to a eucaryotic glycolipid receptor, which is typically globotriaosyl ceramide (Gb_3) [Galα(1-4)-Galβ(1-4) glucosyl ceramide] (Waddell, T. *et al.*, 1988), whereas SLT-IIe preferentially recognises globotetraosyl ceramide (Gb_4), which contains an additional terminal β-(1-3)-linked N-acetylgalactosamine residue, in addition to recognising Gb_3. The B pentamer binds to glycolipids on the target cell surface, and the holotoxin is endocytosed through coated pits. The toxin is then transferred both to lysosomes and to the Golgi apparatus. Transport to the Golgi apparatus appears to be a requisite for intoxication. From the trans-Golgi, Shiga toxin is transported in a retrograde manner to the endoplasmic reticulum, and translocation of the A subunit to the cytoplasm occurs in this organelle (Sandvig, K. *et al.*, 1992). The A subunit (32.2 kDa) is proteolytically nicked with trypsin and reduced, an A_1 portion of ~28 kDa and A_2 peptide of 4 kDa are generated. The A_1 is an N-glycanase (contains the N-glycosidase activity), which removes an adenine at position 4324 of the 28S rRNA of the eucaryotic 60S ribosomes, thus preventing elongation factor-1-dependent binding of aminoacyl-tRNA and causes inhibition of protein synthesis leading to death of the affected cell (Endo, Y. *et al.*, 1988, Saxena, S.F. *et. al.*, 1989). This mode of action is identical to that of the plant lectin ricin. The A_2 peptide is required to noncovalently bind the A_1 polypeptide to the B pentamer (Austin, P.R. *et al.*, 1994).

Although each toxin have the same mode of action, their effect *in vitro* and *in vivo* differs considerably. SLT-I binds more efficiently to the Gb_3 receptor and is more potent on Vero cells. However, SLT-II, when injected intravenously, is 400 fold more lethal for mice than SLT-I (LD_{50} of purified SLT-I for parentally inoculated adult CD-1 mice is ~400 ng), compared to ~1 ng for SLT-II (Tesh, V.L. *et al.*, 1993). Furthermore, SLT-II, but not SLT-I, is responsible for severe necrotic renal tubular lesions and death of streptomycin-treated mice fed an EHEC strain that makes both SLT-I and SLT-II (Wadolkowski, E.A. *et al.*, 1990). This difference in toxicity is also evident when human renal microvascular endothelial cells (HRMEC) are treated with purified SLT-I or SLT-II: SLT-II is about 1000 fold more toxic (Louise, C.B. and T.G. Obrig 1995). Epidemiologically SLT-II containing EHEC O157:H7 strains are more frequently associated with haemolytic uraemic syndrome (HUS) than are strains containing SLT-I (Ostroff, S.M. *et al.*, 1989). Another difference between the Stxs subgroups is that high levels of iron inhibit production of Shiga toxin or SLT-I but not SLT-II (Weinstein, D.L. *et al.*, 1988).

1.2.4.3. Enterotoxins

Enterotoxigenic *E. coli* (ETEC) strains cause watery diarrhea through the action of the enterotoxins, which are known respectively as heat-labile toxins (LTs) and heat-stable toxins (STs). The ETEC strains may express an LT only, an ST only or both an LT and an ST.

1.2.4.3.1. Heat-labile toxins (LTs)

The LTs of *E. coli* are oligomeric toxins that are related in structure and function to cholera enterotoxin expressed by *Vibrio cholerae* (Sixma, T.K. *et al.*, 1993). There are two major serogroups of LT, LT-I and LT-II, which do not cross-react immunologically. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-I produced by ETEC from pigs is designated LTp-I, and LT-I produced by humans is designated LTh-I (Takeda, Y. *et al.*, 1983, Guth,

B.E.C. *et al.*, 1986). LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates, but in neither animals nor humans has it been associated with disease. Two variants of LT-II have been designated LT-IIa and LT-IIb, which have been characterised by Guth and colleagues (Guth, B.E.C. *et al.*, 1986).

LT-I is an oligomeric toxin of ~86 kDa composed of one 28 kDa A subunit (240 to 243 amino acids) and five identical 11.5 kDa B subunits (99 to 103 amino acids) (Geary, S.J. *et al.*, 1982). The B subunits are arranged in a ring or "doughnut" and bind strongly to the ganglioside GM_1 and weakly to GD1b and some intestinal glycoproteins. In contrast to LT-I, the LT-IIa binds best to GD1b ganglioside and LT-IIb binds best to GD1a ganglioside (Fukuta, S. *et al.*, 1988). The A subunit is responsible for enzymatic activity of the toxin and is proteolytically cleaved (with trypsin) to yield A_1 and A_2 peptides joined by a disulphide bond. In contrast to the genes encoding LT-II which are in chromosome (Green, B.A. *et al.*, 1983), the genes encoding LT-I (*elt* or *etx*) reside on plasmids that may also contain genes encoding ST and/or colonisation factor antigens (CFAs) (Gyles, C. *et al.*, 1974). After binding to host cell membranes, the toxin is endocytosed and translocated through the cell in a process involving trans-Golgi vesicular transport (Lencer, W.I. *et al.*, 1995). The cellular target of LT is adenylate cyclase located on the basolateral membrane of polarised intestinal epithelial cells. The A_1 peptide has an ADP-ribosyl transferase activity and acts by transferring an ADP-ribosyl moiety from NAD to the alpha subunit of the GTP-binding protein, G_s, which stimulates adenylate cyclase activity. ADP-ribolysation of the G_s alpha subunit results in adenylate cyclase being permanently activated, leading to increasing levels of cyclic AMP (cAMP). cAMP is an intracellular messenger which regulates several intestinal epithelial cell membrane transporters and other host cell enzymes, as well as having effects on the cytoskeleton. The activation of the cAMP-dependent protein kinase (A kinase) results in phosphorylation of apical membrane transporters (especially the cystic fibrosis transmembrane conductance regulator - CFTR), resulting in secretion of anions (predominantly Cl^- by direct effect, and HCO_3^- indirectly) by crypt cells and decrease in absorption of Na^+ and Cl^- by absorptive villus tip cells (Sears C.L. and

J.B. Kaper 1996). The increased luminal ion content draws water passively through the paracellular pathway, resulting in osmotic diarrhea.

One alternative mechanism by which these toxins could act involves prostaglandins of the E series (PGE_1 and PGE_2). Synthesis and release of arachidonic acid metabolites such as prostaglandins and leukotrienes can stimulate electrolyte transport and intestinal motility. A second alternative mechanism involves the enteric nervous system (ENS), which regulates intestinal motility and ion secretion. Serotonin and vasoactive intestinal polypeptide, both of which can stimulate intestinal epithelial cell secretion via ENS, are released into the human bowel after treatment with toxin. A third mechanism could involve a mild intestinal inflammatory response due to LT (Nataro, J.P. and J.B. Karper 1998).

1.2.4.3.2. Heat-stable toxins (STs)

In contrast to the large oligomeric LTs, the heat stable enterotoxins (STs) are small monomeric toxins that contain multiple cysteine residues, whose disulphide bonds account for the heat stability of these toxins. The STs are low molecular weight, extracellular bacterial polypeptides that alter the movement of fluid and electrolytes across the intestinal epithelium of susceptible animal hosts (Smith, H.W. and C.L. Gyles 1970). The STs are classified into two groups (STa or STI and STb or STII) which differ in structure and mechanism of action.

STa toxins are produced by ETEC and several other Gram-negative bacteria including *Yersinia enterocolitica* and *Vibrio cholerae* non-O1. STa has about 50% protein identity to the EAST1 of EAggEC, which is described further below. Some strains of ETEC may also express EAST1 in addition to STa. STa is methanol-soluble; it can be assayed in new-born mice or new-born pigs, but inactive in older pigs (Burgess, M.N. *et al.*, 1978). The extracellular mature STa toxins consist of an 18 or 19 amino acid peptide and molecular weight about 2 kDa. There are two variants, designated STp (ST porcine or STIa) and STh (ST human or STIb) after their initial discovery in strains isolated from pigs or humans, respectively. Both variants can be found in human ETEC strains. These

two variants are nearly identical in the 13 residues that are necessary and sufficient for enterotoxic activity, and of these 13 residues containing six conserved cysteines which form three intramolecular disulphide bonds. STa is initially produced as a 72 amino acid precursor (a pre region – amino acid residues 1 to 19, a pro region – amino acid residues 20 to 54, and a mature ST region – amino acid residues 55 to 72) that is cleaved by signal peptidase 1 to 54 amino acid peptide (Rasheed, J.K. *et al.,* 1990). This form is transported through the inner membrane to the periplasm, where the disulphide bonds are formed by the chromosomally encoded periplasmic DsbA protein (Yamanaka, H. *et al.*, 1994). An undefined protease processes the pro-STa to the final 18 (STp) or 19 (STh) residue mature toxin which is released by diffusion across the outer membrane. Four amino acids (residues 11 to 14, Asn-Pro-Ala-Cys) are most important to the toxicity of STa and their interaction with the receptor (Osaki, H. *et al.*, 1991). These amino acids are conserved in all ST family members and are partially conserved in the endogenous intestinal peptide hormone guanylin and the EAggEC EAST1 toxin (Ala-Cys) (Sears C.L. and J.B. Kaper 1996).

STa acts by binding to a protein intestinal epithelial receptor localised in the brush border membrane. STa receptors are found throughout the human small intestine and colon, with decreasing receptors along the longitudinal axis of gut (Krause, W.J. *et al.,* 1994). The major receptor for STa is a membrane-spanning enzyme called guanylate cyclase C (GC-C), which belongs to the family of receptor cyclases that includes the atrial natriuretic peptide receptors GC-A and GC-B (Vaandrager, A.B. *et al.,* 1994). Guanylin is the endogenous agonist for GC-C but it is less potent than STa in activating GC-C. Guanylin is presumed to play a role in normal gut homeostasis and GC-C is apparently used opportunistically by STa to cause diarrhea. GC-C is located in the apical membrane of intestinal epithelial cells, and binding of ligands to the extracellular domain stimulates the intracellular enzymatic activity. Binding of STa to GC-C stimulates guanylate cyclase (GC) activity, initiating a cascade, which involves the intracellular accumulation of cyclic guanosine-monophosphate (cGMP), the cGMP-dependent activation of protein kinase A, the protein kinase A-dependent

phosphorylation and activation of the cystic fibrosis transmembrane conductance regulator (CFTR), and the resulting CFTR-dependent secretion of Cl^- ions into the intestinal lumen. Stimulation of chloride secretion leads fluid secretion into the lumen. Alternative mechanisms of action for STa involving prostaglandins, calcium, and the enteric nervous system (ENS) have been proposed, but the evidence for involvement of these factors is inconsistent (Nataro, J.P. and J.B. Kaper 1998).

The toxin is encoded by the transposon-associated *estA* gene, which is located on a plasmid (So, M. and B.J. McCarthy 1980).

The STb is associated primarily with ETEC strains isolated from pigs, although some human ETEC isolates expressing STb have been reported. This toxin is active in a piglet intestinal loop assay. STb is initially synthesised as a 71 amino acid precursor protein, which is processed to a mature 48 amino acid protein with a molecular weight of 5.1 kDa (Dreyfus, L.A. *et al.*, 1992). STb is methanol insoluble and its protein sequence is different from STa, although it does contain four cysteine residues which form disulphide bonds (Arriaga, Y.L. *et al.*, 1995). In contrast to STa, STb induces histological damage in the intestinal epithelium, consisting of loss of villus epithelial cells and partial villus atrophy. The toxin causes intestinal secretion in the absence of elevated levels of cyclic nucleotides. It does stimulate increases in intracellular calcium levels from extracellular sources causing K^+ efflux, releasing arachidonic acid. STb also induces the release of prostaglandin E_2 (PGE_2) and serotonin, suggesting that the enteric nervous system (ENS) may also be involved in the secretory response to this toxin (Hitotsubashi, S. *et al.*, 1992, Fujii, Y. *et al.*, 1995, Arriaga, Y.L. *et al.*, 1995). The receptor for STb is unknown, although it has been suggested that the toxin may bind non-specifically to the plasma membrane prior to endocytosis (Chao, K.L. and L.A. Dreyfus 1997).

1.2.4.3.3. Enteroaggregative *E. coli* (EAggEC) heat-stable enterotoxin 1 (EAST1)

Savarino *et al.* reported that some enteroaggregative *E. coli* (EAggEC)

strains produce an enterotoxin of 38 amino acids named EAggEC heat-stable enterotoxin 1 (EAST1) (Savarino, S.J. *et al.*, 1991, Savarino, S.J. *et al.*, 1993). Although EAST1 of EAggEC and STa of ETEC are genetically and immunogically distinct enterotoxins, EAST1 activates guanylate cyclase as STa does. EAST1 is detected in an *in vitro* rabbit intestinal model, and STa is detected in suckling mice. EAST1 is encoded by the *astA* gene on a plasmid also encoding AAF/I genes in EAggEC strains. The EAST1 gene sequence is more widely distributed among different categories of diarrhea-associated *E. coli*, human and animal colonising ETEC strains (Yamamoto, T. and P. Echeverria 1996, Yamamoto, T. and M. Nakazawa 1997), EAggEC strains, EPEC strains, and diffusely adhering *E. coli* (DAEC) strains (Yamamoto, T. *et al.*, 1997).

1.2.4.3.4. Enteroinvasive *E. coli* (EIEC) enterotoxin (EIET)

EIEC strains are biochemically, genetically, and pathogenetically related closely to *Shigella* species. Both *Shigella* and EIEC infections are characterised by a period of watery diarrhea. The pathogenesis of watery diarrhea has long been unexplained. Fasano *et al.* reported that culture filtrates of EIEC stimulate moderate secretion without histologic damage in 18 h ligated rabbit ileal segments and also increase the transepithelial electrical potential difference in rabbit ileum studied in Ussing chambers *in vitro* (Fasano, A. *et al.*, 1990). The secretory activity present in the EIEC culture filtrates was reported to be partially heat labile and iron regulated. The activity in the EIEC culture filtrates was named EIET (for enteroinvasive enterotoxin). This toxin is also produced by *Shigella flexneri* and was called ShET2 (for Shigella enterotoxin 2). The maximum enterotoxic activity was contained in the 68-80 kDa supernatant fraction. Nataro *et al.* have cloned and sequenced a plasmid born gene from EIEC (designated *sen*), which encodes a novel protein with predicted size of 63 kDa. Mutation in the *sen* gene causes a significant diminution of enterotoxic activity of the parent strain (Nataro, J.P. *et al.*, 1995). A role for enterotoxins is unproven, but their presence may explain the characteristic watery diarrhea attributed to EIEC.

1.3. General introduction of *Neisseria* species

The genus *Neisseria* includes the pathogenic species *Neisseria gonorrhoeae* that causes sexually transmitted disease in humans and *Neisseria meningitidis*, one of the most important agents of bacterial meningitis. Molecular genetic analysis of *Neisseria* species have demonstrated a close relatedness between gonococci, meningococci and to some extent the commensal *Neisseria* species (Aho, E.L. *et al.*, 1987, Stern, A. and T.F. Meyer 1987), which have been shown on occasions to cause meningitis and septicaemia (Johnson, A.P. 1983, Brown, N.M. *et al.*, 1987).

Meningococcal diseases continue to cause devastating illness and world wide health problems. *Neisseria meningitidis* is an exclusively human pathogen, responsible for infections including meningitis, arthritis, acute fulminant septicaemia, and adrenal haemorrhages, so-called "Waterhouse-Fredrichsen syndrome". Vieusseaux, M. was the first to describe an outbreak of an apparently new disease, a cerebrospinal fever, which spread rapidly in and around Geneva in the spring 1805 (Vieusseaux, M. 1805). Several other outbreaks of similar nature were also recorded over the subsequent decades but the organism was first isolated from meningeal exudate of cases of cerebrospinal fever in Vienna in 1887 by Anton Weichselbaum (Weichselbaum, A. 1887).

Infections caused by the meningococcus can have a high morbidity and mortality and occur both endemically and epidemically in the population. For epidemiological purposes *N. meningitidis* has been classified into 13 serogroups (A, B, C, D, H, I, K, L, X, Y, Z, W135, and 29E) on the basis of the immunological specificities of their capsular polysaccharides (Branham, S.E. 1953, Slaterus, K. 1961, Ding, S.Q. *et al.*, 1981, Ashton, F.E. *et al.*, 1983). Meningococcal strains can be subdivided further into serotypes according to the possession of different outer membrane proteins (Frasch, C.E. and S.S Chapman 1972, Frash, C.E. and E.C. Gotschlich 1974). Isolates from patients with meningococcal disease belong almost exclusively to serogroups A, B, C and less frequently W135 and Y (Band, J.D. *et al.*, 1983, Abbott, J.D. *et al.*, 1985,

Spanjaard, L. *et al.*, 1987). The majority of the sporadic cases of infection seen in Britain over the past 15 years were caused by organism belonging to serogroups C and B, with serogroups A, Y and W135 being responsible for less than 10 % of infections (Public Health Laboratory Service of England and Wales, 1999). Most cases occur in children aged under five. However, recently there has been a striking increase in the incidence of infections among teenagers and young adults (PHLS of England and Wales, 1999). In 2000 the number of meningoccal diseases increased in Hungary. The majority of the isolates from patients with meningococcal infection belong to serogroup C (57 %) and B (35.4 %), and only in one case serogroup X meningococcus was found. Most of the cases occured in childhood and in teenagers (Epinfo, 2000). There are effective vaccines to prevent group A, C, Y, and W135 infections. However, vaccination with group A meningococcal vaccine during epidemics in equatorial Africa has little effect on the transmission of group A strains (Hassan-King, M.K. *et al.*, 1988). In addition, the polysaccharide capsule of group B meningococci has been found to be a poor immunogen. *N. meningitidis* group B strains share the same capsular polysaccharide antigen as *E. coli* strain K1, which is the principal agent of bacterial meningitis in the new-born (Robbins, J.B. *et al.*, 1974). The capsule is composed of a homopolymer of N-acetylneuramic acid, that cross reacts immunologically with oligosaccharide side chains of mammalian glycoproteins and glycolipids. It has been suggested that a breakdown of the natural tolerance caused by an artificial vaccine might have adverse effects (Finne, J. *et al.*, 1983, Gregson, N.A. and S. Leibowitz 1985). A vaccine is not currently available to prevent group B infections. The great majority of the studies have focused on development of new vaccines cross-protect against all meningococcal serogroups and serotypes. They are based on non-capsular antigens like immunogenic virulence factors of *N. meningitidis* (e.g. outer membrane proteins, iron regulated protein, transferrin-binding proteins, pili, and protease) (Bjune, G. *et al.*, 1991, Sierra, G.V.G. *et al.*, 1991, Zollinger, W.D. et al., 1991, Ala'Aldeen, D.A.A. and E. Griffiths 1995, Ala'Aldeen, D.A.A. 1996).

The majority of subjects infected with meningococci become

nasopharyngeal carriers, but fail to develop clinical meningococcal disease. Asymptomatic carriage of *N. meningitidis* is an important source of transmission (Broome, C.V. 1986), with 5-10 % of the population harbouring meningococcal isolates in non epidemic periods (Greenfield, S. and H.A. Feldman, 1967, Cartwright, K.A. *et al.*, 1987). In contrast, 17-50 % of household contacts of cases of meningococcal meningitis are found to carry strains of the same serogroups as the index case (Greenfield, S. and H.A. Feldman, 1967, De Wals, P. *et al.*, 1981, Olcen, P. *et al.*, 1981). Carriage rates are apparently dependent on age, sex and carriage of *N. lactamica* (Gold, R. *et al.*, 1978, Baker, C.J. and J.M. Griffiss 1983, Cartwright, K.A. *et al.*, 1987). Stephens *et al.* have demonstrated that meningococci attach selectively to non-ciliated columnar epithelial cells of the nasopharynx. Following attachment, the microvilli of these non-ciliated cells elongate and surround the meningococci to twelve hours after infection, endocytic vacuoles containing meningococci are observed in the apical position of some non-ciliated columnar cells. Later diplococci can be identified in subepithelial tissue adjacent to lymphoid tissue, suggesting that meningococci can penetrate the epithelial cells (Stephens, D.S. *et al.*, 1983). Furthermore, encapsulated viable meningococci have been shown to damage ciliated epithelium of nasopharyngeal organ cultures. Meningococcus-induced ciliary damage has been observed with both piliated and non-piliated isolates and was found to be due to loss of ciliated cells to which meningococci were not attached (Stephens, D.S. *et al.*, 1986). This may be an important first step in meningococcal colonisation of the human nasopharynx.

Protective antibodies may be acquired either passively by transplacental passage of immunoglobulins, or actively as the result of oropharyngeal infection with both capsulated or non-capsulated meningococci (Goldschneider, I. *et al.*, 1969). The effect of passively acquired antibodies is relatively short-lived but is effective in protecting infants against meningococcal disease. The susceptibility to disease increases progressively during the first six months of life (Goldschneider, I. *et al.*, 1969). Protection against meningococcal disease may also result from exposure to cross-reactive antigens from bacteria that bear little or no taxonomic

relationship to meningococci, or from exposure to other commensal *Neisseria* species. A study by Gold *et al.* (Gold, R. *et al.*, 1978) revealed that more than half of the children acquired *N. lactamica*, exhibited cross-reactive antibodies to meningococcal serogroups A, B, and C. Only 5 % of the children who did not become *N. lactamica* carriers developed detectable antibodies against meningococci. According to Goldschneider *et al.* (Goldschneider, I. *et al.*, 1969) individuals who acquired meningococcal diseases were found to lack bactericidal antibodies to the meningococcus. This observation contrasts with the finding of Greenwood *et al.* (Greenwood, B.M. *et al.*, 1987), who noted bactericidal antibodies in all children who subsequently became cases. Furthermore, other studies have shown that although bactericidal antibody apparently fails to protect, its absence does not result in susceptibility to meningococcal infection (Bannister, B. 1988). Additionally, host factors have been implicated in pathogenesis. For example, patients lacking latter components (C6-C9) of complement cascade or having spleen exstirpation are particularly susceptible to meningococcal infections (Nicholson, A. and I.H. Lepow, 1979, Poolman, J.T. 1988). However, it seems that there are other as yet undefined factors also involved in the development of meningococcal disease and there are several undefined protective mechanisms that inhibit development of disease.

1.4. Aims of the thesis

Recently there have been rapid developments in our understanding on different pathogenic mechanisms of *E. coli* strains. Increasing information of virulence factors prompted that their role in the spreading of infectious diseases in the host should be investigated.

In chapter 3, *E. coli* strains expressing different fimbriae are tested for ability to bind to extracellular matrix proteins like fibronectin, laminin, and collagen. The role of fimbriae in ascending urinary infection is investigated. Tissue tropism of fimbriated strains is compared with non-fimbriated derivative after intravenous infection.

In chapter 4, relations between alpha-haemolysin production and lung toxicity are investigated. Anti-haemolysin titres in human and rabbits with infection of haemolytic *E. coli* and *Proteus morganii* are determined and antitoxic effect of these sera was tested against *E. coli* and *P. morganii* lung toxin.

In chapter 5, the influence of the tRNA genes *leuX* and *selC* lost with the pathogenicity islands (Pais) on the virulence of uropathogenic *E. coli* is studied *in vitro* and *in vivo*.

In chapter 6, the influence of RecA protein on the *in vivo* virulence of different *E. coli* pathogens is examined in mice.

Genetic transfer may occur between commensal and pathogenic *Neisseria* species as like as among *E. coli* strains. In chapter 7, I investigated whether some commensal *Neisseria* species may carry virulence genes, which are present in pathogenic *Neisseria* strains.

Chapter 2.
Materials and methods

2.1. Bacterial strains and plasmids

Strains and plasmids used throughout this work are listed in tables 2.1.1. and 2.1.2., respectively.

Table 2.1.1. Bacterial strains used in this work

E. coli K-12-strains	Genotype	Reference
HB 101	F⁻ *ara*-14, *gal*K2, *hsd*S20 (*hsr*⁻, *hsm*⁻), *rec*A13, *sup*E44, *lac*Z4, *leu*B6, *pro*A2, *thi*-1, *rsp*L20 (Sm^R), *xyl*-5, *mtl*-1, λ^-	Boyer, H.W. and D. Roulland-Dussoix 1969
DH5α	F⁻,*end*A1, *hsd*R17, (r_K^-,m_K-), *sup*E44, *thi*-1, *rec*A, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*)U169, λ^-, ϕ80d/*lac*ZΔM15	Bethesda Research Laboratories, 1986
J 53	*met*⁻, *pro*⁻	Datta, N. *et al.*, 1966
C600	*sup*E44, *hsd*R, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21	Appleyard, R.K. 1954

E. coli wild strains and their mutants	Genotype	Reference
536 (575)	O6:K15:H31, *hly*⁺, *fim*⁺, *sfa*⁺, *prf*⁺, *ent*⁺, *fla*⁺, serumresistent, Sm^R	Berger, H. *et al.*, 1982
536-21	O6:K15:H31, Pai I⁻,Pai II⁻, *hly*⁻, *fim*⁻, *sfa*⁻, *prf*⁻, *ent*⁺, *fla*⁺, serumsensitive, Sm^R	Hacker, J. *et al.*, 1983
536-31	O6:K15:H31, *hly*⁻, *fim*⁻, *sfa*⁺, *prf*⁻, *ent*⁺, *fla*⁺, serumresistent, Sm^R, Amp^R	Marre, R. *et al.*, 1986
536-40	O6:K15:H31, *hly*⁻, *fim*⁺, *sfa*⁺, *prf*⁻, *ent*⁺, *fla*⁺, serumresistent, Sm^R, Amp^R	Hacker, J. personal communication
536*rec*A⁻	O6:K15:H31, *hly*⁺, *fim*⁺, *sfa*⁺, *prf*⁺, *ent*⁺, *fla*⁺, serumresistent, Sm^R, insertion of an Cm cassette in the *rec*A gene	Fuchs, S. *et al.*, 1999
536 Δ102	Deletion of the *leu*X gene in 536 strain	Ritter, A. 1996

***E. coli* wild strains and their mutants**	**Genotype**	**Reference**
J96	O4:K6:H$^-$, *hly*$^+$, *prs*$^+$, *pap*$^+$, *pil*$^+$, F1C$^+$, *cnf1*$^+$	Hull, R.A. *et al.*, 1981
J198	O22, *Col*$^-$	Welch, R.A. *et al.*, 1981
IHE 3034	O18:K1:H7, *pil*$^+$	Korhonen, T. *et al.*, 1985
933	O157:H7, *rec*A$^+$, Stx2 converting phage 933W and Stx converting phage 933J	Strockbine, N.A. *et al.*, 1986
933*rec*A$^-$	O157:H7, *rec*A$^-$, Stx2 converting phage 933W and Stx converting phage 933J	Fuchs, S. *et al.*, 1999
86-24	O157:H7, *rec*A$^+$, Stx2 converting phage ϕ86-24	Donnenberg, M.S. *et al.*, 1993
86-24*rec*A$^-$	O157:H7, *rec*A$^-$, Stx2 converting phage ϕ86-24	Fuchs, S. *et al.*, 1999
Pm152	*hly*$^+$	Smith, W.H. (Noegel, A. *et al.*, 1981)
2891/38	O4:K12:H5, *hly*$^+$	
2891/A1	O4:K12:H5, *hly*$^-$	

Proteus strains	**Species**	**Source**	**Genotype**
Pm 290	*P. morganii*	Faecal isolate (Dr Vörös S.)	*hly*$^+$
Pm 290/1	*P. morganii*	Actinomycin D induced mutant	*hly*$^-$

Neisseria strains	Species	Source (where known)	Reference
C501	*N. lactamica*	Throat, contact	
C517	*N. lactamica*	Throat, contact	
C623	*N. lactamica*	Throat, AHCH*	
C628	*N. lactamica*	Throat, AHCH	
C908	*N. lactamica*	PHLS**, Manchester	
C911	*N. lactamica*	PHLS, Manchester	
F302	*N. lactamica*	PHLS, Manchester	
F303	*N. lactamica*	PHLS, Manchester	
F304	*N. lactamica*	PHLS, Manchester	
F305	*N. lactamica*	PHLS, Manchester	
F306	*N. lactamica*	PHLS, Manchester	
F307	*N. lactamica*	PHLS, Manchester	
F308	*N. lactamica*	PHLS, Manchester	
F309	*N. lactamica*	PHLS, Manchester	
F310	*N. lactamica*	PHLS, Manchester	
F311	*N. lactamica*	PHLS, Manchester	
F312	*N. lactamica*	PHLS, Manchester	
F313	*N. lactamica*	PHLS, Manchester	
F314	*N. lactamica*	PHLS, Manchester	
F316	*N. lactamica*	PHLS, Manchester	
C245	*N. pharyngis*	Throat	
C365	*N. pharyngis*	Throat	
C440	*N. subflava* bv *flava*	Throat, contact	
C843	*N. subflava* bv *flava*	Throat	
C351	*N. sicca*	Throat, AHCH	
C394	*N. sicca*	Blood culture, AHCH	
C619	*N. sicca*	AHCH, Liverpool	
C114	*N. meningitidis*	Blood culture	Perry, A.C. *et al.*, 1987
C311	*N. meningitidis*	CSF	Perry, A.C: *et al.*, 1987

* AHCH : Alder Hey Children's Hospital; ** PHLS : Public Health Laboratory Service

Neisseria strains	Species	Source (where known)	Reference
F339	*N. meningitidis*	clinical isolate (Hart, CA)	
F340	*N. meningitidis*	clinical isolate (Hart, CA)	
F341	*N. meningitidis*	clinical isolate (Hart, CA)	
F342	*N. meningitidis*	clinical isolate (Hart, CA)	
F346	*N. meningitidis*	clinical isolate (Hart, CA)	
F343	*N. meningitidis*	clinical isolate (Hart, CA)	
F353	*N. meningitidis*	clinical isolate (Hart, CA)	
F355	*N. meningitidis*	clinical isolate (Hart, CA)	
F357	*N. meningitidis*	clinical isolate (Hart, CA)	
F358	*N. meningitidis*	clinical isolate (Hart, CA)	
F359	*N. meningitidis*	clinical isolate (Hart, CA)	
F360	*N. meningitidis*	clinical isolate (Hart, CA)	
F362	*N. meningitidis*	clinical isolate (Hart, CA)	
F363	*N. meningitidis*	clinical isolate (Hart, CA)	
F375	*N. cinerea*	clinical isolate (Hart, CA)	
F376	*N. cinerea*	clinical isolate (Hart, CA)	
F379	*N. cinerea*	clinical isolate (Hart, CA)	
F381	*N. cinerea*	clinical isolate (Hart, CA)	

Table 2.1.2. Plasmids

Name	Vector	Genotype	Reference
	pUC18	*ori*ColE1, Amp^R, *lacZ*α	Yanisch-Perron, C. *et al.*, 1985
	pUC19	*ori*ColE1, Amp^R, *lacZ*α	Yanisch-Perron, C. *et al.*, 1985
	pSU2716	*ori*P15A, Cm^R, *lacZ*α	Martinez, F. *et al.*, 1988
	pBR322	*ori*ColE1, Amp^R, Tc^R	Bolivar, F. *et al.*, 1977
	pACYC184	*ori*ColE1, Cm^R, Tc^R	Chang, A.C.Y. and S.N. Cohen 1978.
pSF4000	pACYC184	J96 *hlyCABD* operon	Welch, R.A. *et al.*, 1981

Name	Vector	Genotype	Reference
pANN801	pJC74	*fim*$^+$, Amp^R	Hacker, J. *et al.*, 1985
pANN921	pHC79 pBR322	Tc^R, *prf*$^+$	Hacker, J *et al.*, 1986
pANN202-312	pACYC184	*hly*$^+$ (pHly152 of Pm152),Cm^R	Hacker, J. *et al.*, 1983
pSF4000	pACYC184	*hly*$^+$ (J96 *hlyCABD* operon), Amp^R	Welch, R.A. *et al.*, 1981
pBRSol152	pBR322	*hly*$^+$ (pHly152 of Pm152), Amp^R	
pGB30		*fim*$^+$	
pGBB50	pSU2716	*sel*C, Amp^R	Ritter, A. *et al.*, 1995
pGBB51	pSU2716	*leu*X, Amp^R	Ritter, A. *et al.*, 1995
pGBB52	pBR322	*sel*C, Tc^R	Ritter, A. *et al.*, 1995
pIM10	pUC18	*rec*A, Amp^R	Mühldorfer, I. *et al.*, 1996

2.2. Primers

Table 2.2. Primers used in this work

Mark	Long	Sequence 5' – 3'	
N-term	18	ACC CTG ATC CAG CTG ATG	*pilE* class I-II forward
C-II	20	TTC ACG ACC GGG TCA AAC CC	*pilE* class II reverse
1	16	GGC AGG TTG ACG GCA G	*pilE* class I reverse
AR O3	22	GCG GCC GTT GCC GAT GTC AGC C	*porA* outer forward
AR O4	26	GCG GCA TTA ATT TGA GTG TAG TTG CC	*porA* outer reverse
AR O5	17	CAA AGC CGG CGT GGA AG	*porA* inner forward
AR O6	22	GAT CGT AGC TGG TAT TTT CGC C	*porA* inner reverse
IgA PRO1	16	GCA TTG GTC AGA GAC G	*iga* forward
IgA PRO2	17	ATA ATC TTC GAG ACG GC	*iga* reverse

2.3. Chemicals, reagents and buffers

Chemicals were supplied by Sigma Chemical Company, Merck Chemical Company, Unipath (Oxoid) Ltd. and Difco Laboratories Inc. Chemicals were of analytical or reagent grade.

2.3.1. Antibiotics

Antibiotics were supplied by Sigma Chemical Company and were used in the concentrations shown in Table 2.3.1.

Table 2.3.1. Antibiotics

Antibiotics	Concentration	Solvent
Ampicillin (Amp)	100 µg/ml	dH_2O
Chloramphenicol (Cm)	20 µg/ml	Ethanol
Tetracycline (Tc)	15 µg/ml	dH_2O/Ethanol (1:1)
Kanamycin (Km)	50 µg/ml	dH_2O
Gentamicin (Gm)	30 µg/ml	dH_2O
Nalidixic Acid (Nal)	100 µg/ml	1 N NaOH
Streptomycin (Sm)	100 µg/ml	dH_2O

2.3.2. Culture media, buffers, and solutions

Preparation of culture media, buffers, and solutions were performed as described by Sambrook *et al.* (Sambrook, J. *et al.*, 1989) and according to Current Protocol of Molecular Biology (Moore, D.D. *et al.*, 1987). Protocol of culture media can be found in Appendix A.

2.4. Methods for investigation of virulence markers

2.4.1. Particle agglutination assay for detection of fibronectin, fibrinogen and collagen receptors

Strains were grown on various agar media for 24 h. Bacterial colonies were suspended and washed once in 0.02 M potassium phosphate buffer (pH 6.8). Bacterial cells were resuspended in the same buffer to approximately 10^{10} cells per ml and immediately tested for particle agglutination assay (PAA) reactivity.

Preparation of standard latex reagents (Naidu, A.S. *et al.*, 1989): one millilitre of latex particle suspension (bead diameter of 0.8 μm) was mixed with 3 ml of 0.17 M glycine- NaOH (gly) buffer (pH 8.2) and centrifuged at 4500 x *g* for 5 min and the pellet was resuspended in 3 ml of the same buffer. Highly purified protein (100 μg fibronectin for the fibronectin reagent, collagen type I and II for the collagen reagent and fibrinogen for the fibrinogen reagent) was added, and the mixtures were kept at 30°C for 12 h on a horizontal shaker at 50 rotation per min. The mixtures were centrifuged at 9200 x *g* for 5 min at 20°C, and the supernatants were discarded. The pellet was resuspended in 2 ml of gly buffer containing 0.01 % ovalbumin and 0.01 % Merthiolate and kept at 4°C for 12 h.

Latex reagent (1 drop; 20 μl) was placed on a glass slide, and equal volume of bacterial cell suspension was added. The 2 drops were gently mixed, and the agglutination reaction was read after 2 min. The reactions were scored from strongly (+++) to weakly positive (+) or negative (-). Strains were checked for autoaggregation by mixing 1 drop of bacterial cell test suspension with 1 drop of gly buffer.

2.4.2. Extracellular matrix (ECM) protein binding assay

Microtiter plates were coated with extracellular matrix proteins by incubating 100 μl of the appropriate protein, diluted in coating buffer (0.25 M $NaHCO_3$, 0.25 M Na_2CO_3 pH 9.6 or phosphate buffer saline (PBS) to 20 μg of

protein per ml or the concentrations indicated below, in assay wells overnight at 4°C (for collagens) or 35°C (for fibronectin and laminin). Wells were then washed three times with phosphate buffer saline (PBS) pH 7.5 containing 0.05 % Tween 20 (PBST), blocked with 100 µl of 2 % bovine serum albumin (BSA) in PBST for 2 h at room temperature, and washed three times with PBS. Bacteria were grown overnight on Luria-Bertani agar, harvested, washed, and suspended in PBS to the desired optical density at a wavelength of 690 nm (OD_{690}~0.15 ca. 10^9 cells per ml). 100 µl of the bacterial suspensions were added to each well, and the plates were incubated at 37°C for 1 h 30 min. Wells were then washed four times with PBST to remove unbound bacteria. 200 µl of 2 % formaline in PBS was added to each well for fixation. After 1 min, wells were washed once with PBS and 200 µl of 0.13 % Crystal Violet solution was added to each well for 15 min. Wells were washed then three times and 200 µl 1 % Sodium dodecyl sulphate (SDS) in ethanol was added for solving the bacterial cells. The absorbance at a wavelength of 590 nm (A_{590}) was measured for each well (Ljungh, A. and T. Wadström 1995).

2.4.3. Serum bactericidal assay

Normal human serum was obtained from healthy volunteers and was used freshly. Bacteria were grown in 5 ml of Tryptone soy broth (TSB) at 37°C overnight. 1 ml of overnight culture was added to 25 ml of TSB and was incubated at 37°C in shaker thermostat. An early log phase TSB culture was washed three times with PBS and the bacterial cells were suspended in PBS to the desired optical density at a wavelength of 690 nm (OD_{690}~0.15). The bacterial suspension was diluted to 10^6 bacterial cells per ml.

A sample (0.5 ml) of this suspension was added to 1.5 ml of serum, and viable counts were obtained at the beginning of the test and after 1, 2, and 3 h of incubation at 37°C, respectively. Viable bacterial cells were determined in 10 µl sample on Nutrient agar plate (Taylor, P.W. and C. Hughes 1979).

2.4.4. Haemolysin assay

Bacteria were cultured in either Luria-Bertani (LB) broth or Brain-Heart Infusion (BHI) broth (Oxoid) to an optical density of 0.8 at 600 nm (OD_{600}) unless indicated otherwise. Cells were pelleted by centrifugation and the spent media was passed through a 0.45 μm pore size filter.

Sheep, bovine, and human (O, Rh^+) erythrocytes (RBCs) were obtained fresh, defibrinated with glass beads, stored at 4°C and used within 1 week. Haemolysin assays were performed as described previously (Smith, W.H. 1963, Bauer, M.E. and R.A. Welch 1997). Briefly, serial dilutions of culture supernatants were incubated with a 2 % suspension of RBCs in 0.9 % NaCl and 10 mM $CaCl_2$ at 37°C for 1 hour. Intact erythrocytes were removed by centrifugation. The relative haemolytic activity was determined from the release of haemoglobin, as measured spectrophotometrically at a wavelength absorbance 543 nm (A_{543}).

2.4.5. Motility assay

Bacterial cells were inoculated into the centre of 35 mm diameter plastic dishes containing 0.2 % Luria-Bertani agar. Cultures were incubated at 37°C for 16 h. Cultures were examined at different timepoints (0, 3, 6, and 24 h) during growth. Diameter of the swarming area was measured.

2.4.6. Siderophore production assay

Plates of Minimal 9 (M9) medium agar containing a Fe-Chrome Asurol S-hexadecyltrimethylammonium bromide (Fe-CAS-HDTMA) complex were seeded with bacteria and were incubated for 24 h at 37°C. Colonies that produced siderophore changed the colour (blue to orange) of the medium around the colony.

2.4.7. Bacterial growth rate assay

Approximately 10^5 bacterial cells were inoculated into Minimal 9 (M9) liquid media and Luria-Bertani broth. The cultures were incubated at 37°C. 10 µl of cultures and their 10 fold dilutions were plated on Luria-Bertani agar plates for determining the number of colony formings at different timepoints (0, 3, 5, 8, and 24 h) of incubation.

2.4.8. Bacterial haemagglutination assay

Bacterial cells were grown on blood agar plates overnight. Haemagglutinations were performed with 8 % red blood cells (RBCs) in phosphate buffer saline (PBS) pH 7.2 in the presence of 2 % mannose and without mannose on glass slides.

2.5. Animal models

2.5.1. Lung toxicity assay in mice

Carworth Farm Lane Petter (CFLP) specific pathogen-free (SPF) hygienic category outbred mice (Gödöllő, Hungary) weighting 10-12 g were infected intranasally under superficial ether anaesthesia with 0.05 ml overnight nutrient broth culture, containing approx. 3 x 10^9 bacteria per ml. The death rate was recorded hourly up to 8 h and at 24 h and 48 h after infection. Pathomorphological changes such as haemorrhagic lung oedema were checked by autopsy (Kétyi, I. *et al.*, 1978).

2.5.2. Assay for neutralisation of lung toxicity in mice

Sera were collected from immunised rabbits and from patients, who suffered from alpha haemolysin producing *E. coli* infection. Neutralisation was carried out by mixing 0.025 ml volumes of overnight broth cultures with 0.025 ml

of serum dilutions and incubating at 37°C for 30 min. The infection was performed as described in lung toxicity assay. Mice were investigated for 48 h and deaths were recorded hourly up to 8 h and at 24 h and 48 h (Emődy, L. *et al.*, 1979).

2.5.3. Assay for nephrovirulence in adult mice after iv. injection

Approx. 2.5 x 10^8 log phase cells of the strains were injected intravenously into 20-22 g female CFLP outbred mice (Gödöllő, Hungary). The kinetics of bacterial counts in the mouse kidney, blood and spleen was followed at different time intervals (15 min., 3 h, 5 h, and 8 h) up to 8 h after injection (Van den Bosch, J.F. *et al.*, 1979).

2.5.4. Assay for virulence in suckling mice after injection into the urinary bladder

3-5 day old CFLP outbred mice (Gödöllő, Hungary) were injected into the urinary bladder with 0.025 ml volumes of diluted fresh broth cultures containing approx. 10^5 bacteria and 0.05 % Pontamin Sky Blue (6XB Searle, England). The dye indicator of success of inoculation did not have antibacterial activity. Mice were investigated on the 21st day after infection by macroscopic observation and determination of the viable counts in bladder and kidneys. Heart blood was also checked by bacteriological investigation (Kétyi, I. 1981).

2.5.5. Assay for elimination of bacteria in mice after iv. injection

Female CFLP outbred mice (Gödöllő, Hungary) of 20-22 g body weight were injected intravenously with approx. 2.5 x 10^8 bacterium cells. The viable counts were determined in kidneys, spleen, heart blood, and eyes of sacrificed mice in different time intervals (1, 2, 4, 6, 8, 10, 12, and 14 days) after infection (Hacker, J. *et al.*, 1986).

2.5.6. Death rate of mice after intraperitoneal injection

Female CFLP outbred mice (Gödöllő, Hungary) of 20-22 g body weight were injected intraperitoneally with approx. 2.5 x 10^8 bacteria. Mice were investigated over a period of 14 days. Symptoms and death were recorded. Following the death of mice macroscopically visible pathomorphological changes were recorded and pathomorphologically changed organs were investigated by histological methods (Haematoxylin and eosine stain and Periodic-acid Shiff stain).

2.5.7. Death rate of mice after intravenous injection

Female CFLP outbred mice (Gödöllő, Hungary) of 20-22 g body weight were injected intravenously with approx. $2.5x10^8$ bacteria. Observation and investigation was performed as described in assay for virulence in mice after intraperitoneal injection.

2.5.8. Lethality assays (LD_{50})

Overnight grown bacterial strains in nutrient broth were pelleted by centrifugation. The pellets were diluted to approximately 10^9 bacterial cells per ml in 0.9 % saline. Further fivefold dilutions were carried out. 0.5 ml aliquots of the dilutions were given by the intravenous route to 5-5 CFLP outbred mice of 20-22 g body weight. Death was recorded and LD_{50} was calculated by the methods of Kärber (Kärber, G. 1931).

2.5.9. Mouse colonisation experiment

12-week-old CFLP outbred mice (Gödöllő, Hungary) of 25 g body weight were kept separate in sterile jars throughout the experiments. The jars were replaced daily and the animals were given sterile food. 50 mg of streptomycin-sulphate was given through a thin cannula into the oesophagus on two consecutive

days. This antibiotic treatment deprived the faecal *Enterobacteriaceae* flora. On the third day faeces was collected. Faecal samples were homogenised in 1 ml of 0.9 % saline and plated on eosin methylene blue agar plate. If bacterial growth was not detected, the mice were given 0.5 ml of nutrient broth containing approximately 3 x 10^9 bacterial cells trough a cannula into the oesophagus. Faecal samples were collected daily. The samples were homogenised in 0.5 ml of nutrient broth. 10 µl of the homogenisate was plated on eosin methylene blue agar containing antibiotics. The plates were incubated for 24 h at 37 °C. Bacterial colonies were identified by biochemical tests. If the inoculated *E. coli* strain could not be detected in mouse faeces for 3 consecutive days, the animal was considered to be free from colonising strain.

2.5.10. Assay for virulence in chicken embryos

10-day-old Tetra B hybrid stock chicken embryos (Baksa, Hungary) were infected into the allantoic cavity with 0.1 ml volumes of dilutions of overnight nutrient broth cultures. Inocula containing 3 x 10^0, 3 x 10^1, and 3 x 10^2 bacteria were given to groups of 5 eggs. Eggs were checked by candling. Death was recorded at 24 h and 48 h after infection. The results were given in rate of the number of survivors per infected embryos (Powell, C.J. and R.A. Finkelstein 1966, Emődy, L. *et al.*, 1980).

2.5.11. Immunisation of rabbits (antisera production)

Blood for serum was collected from rabbits before the immunisation. Bacterium cells were washed off in 0.9 % saline from nutrient agar cultures. Half of them were inactivated by autoclaving. Inoculations were given by intravenous route. Gradually increasing doses (0.2, 0.5, 1.0, and 2.0 ml) of 10^9 live or inactivated bacteria per ml were inoculated at 4-day intervals. Blood for serum was collected after the booster inoculations and was checked for titre. The rabbits were exsanguinated when appropriate titre (≥1:5120) was achieved. Sera were sterile

filtered and stored at -20°C (Herbert, W.J. 1978, Darling F. and W.J. Herbert 1996).

2.6. *In vitro* DNA manipulation

Standard DNA manipulations were performed as described by Sambrook *et al.* (Sambrook, J. *et al.*, 1989) and according to Current Protocol in Molecular Biology (Moore, D.D. *et al.*, 1987). See Appendix B.

2.7. Immunological screening

Immunological screening was by a modification of the method of Helfman *et al.* (Helfman, D.M. *et al.*, 1984) using the monoclonal antibody SM14 (Virji, M. unpublished).

1. After overnight growth on selective media at 37 °C replicas of colonies were made using sterile nitrocellulose filters that had been keyed to each plate using a series of pinpricks. Original plates were stored at 4 °C until required.
2. Colonies were lysed in chloroform vapour for 30 min.
3. Filters were then incubated for 1 h. in Bovine serum albumin (BSA) blocking buffer (Tris-HCl 0.05 M, pH:7.6; NaCl 0.15 M; Bovine serum albumin 3.0 % [w/v]) at room temperature to block residual binding sites.
4. Filters were washed in 10 ml Tris-Saline (TS) for 5 min.
5. Following removal of TS, filters were incubated in 10 ml of Tris-Saline-albumin (TSA) buffer, containing antibody and incubation continued for 16 h at 30 rpm.
6. Filters were washed twice with TS, prior to incubation for 1.5 h in 10 ml of a solution containing calf intestine alkaline phosphatase-conjugated anti-mouse secondary antibodies diluted in TSA.
7. Finally, unbound antibodies were removed by washing filters 5 times, over a period of 30 min. in TS.
8. BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue-tetrazolium)

one component phosphatase substrate (ICN) was used for colour development. Positive colonies were identified by colour reaction (purple).

2.8. DNA-DNA hybridisation

2.8.1. DNA slot blotting

Genomic DNA (approximately 10 μg) prepared by the rapid method described in Appendix B was applied to the surface of a positively charged nylon membrane filter (Boehringer Mannheim's) that had been presoaked in distilled water and fixed in a Biorad slot blot minifold apparatus. Any minifold holes that were not required for sample application were sealed with parafilm. Suction from a water pump was adjusted so that at least 5 min. was required to aspirate the sample trough the filter. Following removal of the membrane filter from the apparatus it was treated as described in the DIG System User's Guide (Boehringer Mannheim's Roche Diagnostics).

2.8.2. Southern hybridisation

Following agarose gel electrophoresis, DNA was transferred to positively charged nylon membrane filters by the method of Southern (Southern, E.M. 1975) with the modification of Sambrook *et al.* (Sambrook, J. *et al.*, 1989). Southern hybridisation were carried out according to the DIG System User's Guide (Boehringer Mannheim's Roche Diagnostics).

2.9. DNA labeling

PCR DIG Probe Synthesis Kit (Boehringer Mannheim's Roche Diagnostics) was used for DNA labeling as directed by the vendor.

2.10. PCR amplifications

2.10.1. "Hot start" nested PCR for meningococcal *porA* gene

Nested PCR method described by Saunders *et al.* (Saunders, J R *et al* 1993) was used.

First round.

A "master mix" solution was made up as follows;

sterile water	31 µl
10 x Taq Buffer	10 µl
$MgCl_2$ (50 mM)	4 µl
dNTPs	10 µl (20 µmol)
primer (AR03)	5 µl (20 pmol)
primer (AR04)	5 µl (20 pmol)
Total	65 µl

Sixty-five microlitres of master-mix was made for each sample and positive control, plus 65 µl extra for a reagent control. The master-mix was placed on ice.

Sample preparation

The bacteria were suspended in 25 µl sterile water and then denatured at 95°C for 10 minutes. Samples were then centrifuged and placed on ice. 65 µl of master-mix was added to each sample and overlaid with mineral oil. Tubes were placed in thermal cycler and heated to 90°C for 5 min. Taq DNA polymerase (2.5 units in 0.5 µl) was diluted 1 in 20 with sterile water, and 10 µl was added to each sample after they had been heated for 5 min. ("hot-start").

This was followed by 15 cycles of;

95°C for 1.5 min.
70°C for 3 min.

then 15 cycles of;

95°C for 1.5 min
70°C for 4 min.

Second round

A master-mix solution was made up as follows;

sterile water	46 µl
10 x Taq Buffer	10 µl
$MgCl_2$ (50 mM)	4 µl
dNTPs	10 µl (20 µmol)
primer (AR05)	5 µl (20 pmol)
primer (AR06)	5 µl (20 pmol)
Total	80 µl

Eighty microlitres of master-mix was placed in each sample tube and stored on ice.

10 µl of the first round product was added to the master-mix in each sample tube and overlaid with mineral oil. Tubes were placed in a thermal cycler and heated to 90°C for 5 min and then had a "hot-start" with the addition of diluted Taq DNA polymerase, as before.

This followed by 30 cycles of ;

95°C for 3 min.
50°C for 2 min
72°C for 2 min.

Samples were analysed by electrophoresis on a 1.2 % agarose gel.

2.10.2. PCR amplification for *pilE* gene

Amplification was performed with;

Taq DNA polymerase (5U/µl)	0.5 µl
dNTPs (10mM)	1.0 µl
primer (N-term) (10 pmol)	2.0 µl
primer (C-II) or (1) (10pmol)	2.0 µl
sterile water	73.0 µl
$MgCl_2$	12.0 µl
10 x Taq buffer	10.0 µl
Bacterial suspension	5.0 µl

Each sample was overlaid with mineral oil. Tubes were placed in a thermal cycler and heated to 90°C for 5 min. and this followed by 30 cycles of;

95°C for 1 min.

45°C for 1 min
72°C for 1 min.

Samples were analysed by electrophoresis on a 1 % agarose gel.

2.10.3. PCR amplification for IgA protease encoding gene

Amplification was performed with:

Taq DNA polymerase (5U/µl)	0.5 µl
dNTPs (10mM)	1.0 µl
primer (IgA Pro1) (10 pmol)	2.0 µl
primer (IgA Pro2) (10pmol)	2.0 µl
sterile water	73.0 µl
$MgCl_2$	12.0 µl
10 x Taq buffer	10.0 µl
Bacterial suspension	5.0 µl

Each sample was overlaid with mineral oil. Tubes were placed in a thermal cycler and heated to 90°C for 5 min. and this followed by 30 cycles of;

95°C for 1 min.
48°C for 1 min
72°C for 1 min.

Samples were analysed by electrophoresis on a 1 % agarose gel.

2.11. Electron microscopy

The piliation state of each of the neisserial isolates was determined by electron microscopy (Prof Hart, C.A., personal communication).

2.12. Statistical analysis

Statistical analysis was performed according to Horvath (Horvath, I. 1974) and Dawson-Saunders and Trapp (Dawson-Saunders, B. and R.G. Trapp 1994). Poisson and gaussian distribution, chi-square (X^2) and t tests were used for calculation. Standard computer program was used to produce statistical analysis and to illustrate the results.

Chapter 3.

Fimbrial expression, matrix protein binding and organotropy of *Escherichia coli*

3.1. Introduction

Fimbriae or pili, assembled and expressed on the surface of Gram negative bacteria, have been studied in numerous organisms and have been shown to establish a critical link in communication among bacteria, and between bacteria and eucaryotic cells. The initial step in most bacterial attachment to the host epithelium is adherence by fimbriae. The fimbrial adhesins bind to receptors on epithelial cells. This interaction of fimbriated pathogenic bacteria has been studied extensively.

One of the main host defences of the urinary tract is the washing action of urine. Bacteria that do not adhere will be washed out of the bladder faster then they can multiply. Thus the key feature of uropathogenic *E. coli* strains is their ability to adhere to bladder mucosa. Also, adherent bacteria are in close proximity to mucosal cells and are thus in a better position to provoke an inflammatory response than bacteria growing in the lumen of the bladder. A variety of fimbrial adhesins, such as P, S, Dr, and type 1 fimbriae may be expressed on the surface of uropathogenic *E. coli* strains. Although type 1 fimbriae are commonly found on *E. coli* strains from resident intestinal microflora and are not limited to virulent strains, they are thought to contribute primarily to the colonisation of the bladder. A type 1 fimbria is a thin, 7 nm-wide and approximately 1 μm-long, rod-shaped surface organelle. It is a heteropolymer consisting of four different subunits. Approximately 1000 copies of the major building element, FimA are polymerised into right-handed helical structure also containing small percentages of the minor components, FimF, FimG, and FimH (Klemm, P. *et al.*, 1988). The receptor-recognising element of type 1 fimbriae is the 30 kDa FimH protein (Krogfelt, K.A. *et al.*, 1990). By virtue of the FimH adhesin, type 1 fimbriated bacteria confer α-D-mannose-sensitive agglutination of a number of eucaryotic cell types displaying this molecular motif, such as certain erythrocytes and yeast cells. FimH recognises terminally located D-mannose moieties on cells that bind and secret glycoproteins (Wold, A.E., *et al.*, 1990) and can bind to nonglycosylated peptide epitopes (Sokurenko, E.V., *et al.*, 1994.). Receptors for type 1 fimbriae are present in blood vessel walls and in the muscular layers but not on the epithelium of the

human bladder. Type 1 fimbriated bacteria bind to ureteral epithelium and to human and some animal kidney cell lines. However, the scanty binding sites for type 1 fimbriated cells within the human kidney are limited to vascular connective tissue layers of vessels and the cytoplasm and lumenal surfaces of proximal tubular cells; the distal tubules, collecting ducts, glomeruli, and vascular endothelium are devoid of receptors (Virkola, R. 1987). Immunisation with FimH was shown to prevent urogenital mucosal infection by *E. coli* in mouse model (Svanborg Eden, C. *et al.*, 1982). The chromosomally located *fim* gene encodes the components of the fimbrial organelle (Klemm, P. *et al.*, 1987). Expression is phase variable, with individual cells switching between fimbriated and nonfimbriated states. Two recombinases, FimB and FimE mediate the inversion of the phase switch (Gally, D.L. *et al.*, 1996). FimB and FimE are members of the tyrosine recombinase family (Esposito, D. and J.J. Scocca 1997).

Perhaps the most important type of adhesin, especially in strains that cause kidney infections, is P fimbria (Vaisanen, V. *et al.*, 1981). This adhesin is mannose-resistant and recognises the disaccharide α-D-galactosyl-(1-4)β-D-galactose on the P blood group antigen (enabling it to bind red blood cells) and on uroepithelial cells from the majority of the population (Kallenius, G. *et al.*, 1981). α-D-galactosyl-(1-4)β-D-galactose moiety is present on certain mammalian cells primarily as the carbohydrate component of glycosphingolipids. P-fimbriated strains differ slightly in their binding specificity, depending on expressed adhesins. In some cases, Forsmann antigen recognition is due to the presence of a variant adhesin termed Prs (for P-related sequence) or Pap-2 (for pilus associated with pyelonephritis). Purified P fimbriae or whole P fimbriated bacteria adhere to proximal tubular epithelial cells, mesangial or endothelial cells, distal tubule, collecting ducts, or Bowman's capsule (Korhonen, T.K. *et al.*, 1986). P fimbriae also bind to epithelial and muscular layers of the bladder.

P fimbriae are composed of approximately 10^3 helically polymerised subunits, with one major subunit species (PapA – 19.5 kDa) constituting the bulk of the fimbria. Three minor adherence-related fimbrial subunits (PapE – 16.5 kDa, PapF – 15 kDa, and PapG – 35 kDa) are present in minute amounts at the fimbrial tips. PapF-PapG constitutes the minimal adhesin complex, which is linked to

fimbriae by PapE. An alternative adhesin is (PrsG). These fimbrial proteins are encoded by a chromosomal multicistronic gene cluster termed *pap*. P fimbriae are subject both to rapid, random phase variation and to environmental influences. P fimbriation is favoured by growth at 37 °C and inhibited by growth at 18-22°C.

S fimbriae are more closely associated with *E. coli* causing neonatal septicaemia and meningitis, than with urinary tract infection (Ott, M. *et al.*, 1985). S fimbria is the other mannose-resistant adhesin with a binding specificity for terminal sialyl((2-3)galactoside, which is found on many epithelial surfaces as a structural component of glycoproteins or glycolipids. Binding sites for S fimbriae are found on epithelial cells of proximal and distal tubules, collecting ducts, and glomerulus; in the renal interstitium; and on renal vascular endothelium (Korhonen, T.K. *et al.*, 1986). In the neonatal rat brain, S fimbriae bind efficiently to epithelial cells lining the choroid plexuses and brain ventricles and to subarachnoid endothelium (Parkkinen, J. *et al.*, 1988.). Expression of S fimbriae exhibits phase variation (Nowicki, B. *et al.*, 1985). Nine chromosomally encoded *sfa* genes are involved in the production of the S fimbriae, and the fimbrial filament is composed of four subunits, the major subunit being termed SfaA (16 kDa) and the tree minor components being termed SfaG (17 kDa), SfaH (29 kDa), and SfaS (15 kDa). SfaS is the sialylgalactoside-binding adhesive subunit present probably in a single copy at the tip of the fimbrial filament (Schmoll, T. *et al.*, 1989).

The urine contains a variety of free oligosaccharides and a considerable amount of protein bound saccharides, those soluble receptorlike carbohydrates, which may inhibit the bacterial adhesion to the urinary tract epithelial cells. The major inhibitor of S fimbriae was identified as Tamm-Horsfall (T-H) glycoprotein. The urinary Tamm-Horsfall protein may serve as a clearence factor for *E. coli* expressing S fimbriae (Parkkinen, J. *et al.*, 1988). This may give explanation for the low clinical relevance of S-fimbriated *E. coli* in urinary tract infections.

Previously purified S fimbriae and S fimbriated *E. coli* strains were given to the target cells of frozen section of human kidney for showing tissue tropism. In this thesis the tissue tropism and organ tropism of fimbriated *E. coli* strains in

haematogenous spread and through the ascending route from the bladder is described. S fimbriated uropathogenic *E. coli* strain evoked symptoms of brain damage in some mice after intravenous infection, but the symptoms are different from those that caused by O18ac:K1:H7 *E. coli* strains, which are associated with new-born meningitis and carrying S fimbriae. We tested the binding of different fimbriated *E. coli* strains to extracellular matrix proteins. The bacterial binding of extracellular matrix protein may induce structural and functional alterations of the proteins, which in turn may activate other cellular mechanisms and enhance tissue invasion of bacterial pathogens. These mechanisms may contribute to dissemination of infection.

All the strains used were derived from the wild-type isolate *E. coli* 536. The strain 536 was isolated from a patient suffering from urinary tract infection. The strain belongs to the serotype O6:K15:H31 and exhibits S fimbriae with Sfa adhesin, type 1 fimbriae, and P fimbriae with Prs adhesin. In addition the strain is serum resistant, and produces alpha-haemolysin and enterobactin.

A spontaneous mutant, 536-21, has lost the pathogenicity islands and became nonhaemolytic, sensitive to human serum, and unable to express the fimbriae and to produce the siderophore. This strain was used as recipient for the introduction of different cloned fimbrial adhesin determinants.

Both S fimbriae and serum resistance were regained following transformation and subsequent integration of a *sfa*-cosmid into the chromosome of mutant 536-21 (Marre, R. *et al.*, 1986). The resulting strain 536-31 was used in assays.

E. coli 536-40 is the derivative of 536-21 with chromosomal integration of the plasmid pGB30 coding for type 1 fimbriae (Hacker, J. personal communication).

The genetic determinant coding for the P specific F8 fimbriae was ligated into the cosmid vector pHC79 and transduced into 536-21 with the help of the cosmid packaging system and subcloning was done into pBR322. The resulting recombinant plasmid, pANN921, which had lost ampicillin resistance, still expressed resistance to tetracycline and conferred P-specific haemagglutination and fimbria formation on 536-21 (Hacker, J. *et al.*, 1986).

All these strains and the wild type strain were used for *in vivo* and *in vitro* tests. Characteristics of *E. coli* 536 strain and its derivates are shown in Table 3.1.

Table 3.1. Characteristics of *E. coli* and its derivates

	Phenotypic characters				
Derivates	Hly	Prf	Fim	Sfa	HA
Wild type 536	+	+	+	+	MRHA, MSHA
536-21	-	-	-	-	-
536-31	-	-	-	+	MRHA
536-40	-	-	+	-	MSHA
5362-21pANN921	-	+	-	-	MRHA

Hly: haemolysin; Prf: P-related fimbriae; Fim: type 1 fimbriae; Sfa: S fimbrial adhesin; HA: haemagglutination;
MRHA: mannose-resistant haemagglutination with human A, Rh^+ erythrocytes;
MSHA: mannose-sensitive haemagglutination with guinea pig erythrocytes.

3.2. Extracellular matrix protein binding

The uropathogenic *E. coli* strain and its derivatives were tested for binding to subepithelial connective tissue proteins, fibronectin, type I collagen, and laminin. Strain with the ability to bind these proteins may have selective advantage to colonize the tissue.

The results of extracellular matrix protein binding are shown in Table 3.2. As seen, binding to fibronectin, type I collagen, and laminin was expressed only in the wild type strain and its fimbriated derivatives.

Table 3.2. Expression of binding of extracellular matrix proteins in *E. coli* 536 and its derivatives

	Extracellular matrix protein		
Derivates	fibronectin	Type I collagen	Laminin
Wild type 536	+	+	+
536-21	-	-	-
536-31	+	+	+
536-40	+	+	+
5362-21pANN921	+	+	+

3.3. Animal assays with uropathogenic *E. coli* strain 536 and its derivatives

The wild type isolate and its derivatives were tested in three short term assays and a long-term mouse assay.

The lung toxicity assay, intravenous lethality and 8 hours intravenous nephrovirulence assays are represented as short-term mouse assays.

The uropathogenic wild type isolate was lethal after intranasal infection to mice with 2.5 x 10^8 bacteria. All mice infected with wild type strain exhibited clinical symptoms of the lung disease and 80 % of the animals died. All of the derivatives were avirulent for mice in this assay.

All the killed mice exhibited pathomorphological changes in the lung tissue including several haemorrhagic lesions.

After intravenous injections of the wild type strain the mice died within one day. Haemoglobinuria was observed more than six hours after infection indicating the presence of intravasal haemolysis. The deletion mutant 536-21 was avirulent causing no death of mice. No symptoms developed in animals during the observation. Strains 536-31, 536-40, and 536-21pANN921 exhibited an equal level of intermediate virulence. After 24 hours obvious symptoms of septicaemia were observed in the majority of the animals. Symptoms of brain damage were found in some mice infected with strain 536-31. The septicaemia either subsided

or led to death in a comparable ratio for the three strains. Haemoglobinuria could not be observed.

In the short term nephropathogenicity assay only the wild type strain showed the pattern characteristic for nephropathogenic group II strains according to van den Bosch *et al.* (van den Bosch, J.F. *et al.*, 1982). The other strains belonged to group I, that means they proved to be avirulent in this test system. Table 3.3. presents the results of the short term mouse virulence assays.

Table 3.3. Short term virulence test in mice with *E. coli* 536 and its derivatives

Derivative	Virulence test		
	Lung toxicity (% killing)	Intravenous lethality killed/infected	Nephrovirulence (I, II)*
Wild type 536	80	50/50	II
536-21	0	0/50	I
536-31	0	19/50	I
536-40	0	16/50	I
536-21pANN921	0	17/50	I

*I, avirulent; II, nephrovirulent; according van den Bosch *et al.*, 1982

Suckling mice were given 10^5 bacterial cells intravesically in the long-term mouse virulence assays. The wild type strains proved to be highly virulent in the ascendent model. All mice died within two days after infection. Strain 536-21 did not kill the animals and could be isolated from the site of the infection only in half of the animals after three weeks (Table 3.4.). Strains 536-31, 536-40, and 536-21pANN921 caused long lasting infection of the bladder more frequently, and each strain was able to ascend to the kidney in most of the animals. The median values of the bladder and renal bacterial counts remained lower than the infective dose. None of the animals with bladder and kidney infection produced a positive culture from the heart blood indicating that the presence of bacteria in the bladder and kidney was not the part of a systemic infection. It means the infection caused by derivatives remained restricted to the urinary tract in this assay.

Table 3.4. Virulence of *E. coli* 536 and its derivatives in mice infected intravesically

Derivative	Rate of bladder infection*	Rate of kidney infection*
Wild type 536	All of the 14 infected mice died within 48 h after infection	
536-21	12/25 (7.5×10^2)	0/25
536-31	16/16 (1.4×10^3)	14/16 (1.2×10^2)
536-40	23/28 (5.7×10^3)	17/28 (3.9×10^2)
536-21pANN921	15/15 (6.8×10^2)	14/15 (1.6×10^2)

*positive culture 21 days after intravesical injection/total infected
in parenthesis: median number of viable bacterial cells in positive organs

3.4. Discussion and conclusion

E. coli 536 strain is a urinary isolate, and it has been shown to possess several virulence factors encoded by DNA segment located on four pathogenicity islands of the bacterial chromosome (Hacker, J. *et al.*, 1999). The spontaneous loss of two pathogenicity islands and construction of recombinant strains expressing one or more of the virulence factors provided us with the possibility to assess the pathogenic role of the individual virulence traits in an isogenic bacterial background. In this study we show that an acute toxic effect by haemolysin production plays a pivotal role in the lethality of the wild type strain in mouse models regardless of whether the route of the infection was ascendent, haematogenous, or bacteria were administered intranasally. It is also sown that non-haemolytic derivatives expressing S or Type-1 or P fimbriae are more virulent in our mouse assays - including the ascendent one - than the background strain 536-21. Data concerning the role of S, Type-1, and P fimbriae in an ascendent urinary infection are not unequivocal. Schaffer *et al.* (Schaffer, A.J. *et al.*, 1987) found that *E. coli* producing Type-1 fimbriae colonised the bladder and kidney of mice with a higher frequency than non-fimbriated strains. It was also published that Type-1 and S fimbriae enabled bacteria to bind not only to cell surface structures but also to the components of tissue matrix (Korhonen, T.K. *et al.*, 1992). Matrix protein binding was also expressed by *E. coli* 536 and its

fimbriae producing derivatives in our experiments. Although inhibitory compounds affecting the binding to cell surface and matrix components are present in the urinary tract (Korhonen, T.K. *et al.*, 1992), a high density of receptors in individuals prone to urinary infections may direct the process of host-parasite interaction towards an effective bacterial colonisation. Also in the less frequent cases of haematogenous pyelonephritis fimbrial binding to target tissue cell surface and interaction with matrix components might be involved in tissue colonisation. This assumption is supported by the finding that tissue tropism toward the kidney could be observed when S-fimbriae producing bacterial cells were administered intravenously. Further investigations are needed to elucidate the significance of these interactions in the process of urinary tract infections in humans.

Chapter 4.

Relations between alpha-haemolysin production and lung toxicity; antitoxic effect of human and rabbit sera against *Escherichia coli* and *Proteus morganii* lung toxin

4.1. Introduction

Haemolytic *Escherichia coli* strains were isolated from the normal stool flora and both extraintestinal and intestinal infections of man. These strains characteristically carry the haemolysin determinant on the chromosome while in *E. coli* strains of animal origin the genes encoding haemolysin production are located on large transmissible plasmids(Müller, D. *et al.*, 1983, Hacker, J. and C. Hughes, 1985)

Alpha-haemolysin producing *E. coli* strains occur in high percentage among those strains causing urinary tract infection and bacteraemia. Recently there have been rapid developments in our understanding of the role of α-haemolysin in the pathogenesis of human disease. The effects of this protein were described on leukocytes, red blood cells (RBCs), and different cell lines *in vitro* and on renal tubular cells *in vivo* (Keane, W.F. *et al.*, 1987). Intranasal application of α-haemolytic *E. coli* strains in mice exhibit strong toxic effect in the lung causing haemorrhagic lung oedema and death within a couple of hours (Figure 4.1.) (Kétyi, I. *et al.*, 1978).

Figure 4.1. Haemorrhagic lung oedema in the mouse caused by *E. coli* J96.

Investigating 146 *E. coli* isolates we could find an almost 100 percent positive correlation between α-haemolysin production and lung toxicity. All of the 73 *Hly*⁻ strains were negative in the lung toxicity assay, and only a single α-haemolytic strain out of 73 failed to evoke oedema and death. It is remarkable that in contrast to the extracellular α-haemolysin, the lung toxin seems to be cell associated because its activity can be evoked only by bacterial cells or sonicates thereof (Kétyi I., *et al.*, 1978). These incongruities prompted us to check the lung toxicity of recombinant *E. coli* strains with cloned haemolysin determinants. At the same time we also investigated if antitoxic antibodies are induced in experimental infections in rabbits or during natural infections in human beings.

Proteus morganii and *Proteus penneri* strains can produce haemolysin, which is related to the α-haemolysin of *E. coli* (Koronakis, V. *et al.*, 1987) and also belong to the RTX family. Haemolytic *Proteus* strains similarly to haemolytic *E. coli* strains exhibit a toxic effect in the lung of mice (Emődy, L. *et al.*, 1982). We investigated if antitoxic antibodies induced by haemolytic *E. coli* strains neutralise the lung toxic effect of *Proteus morganii*.

Wild type *E. coli* strains were from various European countries, including human and animal isolates from intestinal and extraintestinal sources. *P. morganii* strains were from the collection of Dr Vörös in our department. The single haemolytic but non lung toxic *E. coli* strain Pm152 was isolated from mouse faeces in England (collection of Smith, H.W.).

The strain J96 was isolated from a patient suffering from urinary tract infection. The strain belongs to serogroup O4:K6 and exhibits Type-1 fimbriae and two types of P fimbriae (Pap and Prs), produces alpha-haemolysin and CNF-1 toxin. The strain is serum resistant.

The strain J198 is a nonhaemolytic faecal isolate from a healthy human. This strain was used as recipient for the introduction of various cloned haemolysin determinants.

Chromosomal *hly* determinant of the urinary tract isolate J96 was cloned in cosmid and an 11.7 kb *Sal*I restriction endonuclease fragment encoding haemolysin production was subcloned into a vector plasmid pACYC184. The resulting recombinant plasmid pSF4000 was transformed into J198. The strain

WAF107 is this derivative of J198. The strain WAF108 is a transposon mediated haemolysin-negative mutant with Tn1 integrated in the structural haemolysin gene.

HlyCAB genes from pHly152 were cloned into the vector plasmid pACYC184 resulting pANN202-312. Strain J198 was transformed with this recombinant plasmid and renamed WAF111.

All these strains were used for *in vivo* and *in vitro* assays.

4.2. Animal assays with haemolytic and nonhaemolytic *E. coli* strains transformed with haemolysin determinants of chromosomal and plasmid origin

Two wild type haemolytic *E. coli* strains and recombinants harbouring their determinants were compared in the mouse lung toxicity assay. The strain J96 was earlier shown to be positive in this test. As shown in Table 4.1. the strain WAF107 that is constructed from the nonhaemolytic faecal isolate J198 by introducing haemolysin determinants of the strain J96 became not only haemolytic but also lung toxic. *E. coli* Pm152 was the single wild type haemolytic strain that failed to show toxic effect in the lung. The strain WAF111 which is J198 carrying cloned plasmid *hly* determinant of *E. coli* Pm152 was also haemolytic but non lung toxic. The DNA inserts in the cloning vector were different in strains WAF107 and WAF111. A transposon mutation in the haemolysin structural gene of *E. coli* WAF107 resulting the haemolysin negative mutant *E. coli* WAF108 abolishes the lung toxic activity (Figure 4.2, 4.3.).

Results of the intraperitoneal mouse virulence test, mouse nephrovirulence test, and chicken embryo virulence model are consistent with the results of lung toxicity assay (Table 4.1.).

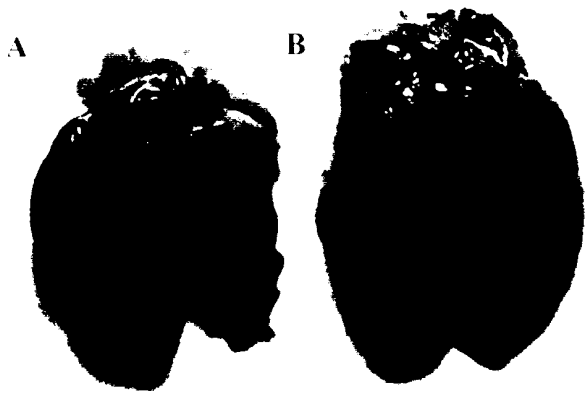


Figure 4.2. Lung toxicity assay. A: haemolysin negative mutant *E. coli* WAF108 did not cause changes in lung morphology. B: *E. coli* WAF107 (Hly^+) caused haemorrhagic lung oedema in the mouse.

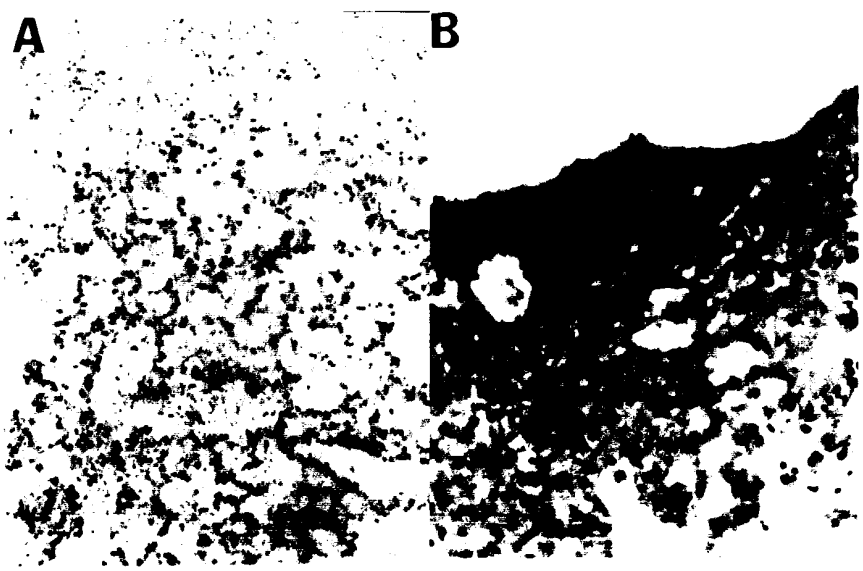


Figure 4.3. Photomicrographs of haemorrhagic lung oedema. /A/. Haematoxin (H) and eosine (E) stain and /B/. Periodic-acid Shiff stain of lung tissue.

Table 4.1. Virulence of *E. coli* strains harbouring isogenic haemolysin determinants

Virulence model	Bacterial clones					
	J96 *Hly*$^+$	J198 *Hly*$^-$	WAF107 *Hly*$^+$	WAF108 *Hly*$^-$	PM152 *Hly*$^+$	WAF111 *Hly*$^+$
Lung toxicity	8/10*	0/10*	3/10*	0/10*	0/10*	0/10*
Chicken embryo	10/10*#	1/10*#	7/10*#	1/10*#	0/10*#	2/10*#
i.p. LD_{50} in mouse	$5.5x10^8$	$>5x10^9$	$2.4x10^8$	$>5x10^9$	$3x10^9$	$3x10^9$
Nephrovirulence	yes	no	yes	no	no	no

*died/infected # allantoic infection with ~10^2cells

4.3. Neutralisation of *E coli* lung toxicity by rabbit and patient immune sera

It has been known that E. *coli* alpha-haemolysin is antigenic and induces the production of anti-haemolytic antibodies in experimental animals (Smith, W.H. 1963). We have also shown that infections with alpha-haemolytic *E. coli* may evoke the production of such antibodies in patients (Emődy, L. *et al.*, 1982). The immune sera of rabbits raised with live haemolytic *E. coli* cells elicit a neutralising effect against the toxicity of both the homologous and serologically heterologous strain (Table 4.2.). Immunisation with boiled cells resulted in the appearance of lower level antibacterial antibodies acting only in the homologous system.

Table 4.2. Neutralisation of *E. coli* lung toxicity by rabbit immune sera

Serum	Bacterium	ED_{50} (ml serum)
Anti O4:K12:H5 "live"	O4:K12:H5	$2.5x10^{-4}$
	O6:K?:H?	$2.5x10^{-3}$
Anti O4:K12:H5 "boiled"	O4:K12:H5	$1.0x10^{-2}$
	O6:K?:H?	$>2.5x10^{-2*}$

*Maximum amount of serum applicable in this assay

Immunisation with live cells of lung toxic *E. coli* induces neutralising antibodies against the toxic effect of *P. morganii*. The cross neutralising effect was also present when sera raised with live toxic *P. morganii* cells were tested against *E. coli* (Table 4.3.). It is remarkable that live non-toxic cells elicited antibodies acting also only in the homologous system.

Table 4.3. Cross-neutralisation of lung toxicity of *E. coli* and *P. morganii*

Immune sera #	Bacteria	ED_{50} (ml serum)
Anti-*E. coli* 2891/38	*E. coli* 2891/38	1.0×10^{-4}
	P. morganii 290	1.4×10^{-4}
Anti-*E. coli* 2891/A1*	*E. coli* 2891/38	1.6×10^{-2}
	P. morganii 290	$>2.5 \times 10^{-2}$
Anti-P. *morganii* 290	*E. coli* 2891/38	3.6×10^{-3}
	P. morganii 290	1.4×10^{-3}
Anti-P. *morganii* 290/1*	*E. coli* 2891/38	$>2.5 \times 10^{-2}$
	P. morganii 290	2.0×10^{-3}

Immune sera were produced with live bacterial cells
*Non-toxic derivatives

The experiments with sera from patients suffering from haemolytic *E. coli* infection compared with sera from healthy humans proved a high titre antitoxic antibody production during the disease. These antitoxin titres are comparable to anti-haemolysin titres (Table 4.4.). Changes in titres could be observed during the infectious process and recovery period (Figure 4.4.).

Figure 4.4. Anti-haemolytic and lung-toxicity neutralising antibodies in a patient's sera after infection with alpha-haemolytic *E. coli*

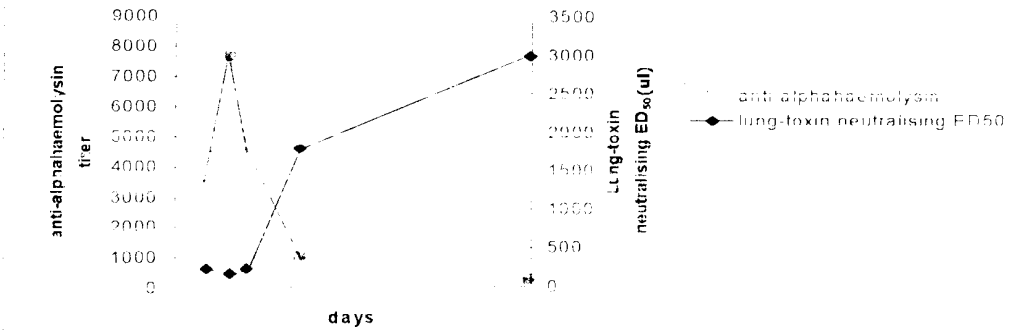


Table 4.4. Anti-haemolytic and anti-lung toxic effect of human sera

	Anti-Hly titre	Anti-lung toxic ED_{50} (ml)
Controls		
1.	1:34	$>2.5 10^{-2}$
2.	1:44	$>2.5 10^{-2}$
3.	1:56	$>2.5 10^{-2}$
4.	1:1088	7.2×10^{-4}
5.	1:1120	5.0×10^{-4}
Patients		
1.	1:896	1.8×10^{-4}
2.	1:1728	7.9×10^{-5}
3.	1:864	2.4×10^{-4}
4.	1:4352	7.9×10^{-5}
5.	1:2176	1.8×10^{-4}

4.5. Discussion and conclusion

The relation between haemolysin production and lung toxicity of *E. coli* and *P. morganii* was described (Emődy, L. *et al.*, 1981) but a single wild type haemolytic *E. coli* (Pm152) isolate from mouse faeces was found which failed to evoke haemorrhagic lung oedema. This strain and the haemolytic clinical isolate *E. coli* (J96) together with recombinants harbouring their haemolysin determinants were compared in different virulence tests and mouse lung toxicity assay. The haemolysin determinants were cloned into a non-haemolytic *E. coli* (J198), and changed the phenotype of the strain. The recombinant (WAF107) carrying haemolysin genes from the clinical isolate (J96) is haemolytic and lung toxic, while WAF108 strain including transposon mutation in the haemolysin structural cistron of WAF107 showed no haemolytic and lung toxic activity. The other recombinant strain (WAF111) containing haemolysin determinant from an animal (mouse) isolate (Pm152) produced haemolysin and was negative in the

lung toxicity assay similarly to the animal isolate (Pm152). The two recombinant *E. coli* strains (WAF107 and WAF111) differ only in the DNA insert in the cloning vector. This means that the non toxic character of animal isolate *E. coli* (Pm152) is not due to the bacterial background as its haemolysin determinant did not mediate toxicity in the *E. coli* (J198) background while that of the corresponding clinical isolate *E. coli* (J96) DNA did. Transposon mutation in the haemolysin structural gene abolishes haemolysis and lung toxicity. This suggests that the transposon insert is located in DNA segment which is necessary not only to haemolysin production but also to lung toxicity.

Results of the different virulence tests indicated that the difference in the DNA inserts of the two recombinants (WAF107 and WAF111) was involved in the expression of all tested virulence characters. Further analysis of the two inserts may identify the exact sequences representing the full genetic information of lung toxicity and the other virulence properties tested.

E. coli haemolysin induces the production of anti-haemolytic antibodies as antigen. The presence of such antibodies in patients suffering from haemolytic *E. coli* infection was proved. In our study the existence of antibodies neutralising lung toxicity was demonstrated. Antibodies induced in rabbit by live haemolytic *E. coli* cells elicited a neutralising effect against the toxicity of both the homologous and serologically heterologous haemolytic strain. Immune sera raised with boiled cells showed lower level protectivity acting only in the homologous system. This effect might be explained by the presence of antibacterial antibodies interacting with bacterial surface and thus blocking the close contact between the toxic strain and the mucosal surface. The lung toxic effect of *Proteus morganii* was neutralised by antibodies produced with lung toxic *E. coli*. Sera raised with a live toxic *Proteus* strain had cross-neutralising effects against lung toxic *E. coli*. Antibodies induced by live non-toxic cells act only in the homologous system. These results indicate that besides the already known relationship between the haemolysin of *E. coli* and *Proteus* species (Koronakis, V. *et al.*, 1987, Eberspächer, B. *et al.*, 1990) lung toxicity might also be induced in a similar way in these strains. However, it is not yet exactly known the contribution

of which bacterial factor(s) other than alpha-haemolysin is/are necessary to the development of this effect.

The rising titre of anti-toxic antibodies that were detected in sera of patients suffering from haemolytic *E. coli* infection during the disease were similar to anti-haemolysin titres. It is tempting to speculate that antibodies against this toxin were effective means of antitoxic immunity in the infected person. However, further studies are needed to exactly characterise the procedure leading to lung damage, and to evaluate its role in the pathogenesis and immunology of human infection.

Chapter 5.

The influence of the tRNA genes *leuX* and *selC* on the virulence of uropathogenic *Escherichia coli*

5.1. Introduction

On the chromosome the pathogenic determinants may be organised on so-called 'pathogenicity islands' (Pais), which represent large fragments of DNA. The uropathogenic strain 536 (O6:K15:H31) has four Pais. The sizes of the Pais vary significantly being between ~25 and 190 kb. While Pai I and II carry two haemolysin determinants (*hly* I, *hly* II) as well as P related fimbria-encoding genes (*prf*) and are located at map positions 82 and 97, respectively (Blum, G. *et al.*, 1994), Pai III encodes the S fimbrial adhesin. Pai IV is characterised by the presence of the *fyuA* (ferric yersiniabactin uptake) and *irp*1 through *irp*5 (iron-repressible protein) genes, which encode the yersiniabactin iron uptake (*ybt*) system originally found in the genome of different *Yersinia* species (Hacker, J. *et al.*, 1999). Interestingly, the four Pais of the strain 536 are associated with tRNA-specific loci as flanking sequences. While Pai I is associated with the *selC* gene that codes for $tRNA^{Sec}$ specific for selenocysteine (Böck, A. *et al.*, 1991), Pai II carries the leucin-specific tRNA gene *leuX* encoding $tRNA^{Leu}$ (Komine, Y. *et al.*, 1990).

The pathogenicity islands I and II of the uropathogenic strains have the capability to be deleted from the chromosome at high frequencies (Knapp, S. *et al.*, 1986, Blum, G. *et al.*, 1995) in contrast to Pais III and IV the deletion of which has not been detected yet. The deletion mutants such as mutant strain 536-21 have lost their pathogenic properties (Hacker, J. *et al.*, 1983,1986). In the strain 536-21, the loss of pathogenic determinants (*hly, prf*) and prf-specific activators, which have the capacity to trans-activate S fimbrial adhesin-specific genes, accounts for non-pathogenic phenotypes (Morschhäuser, J. *et al.*, 1994). In addition this mutant has lost the ability to express type 1 fimbriae, flagella, and to produce enterobactin, and survive in human serum (Hughes, C. *et al.*, 1987). As a consequence of the deletion of the Pais of strain 536, the tRNA loci *selC* and *leuX* were also destroyed (Blum, G. *et al.*, 1994). In the present thesis the influence of tRNA loci associated with pathogenicity islands on the production of pathogenicity traits will be demonstrated.

The *E. coli* strain 536 as described previously carries four pathogenicity islands. The mutant strain 536-21 has lost the Pais I and II including the structural genes of two haemolysins and P-related fimbria. In addition this strain is unable to synthesise the tRNA-specific transcripts encoded by the genes *selC* and *leuX*. In order to investigate whether the tRNAs have an impact on the production of virulence markers and metabolic enzymes, the structural genes for *selC* and *leuX* were cloned from strain 536. The DNA fragments coding for $tRNA^{leu}$ and $tRNA^{Sec}$ were amplificated by the polymerase chain reaction (PCR) (*leuX*) or were isolated as a 390 bp *Eco*RV-*Hinc*II fragment of plasmid p15-65/2 (*selC* in pUC 18) and cloned into the plasmid vectors pSU2716 and pBR322, respectively. The *selC*- and *leuX*-coding plasmids (pGBB50 and pGBB51, respectively) were transformed into *E. coli* strain 536-21.

A *leuX*$^-$ mutant of strain 536 was constructed by the help of a suicide vector comprising *leuX* promoter and downstream segment. The mutant strain was termed 536Δ102 in which 102 bp was deleted in such way in the *leuX* gene. This strain was complemented with pGBB51 (*leuX*$^+$) plasmid and plasmid vector, respectively.

5.2. Influence of tRNAs on expression of fimbriae and flagella

The uropathogenic *E. coli* strain 536 produces three different types of fimbria: S fimbria (Sfa), P-related fimbria (Prf) and Type1 fimbria (Fim). In addition, strain 536 expresses flagella of serotype H31 (Hacker, J. *et al.*, 1983) and shows motility on 0.2 % Luria-Bertani (LB)-agar plates. In contrast to the wild type isolate, the mutant strain 536-21 is not able to produce any fimbria or flagella. While the genes responsible for P-related fimbria (*prf*) are deleted from the chromosome of strain 536-21, the genes coding for other fimbriae and for flagella are still present in the genome of 536-21 (Morschhäuser, J. *et al.*, 1994). The *leuX*$^-$ mutant strain kept the pathogenicity islands and the other genes coding for fimbria and flagella, thus any change in expression in virulence traits may be attributed to regulatory defects.

Surface structures of the strains were visualised by electron microscopy. The wild type strain 536 has fimbriae, in contrast to strain 536-21, on which surface fimbriae and flagella were not detected. The strain 536-21 pGBB51 (*leuX*$^+$) carrying the *leuX* gene has regained the ability to produce fimbria. This was also found with strains 536-21 pGBB51,pGBB52 (*leuX*$^+$, *selC*$^+$), and 536Δ102 pGBB51 (*leuX*$^+$). Agglutination assay with yeast cells and haemagglutination tests demonstrated that the *leuX* gene is necessery to type 1-specific fimbria formation. S fimbria is not expressed in the clones, which have not pathogenicity islands. The motility test demonstrated that *leuX*$^+$ can readily produce flagella (Figure 5.1.). As can be seen in Figure 5.1. only the *leuX*$^+$ strains but not the *leuX*$^-$ clones are able to move on 0.2 % LB-agar surfaces within 8 hours after inoculation. From these results, it is obvious that the leuX-specific tRNA is necessary for assembly and function of type 1 fimbria and for complete flagellar motility of strain 536.

Figure 5.1. Motility of *E.coli* 536 strain and its derivatives in 0.2 % LB agar (Strains: A1: 536; A2: 536-21; A3: 536-21 pSU2716,pBR322; A4: 536-21 pGBB50; B1: 536-21 pGBB51; B2: 536-21 pGBB51,pGBB52; B3: 536Δ102 pGBB51; B4: 536Δ102; C1: 536Δ102 pSU2716)

5.3. Influence of tRNAs on production of siderophores

The production of siderophores was detected on the chrome Asurol sulfonate (CAS) indicator plates. These plates contain an iron-dye complex, which causes blue colour background. Siderophore producing strains utilise the iron from the complex and the medium surround of positive colonies becomes orange. As can be seen in Figure 5.2. the strain 536 produces siderophore, while strain 536-21 has lost this capacity. In order to investigate whether the tRNAs have any influence on siderophore production, we tested the proper clones. Only the *leuX* strains were able to produce siderophore, while *selC* had no influence on iron uptake

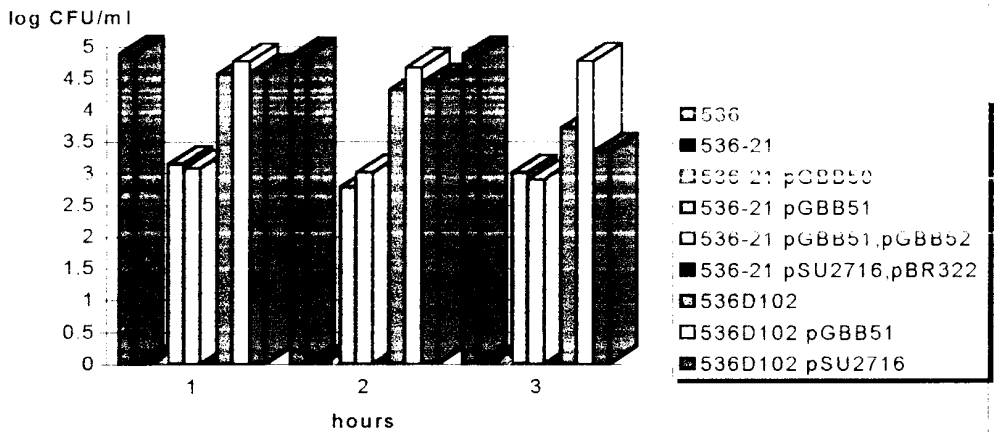
Figure 5.2. Phenotypic test for production of siderophores. *E. coli* strain 536 and its derivatives were tested on the CAS indicator plates. (Strains: A1: negative control; A2: 536; A3: 536; A4: 536-21; A5: 536-21 pSU2716,pBR322; A6: 536-21 pGBB50; A7: negative control; B1: 536-21 pGBB51; B2: 536-21 pGBB51,pGBB52; B3: 536Δ102; B4: 536Δ102 pSU2716; B5: 536Δ102 pGBB51; B6: 536 (*rfaH*)

5.4. Influence of tRNAs on serum resistance

The strain 536 was previously described as serum resistant, while strain 536-21 has lost the capability to grow in 90 % human serum (Hughes, C. *et al.*, 1987). In order to investigate whether the Pais associated tRNAs influence the serum resistance of the uropathogenic strain the wild type strain 536, mutant 536-

21, *leuX*⁻ mutant 536Δ102 and trans-complemented clones were tested for their ability to survive in 90 % human serum. As shown in Figure 5.3., strain 536 and the *leuX*⁺ clones are able to grown in human serum after 3h incubation while the *leuX*⁻ variants 536-21, 536-21 pSU2716,pBR322, 536-21 pGBB50, 536Δ102, and 536Δ102 pSU2716 are serum sensitive. It can be concluded that *leuX*, which is associated with Pai II, influences the serum resistance of the strains.

Figure 5.3. Growth capacity of *E. coli* strains in 90 % human serum



Properties of *E. coli* strain 536 and its derivatives are described in Table 5.1.

5.5. Influence of tRNAs on *in vivo* virulence of uropathogenic *E. coli*

Animal tests were performed to establish whether Pai-associated tRNAs may influence the *in vivo* virulence of the uropathogenic strain 536. The *E. coli* strains were injected into mice via the intravenous (i.v.) route. The wild type *E. coli* strain 536 was able to kill 100 % of mice following injection of 2.5×10^8 bacteria. In contrast, the strain 536-21, the *selC*⁻ positive clone 536-21 pGBB50, and 536Δ102 *leuX*⁻ negative clone were avirulent following i.v. injection. However, the *leuX*⁺ clone 536-21 pGBB51 killed 47 % of mice, while the strain

Table 5.1. Properties of *E. coli* strain 536 and its derivatives

Properties	Strains (Clones)								
	536	536-21	536-21 pSU2716 pBR322	536-21 pGBB50	536-21 pGBB51	536-21 pGBB51 pGBB52	536Δ 102	536Δ102 pSU2716	536Δ102 pGBB51
selC	+	-	-	+	-	+	+	+	+
leuX	+	-	-	-	+	+	-	-	+
Pai I	+	-	-	-	-	-	+	+	+
Pai II	+	-	-	-	-	-	+	+	+
Hly	+	-	-	-	-	-	+	+	+
Fimbria (EM)	+	-	-	-	+	+	+	+	+
Prf fimbria	+	-	-	-	-	-	+	+	+
S fimbria	+	-	-	-	-	-	+	+	+
Type 1 fimbria	+	-	-	-	+	+	-	-	+
Flagella (EM)	+	-	-	-	+	+	-	-	+
Motility	+	-	-	-	+	+	-	-	+
Iron uptake*	+	-	-	-	+	+	-	-	+
serum resistance	+	-	-	-	+	+	-	-	+

536-21 pGBB51, pGBB52 was even more virulent killing 76 % of the animals, suggesting an additional role of *selC* on the *in vivo* virulence in presence of *leuX*. The *in vivo* virulence of the *leuX*$^+$ clones 536-21 pGBB51 and 536-21 pGBB51, pGBB52 did not reach the virulence level of the wild type strain 536, presumably because of the deletion of all of Pai I and Pai II. In spite of the fact that the strain 536Δ102 pGBB51 is a *leuX* positive clone and carries pathogenicity islands (Hly$^+$, Prf$^+$), unlikely to the wild type strain it did not kill all of the infected mice. In the lung toxicity assay this strain was positive in contrast to the *leuX* negative strain 536Δ102 which also has haemolysin determinants.

Table 5.2. Killing rate, nephrovirulence, and lung toxicity in mice after infection with *E. coli* 536 and its derivatives

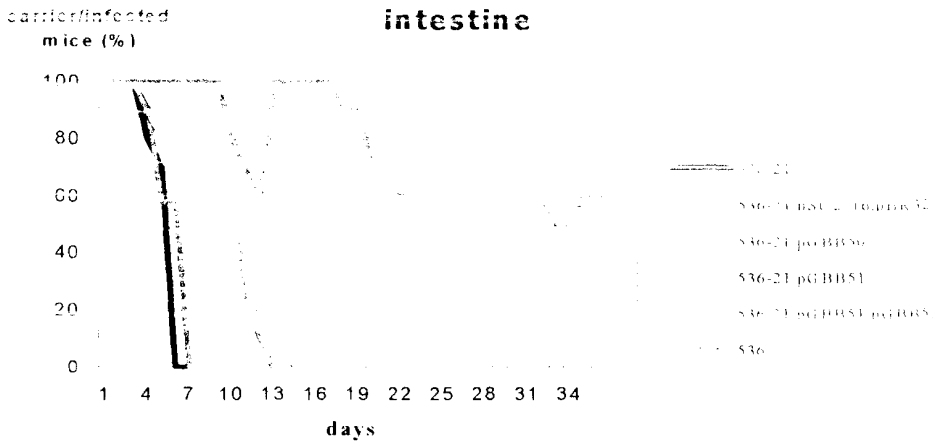
Strains	selC	leuX	hly	Death rate (%)[a]	Virulence in mice[b]	Lung toxicity(%)[a]
536	+	+	+	100	II	100
536-21	-	-	-	0	I	0
536-21 pSU2716,pBR322	-	-	-	0	I	0
536-21 pGBB50	+	-	-	0	I	0
536-21 pGBB51	-	+	-	47	II	0
536-21 pGBB51,pGBB52	+	+	-	76	II	0
536Δ102	+	-	+	0	II	0
536Δ102 pSU2716	+	-	+	0	II	0
536Δ102 pGBB51	+	+	+	66	II	90

[a] Dead mice/infected mice
[b] Group I, avirulent; II, mouse nephropathogenic; (Van den Bosch, J. 1979)
The significance in the virulence in the lethality assay was as follows: 536: $P<0.001$; 536-21 pGBB51, 536-21 pGBB51,pGBB52, 536Δ102 pGBB51: $P<0.01$ and in the lung toxicty assay: 536, 536Δ102 pGBB51: $P<0.001$

5.6. Influence of tRNAs on colonisation of uropathogenic *E. coli* in mouse large intestine

Influence of Pais associated tRNAs on colonisation in intestine was investigated when *E. coli* strain 536 and its derivatives (5 x 10^8 CFU) were fed to streptomycin treated mice. *Escherichia coli* 536-21 and 536-21 pSU2716,pBR322 were essentially eliminated from the intestine by day 6. They failed to colonise the mouse intestine in contrast to wild strain 536 and *leuX*$^+$, *selC*$^+$ strain 536 pGBB51,pGBB52 (Fig.5.4.). However, *E. coli* 536 pGBB50 *selC* positive and 536 pGBB51 *leuX* positive derivatives carrying tRNA on plasmid colonised less effectively than the wild type and the mutant strain carrying both tRNAs. It is suggested that both tRNAs are needed to develop factors to wild type level colonisation.

Figure 5.4. Colonisation of *E. coli* strain 536 and its derivatives in streptomycin-treated mouse intestine



5.7. Discussion and conclusion

Pathogenic determinants, genes that code for virulence factors of pathogenic microorganisms, may be located on plasmid, phage genome or on the chromosome (Finley, B.B. and S. Falkow, 1989, Mühldorfer, I. and J. Hacker, 1994). The specific regions of chromosomal DNA termed 'pathogenicity islands' (Pais) have been recently described by Hacker and Blum (Hacker, J. *et al.*, 1990, Blum, G. *et al.*, 1994, Hacker, J. *et al.*, 1999). Pais are large fragments of the chromosome, which comprise more than 30 kb of DNA. They carry virulence-associated gene clusters. The Pais can be deleted from the genomes of pathogenic bacteria at high frequencies (Knapp, S. *et al.*, 1986, Hacker, J. *et al.*, 1990, Blum, G. *et al.*, 1994). The Pais of the uropathogenic *E. coli* strain 536 are associated with tRNA loci. Linkage of virulence-associated gene clusters and their location on genetic elements, which potentially have the ability to move from one strain to another, may be of evolutionary significance because it may generate new variants of strains in a certain population by means of 'genetic quantum leaps' rather than by slow adaptive evolution via point mutations.

In general tRNAs may act as target structures for foreign DNA species. Thus certain plasmids of *Streptomyces coelicolor* and *Streptomyces griseus* are

able to integrate into the chromosome via tyrosine- and serine-specific tRNA genes (Reiter, W. D. *et al.*, 1989, Vögtli, M. and S.N. Cohen 1992). Interestingly, the *selC* gene encoding a selenocysteine-specific tRNA, represents the target structure for the retronphage ΦR73 of *E. coli* (Inouye, S. *et al.*, 1991). It is also associated with one of the Pais present in uropathogenic *E. coli* strain 536.

The contribution of Pais of the uropathogenic strain 536 to virulence was analysed in previous studies (Knapp, S. *et al.*, 1986, Hacker, J. *et al.*, 1990, Morschhäuser, J. *et al.*, 1994, Blum, G. *et al.*, 1994). The mutant strain 536-21, which has lost the two Pais, gave the possibility of studying the structure and function of pathogenicity islands. The Pai-positive wild type strain 536 is virulent, serum resistant, and able to produce haemolysins (Hly I, Hly II), three different adhesins (Sfa, Prf, and Fim), the iron chelator enterobactin, and flagella. In contrast, the Pai I-II-negative, non-virulent, serum sensitive mutant strain 536-21 has lost the capacity to produce haemolysins, fimbrial adhesins, flagella, and enterobactin. It was earlier published that the Pais contribute to virulence of the uropathogenic strain 536 by two mechanisms: (i) the Pais have an impact on virulence by the action of virulence factors which are directly encoded by Pai I (*hly* I) and Pai II (*hly* II, *prf*) specific genes (Hacker, J. *et al.*, 1990); and (ii) Pais influence virulence by cross-talk of the Pai II-encoded regulators PrfB and PrfC which positively regulate Sfa production (Morschhäuser, J. *et al.*, 1994). Here we describe a third Pai-dependent mechanism of virulence modulation: stimulation of gene expression by particular tRNAs.

By performing trans-complementation studies with the non-virulent mutant strain 536-21, the *leuX* negative mutant strain 536Δ102, and the cloned tRNA-specific genes *selC* and *leuX*, which are associated with Pais I and II of strain 536, we demonstrated, that the *selC* product is necessary for anaerobic growth.

In addition to *selC*, the leucine-specific tRNA gene *leuX* influences the expression of important bacterial gene products (see Table 5.1.). In contrast to selenocysteine, which is transported exclusively by $tRNA^{Sec}$, the amino acid leucine can be transferred by six different tRNA species (Sprinzl, M. *et al.*, 1989, Komine, Y. *et al.*, 1990). One of those, $tRNA^{Leu}$, is produced by gene *leuX* which

is located at the right boundary of Pai II of strain 536. As noted previously, *leuX* contributes to a number of effects in strain 536, including *in vivo* virulence in the mouse assays. Newman and co-workers reported that *leuX* also stimulates fim production in the *E. coli* isolate F-18 (R:K1:H5) (Newman, J.V. *et al.*, 1994). Furthermore, the production of three outer membrane proteins of 69, 71, and 74 kDa, is stimulated by *leuX* or *leuX*-dependent regulators (Burghoff, R.L. *et al.*, 1993). Moreover the intact *leuX* and *selC* locus is necessary for the colonisation of strain 536 in the mouse large intestine.

The *leuX*-specific codon TTG is preferentially present in low-bias genes (Anderson, S.G.E. and C.G. Kurland 1990). The structural genes *fimA* and *fliC* (subunit gene of flagella) do not contain any or contain only two TTG codons, respectively (Klemm, P. 1984, Macnab, R.M. 1992). In contrast, in the genes *fimB* and *flhC*, which act as positive regulators of type 1 fimbria production (Klemm, P. 1986) and flagella formation (Bartlett, D.H. *et al.*, 1988), 28,6 % and 26,3 % of the leucin-specific codons are TTG. Thus, it appears that genes which contain only limited number of leucin specific codon TTG can be translated in *leuX* negative background by another $tRNA^{Leu}$ encoded by *leuZ*, which usually recognise UUG codons. This mechanism may explain the viability of the Pai$^-$, *leuX*$^-$ mutant 536-21. However, genes such as *fimB* and *flhC*, which contain a large number of *leuX* codons, are presumably not further translated in a *leuX*-negative background. This may result in negative phenotype of corresponding type 1 fimbrial and flagella structures.

The production of enterobactin is also stimulated by *leuX* (see Table 5.1.). Enterobactin is a cyclic trimer of 2,3-dihydroxy-*N*-bensoylserine (2,3-DHBS). Its biosynthesis originates from chorismic acid and is thought to require the products of seven genes, designated *entA-G*. The *entC*, *entB* and *entA* gene products convert chorismic acid to 2,3 dihydroxybenzoic acid (DHBA), and the *entD-G* gene products synthesise enterobactin from 2,3-DHBA and serine. These gene products act as a multienzyme complex and are membrane associated (Earhart, C.F. 1987, Nahlik, M.S. *et al.*, 1987). The products of *entDEF* or one of them must be regulated by *leuX*. Interestingly, 16 %, 14 % and 12,5 % of the leucin codons in *entE*, *entF*, and *entD*, respectively, are *leuX* specific, suggesting a major

influence of *leuX* on the synthesis of these proteins. While type 1 fimbria, flagella and enterobactin are encoded by distinct set of genes, serum resistance represents a complex phenomenon leading to survival of bacterial cells following an attack of the complement system (Joiner, K.A. 1988). Several factors, including capsules, the length of O side chains or certain proteins of the outer or even the cytoplasmic membrane (Fernandez, R.C. and A.A. Weiss 1994) may contribute to serum resistance of pathogenic bacteria. Similarly, the lung toxic effect of bacteria may depend on not only haemolysin but also certain proteins of the outer membrane, which may be influenced by the *leuX* gene. Future studies will focus on the impact of particular gene products on *leuX*-dependent serum resistance and mouse lung toxicity of the uropathogenic strain 536.

It can be concluded that Pais of the pathogenic strain 536 influence virulence traits and metabolic activity by three mechanisms: (i) a direct contribution of Pai-encoded genes (Hacker, J. *et al.*, 1990); (ii) a transregulation of *sfa* genes by Pai encoded regulators (Morschhäuser, J. *et al.*, 1994); and (iii) the action of Pai-linked tRNA genes. These processes perfectly illustrate the interdependency of virulence and metabolic activities in pathogenic bacteria. By using the pathogenic network of strain 536, it has been possible to demonstrate a key role of tRNA-specific loci in a global regulatory cascade of pathogenic bacteria.

Chapter 6.

Influence of the RecA protein on the *in vivo* virulence of different *Escherichia coli* pathogens in mice

6.1. Introduction

Bacterial pathogenicity strongly depends on the presence and expression of various virulence factors and on the influence of other determinants such as regulator proteins, gene products involved in metabolism and cell surface structures. Thus it was shown that the RecA protein has an influence on virulence in certain *Enterobacteriaceae,* as was demonstrated for *Salmonella*, *Shigella*, enteroinvasive *Escherichia coli* (EIEC) (Buchmeier, N.A. *et al.*, 1993, Zagaglia, C. *et al.,* 1991). The RecA protein plays a key role in the so-called SOS response of bacteria to DNA damage. In bacterial lysogens, it includes not only the inhibition of bacterial cell division and an increased ability to repair and tolerate DNA damage by genetic recombination, but also implies the conversion from lysogenic to lytic bacteriophage replication by inactivating the lysogenic phage repressor (Roca, A.I. and M.M. Cox 1997, Kuzminov, A. 1999). Shiga-like toxins (SLT-I and SLT-II) produced by enterohaemorrhagic *E. coli* (EHEC), are phage-encoded cytotoxins. It has previously been shown that phage induction, which can be elicited by treatment of the EHEC lysogens with the alkylating antibiotic mitomycin C or UV irradiation (Giacomoni, P.U. 1983, Yamamoto, K. *et al.,* 1985), results in dramatic increase of SLT production involving two mechanisms. Firstly, induction of the SLT converting phages brings about an increase in toxin production due to a concomitant multiplication of toxin gene copies (Mühldorfer, I. *et al.*, 1996). Secondly, an influence of the phage-encoded regulatory factor, recently characterised as the Q transcription activator protein, was demonstrated (Mühldorfer, I. *et al.*, 1996, Neely, M.N. and D.I. Friedman 1998).

This study investigated the impact of RecA on the virulence of various *E. coli* pathotypes. *RecA*-mutations were introduced into the two wild type EHEC strains 933 and 8624, producing phage mediated SLT as well as into the uropathogenic *E. coli* strain 536, producing chromosomally encoded haemolysin. The wild type strains were compared with their isogenic mutants and *recA* transcomplemented derivatives for their *in vivo* virulence potential in intravenous lethality and lung toxicity assays in mice.

6.2. Influence of RecA protein on the *in vivo* virulence in mice

Isogenic *recA* mutants of EHEC and UPEC strains were constructed and transcomplemented with the cloned *recA* gene derived from *E. coli* K-12 strain C600. The wild type strains were compared with their isogenic *recA* mutants and *recA* transcomplemented derivatives for their virulence potential in intravenous lethality and lung toxicity assays. Table 6.1. describes the results of these *in vivo* virulence tests.

Table 6.1. *In vivo* virulence assays in mice

Strains	*RecA*	Intravenous lethality assay dead/infected (%)	Lung toxicity ass dead/infected (%
EHEC O157:H7 strain 933	+	20/20 (100)	16/20 (80)
EHEC O157:H7 strain 933r	-	0/20 (0)	3/20 (15)
EHEC O157:H7 strain 933r(pIM10)	+	14/20 (70)	10/10 (100)
EHEC O157:H7 strain 933r(pUC18)	-	0/20 (0)	1/10 (10)
EHEC O157:H7 strain 8624	+	20/20 (100)	20/20 (100)
EHEC O157:H7 strain 8624r	-	0/20 (0)	3/20 (15)
EHEC O157:H7 strain 8624r(pIM10)	+	20/20 (100)	10/10 (100)
EHEC O157:H7 strain 8624r(pUC18)	-	1/20 (5)	0/10 (0)
EHEC TUV862 (SLT-II$^-$)	+	0/20 (0)	0/10 (0)
EHEC TUV862r (SLT-II$^-$)	-	0/20 (0)	0/10 (0)
UPEC O6:K15 strain 536	+	20/20 (100)	20/20 (100)
UPEC O6:K15 strain 536r	-	20/20 (100)	20/20 (100)
UPEC O6:K15 strain 536r(pIM10)	+	20/20 (100)	10/10 (100)
UPEC O6:K15 strain 536r(pUC18)	-	20/20 (100)	10/10 (100)

It became obvious that RecA protein had no influence on the virulence of UPEC strain 536, which caused death within a couple of hours in both assays. Haemorrhagic lung oedema and haemoglobinuria, reflecting *in vivo* lysis of erythrocytes showed the acute toxic effect. Haemolysin production of UPEC

strain 536 did not differ from that of its isogenic *recA* mutant. In contrast, the virulence properties of the EHEC O157:H7 strains 933 and 8624, producing either SLT-I and SLT-II or only SLT-II, respectively, were strongly influenced by *recA* expression. The introduction of *recA* mutations into the genomes of the EHEC strains abolished either completely or nearly their virulence potentials. Trans-complementation of the EHEC *recA* mutants with cloned *E. coli recA* gene restored the virulence in all cases while the presence of vector plasmid pUC18 had no effect. In contrast to the acute toxic effect of the UPEC strain, the EHEC infected mice were free of symptoms in the first two days, and death started to occur only after the third day following infection in both assays.

6.3. Discussion and conclusion

In this study, the effect of the deletion of the *recA* gene from different pathogenic *E. coli* strains on the *in vivo* virulence of the respective pathogens was demonstrated. Previous investigations on the impact of *recA* on the virulence of pathogens have shown that in several cases, the virulence of the tested organisms is directly or indirectly controlled by *recA* involving different mechanisms (Buchmeier, N.A. *et al.*, 1993, Zagaglia, C. *et al.*, 1991).

In order to determine the effect of RecA on *E. coli* pathogenesis, the *in vivo* virulence potentials of the wild type EHEC strains 933 and 8624, their isogenic *recA* mutants, and *recA* trans-complemented mutant strains as well as equivalent mutant and complementant strains of UPEC strain 536 were tested by performance in intravenous lethality assays and lung toxicity assays with CFLP specific-pathogen-free (SPF) hygienic category outbred mice. It became evident that introduction of *recA* mutations into the EHEC strains 933 and 8624 resulted in loss of their virulence potency which was restored following trans-complementation with cloned *E. coli recA* gene. The SLT-II negative EHEC mutant TUV862 as well as its isogenic *recA* mutant were completely avirulent in both assays. We therefore conclude that the lethality observed with the EHEC wild type strains is mainly due to the production of Shiga-like toxins which are severely down regulated in the *recA* mutants as a result of lacking spontaneous

phage induction. The mouse model is an assay system in which toxic effect of Shiga-like toxins can be monitored. Thus, it reflects the change in virulence of our tested mutants due to toxin production, but it is not an appropriate model for the accurate reproduction of disease caused by EHEC upon oral ingestion. Therefore, the role of RecA on the pathogenesis of EHEC should further be studied in animal models like the gnotobiotic piglet model reproducing more specific aspects of EHEC infection (e.g. intestinal colonisation). In accordance with the above-mentioned data, the introduction of *recA* mutations did not affect the virulence potential of the uropathogenic *E. coli* strain 536. We presume that as long as the viability of the UPEC *recA* mutant is not affected, its virulence potential remains equivalent to that of the wild type strain, as it does not harbour any inducible bacteriophages encoding either virulence genes or virulence regulatory functions.

The present data demonstrate that the deletion of *recA* from EHEC strains clearly attenuates the respective strains by significantly down-regulating the production of their major virulence factor, STL-II. Moreover, in respect to the development of bacterial live vaccines; the introduction of *recA* mutations into vaccine strains is an important safety measurement as prevents recombination with virulence genes of a pathogen being present in the host.

Chapter 7.

Virulence genes of *Neisseria meningitidis* in commensal *Neisseria* species

7.1. Introduction

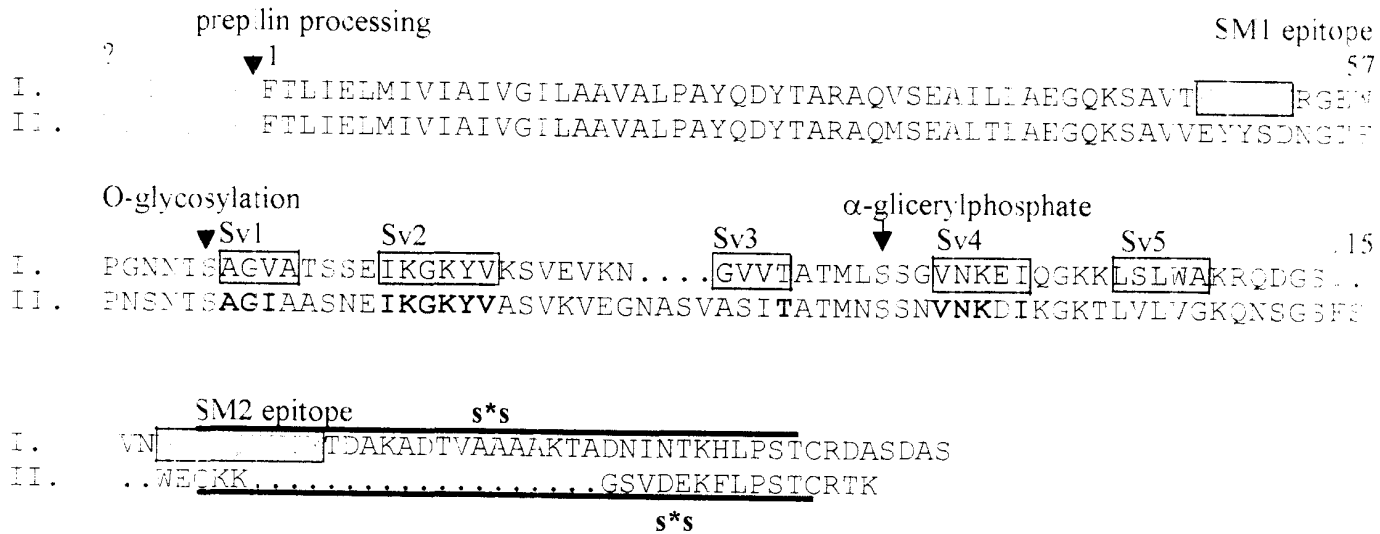
Pathogenic *Neisseria* species are responsible for causing bacterial meningitis and gonorrhoea. Their surface structures have been extensively studied. Common structures and functions of *N. gonorrhoeae* and *N. meningitidis* have been implicated in virulence, including the capsule, heat-modifiable opacity protein (identified as protein II for gonococci and class 5 protein for meningococci), PorA/PorB (identified as protein I for gonococci and class 1 protein for meningococci), pili, and a secreted protease that is specific for human IgA (Heckels, J.E. 1977, Frasch, L.E. and L.F. Mocca 1978, Koomey, J.M. *et al.*, 1982, Pohlner *et al.*, 1987).

The pilus is one of several virulence factors identified as crucial to colonisation and infection of the human host by the pathogenic *Neisseria* species *N. meningitidis* and *N. gonorrhoeae*. Neisserial pili share a common structural pilin subunit (PilE), that directly influences their adhesive properties (Virji, M. *et al.*, 1993, Nassif, X. *et al.*, 1993).

Strains of *N. meningitidis* may express one of two pilin types termed class I and II (Figure 7.1.), although the majority of disease isolates express class I pili, both classes mediate adhesion to various human cell lines *in vitro* (Diaz, J.L. and J.E. Heckels 1984). The class I pilin subunit is closely related to that of *N. gonorrhoeae* sharing an ability to bind the monoclonal antibody SM1, and a common gene structure consisting of conserved N-terminal domain followed by semivariable and hypervariable regions (Potts, W.J. and J.R. Saunders 1988). Such pilin subunits show a propensity for both phase variation and antigenic variation through intragenic recombination involving the *pilE* locus and variant silent (*pilS*) gene copies lacking the promoter and conserved 5' regions.

Although class II pilin subunits share considerable peptide sequence homology with the conserved region of meningococcal class I and gonococcal pilins, they do not react with the SM1 monoclonal antibody and are encoded by structurally distinct *pilE* loci with unrelated flanking sequences (Saunders, J.R. *et al.*, 1993, Nassif, X. *et al.*, 1993, Aho, E.L. *et al.*, 1997). Pili of strains encoding a class II *pilE* undergo phase variation characteristic of Neisserial pili. However,

Figure 7.1. Class I and II meningococcal pilins



76.4 % Similarity, 66.7 % Identity
I. : Class I from *N. meningitidis* C311; II. : Class II from *N. meningitidis* C114
Sv -Sv5: semivariable regions
s*s disulfide region
Numbering begins with the first amino acid of the mature proteins, indicated by 1
Serine 63, which represents a potential glycosylation site
Serine 97, which represents a potential site of α-glicerylphosphate modification

despite containing the class I related *pilS* gene copies associated with antigenic variation (Perry, A.C. *et al.*, 1989), strains expressing class II pili appear to exhibit little antigenic variation within the *pilE* locus (Virji, M., G. Payne, Z. Marschall and J.R Saunders unpublished observation). This may be due to the divergent 3' and downstream sequences of *pilE* and *pilS* loci in class II meningococci providing insufficient homology for recombination events.

Fundamental structural differences between the meningococcal pilin classes prompted the suggestions that they may have arisen by horizontal gene transfer (Saunders, J.R. *et al.*, 1993, Aho, E.L. *et al.*, 1997). Variation at several meningococcal loci including *penA* (Spratt, B.G. *et al.*, 1992), *aroE*, *glnA* (Zhou, J. *et al.*, 1997), and *adk* (Feil, E. *et al.*, 1996) has been attributed to interspecies recombination involving sequences introduced by transformation with various commensal *Neisseria* species identified as donor organisms.

It is believed that the carriage of commensal *Neisseria* contributes to acquired immunity to *N. meningitidis* (Kim, J.J. *et al.*, 1989). Pili are potent immunogens and those from both pathogenic and commensal species have been shown to potentiate LPS-mediated toxicity for human epithelial and endothelial cells (Dunn, K.L. *et al.*, 1995). Expression of homologous surface structures could therefore be important in such immunity. In order to determinate whether class II pili were acquired from commensal *Neisseria* strains of *N. lactamica*, *N. sicca*, *N. subflava*, and *N. pharyngis* that had been typed genomically by pulsed field electrophoresis (PGFE), strains were screened for the presence of *pilE* sequences and pilin expression. Deduced amino acid sequences were then compared to the classes of pilin sequence found in pathogenic *Neisseria* species.

Virtually all meningococci possess a major surface component such as class 1 outer membrane porin protein (PorA) which is encoded by the *porA* gene. This protein is nowadays considered as potential vaccine candidate. The porins of all *Neisseria* species were determined and sequenced. The *porA* genes were cloned and sequenced, and a topology model was proposed. This model predicts a structure composed of 16 amphipathic β-strands, which traverse the outer membrane and generate eight surface-exposed loops. The longest surface-exposed loops, 1 and 4, correspond to two variable regions (VR1 and VR2, respectively)

on which subtyping of meningococci is based (Frasch, C.E. *et al.*, 1985). The PorA proteins are sufficiently variable to be used as sero-subtyping antigen for meningococci (Abdillahi, H. and T. Poolman, 1987, 1988). Class 1 outer membrane protein (PorA) may influence virulence of the bacterium as mutation of one amino acid in variable region 2 induces an increased resistance to bactericidal antibodies (McGuinness, B.T. *et al.*, 1991, Rosenqvist, E. *et al.*, 1993). In order to determine whether *porA* gene was acquired from commensal *Neisseria* or this gene could be found in commensals, neisserial isolates were screened by PCR for the presence of *porA* gene and *pilE* (class I). Deduced amino acids were compared to the published sequences.

Pathogenic *Neisseria* strains produce IgA proteases that cleave secretory and serum IgA1 from the hinge region, thus separating the antigen binding fragment (Fab) and constant regions (Mulks, M.H. and R.J. Shoberg 1994). The released Fab fragment is able to bind cognate antigen and is thus capable of masking epitopes from subsequent recognition by other intact immunoglobulins (Mansa, B. and M. Kilian 1986). This suggests that IgA1 protease is a virulence determinant of disease and its role in the pathogenesis has been reported. IgA protease is encoded by the *iga* gene, which occurs in meningococcal and gonococcal strains. Nonpathogenic species of *Neisseria* lack IgA1 protease activity. In order to determine whether the *iga* gene or a similar gene sequence is found in commensals without expression, hybridisation technique was used to analyse *Neisseria* species for the presence of DNA sequences of the gene for the IgA1 protease.

7.2. Amplification and sequencing of *pilE* loci from commensal *Neisseria*

A series of commensal *Neisseria* isolates from various sources were typed by PFGE of *Nhe*I-cut preparation of chromosomal DNA. Method was previously described by Ledson (Ledson, M.J. *et al.*, 1998). The relationships between isolates of *N. lactamica* are shown in Figure 7.2.

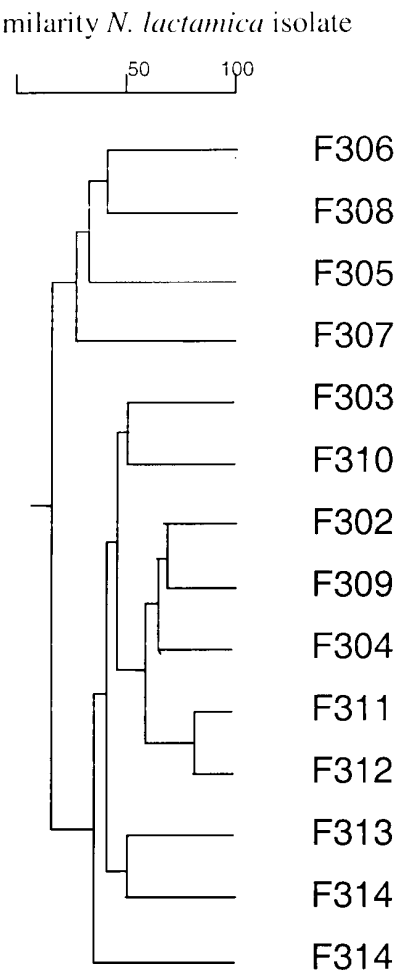


Figure 7.2. Percentage Relatedness Diagram for PFGE patterns of DNA from *N. lactamica* isolates examined. *Nhe*I handing patterns (Ledson, M.J. *et al.*, 1998) were analysed using the Biorad fingerprinting program.

Oligonucleotide primers were designed against codon 9-14 of pilE (5' ACC CTG ATC CAG CTG ATG), a region conserved in all known *Neisseria* pilin genes, and against a class II-specific region immediately downstream of the coding sequence (5' TTC ACG ACC GGG TCA AAC CC). PCR amplification with these primers was used to screen strains from various species and isolates of commensal *Neisseriae* (Table 7.1.).

Where present, amplified products of approximately 500 bp (Figure 7.3.) were selected for automated sequencing using dye terminators on an Applied

Biosystems 373a DNA sequencer. Alignment of deduced peptide sequences with representatives of the known meningococcal pilin types, revealed considerable homology between commensal and class II meningococcal pilins (Figure 7.4.).

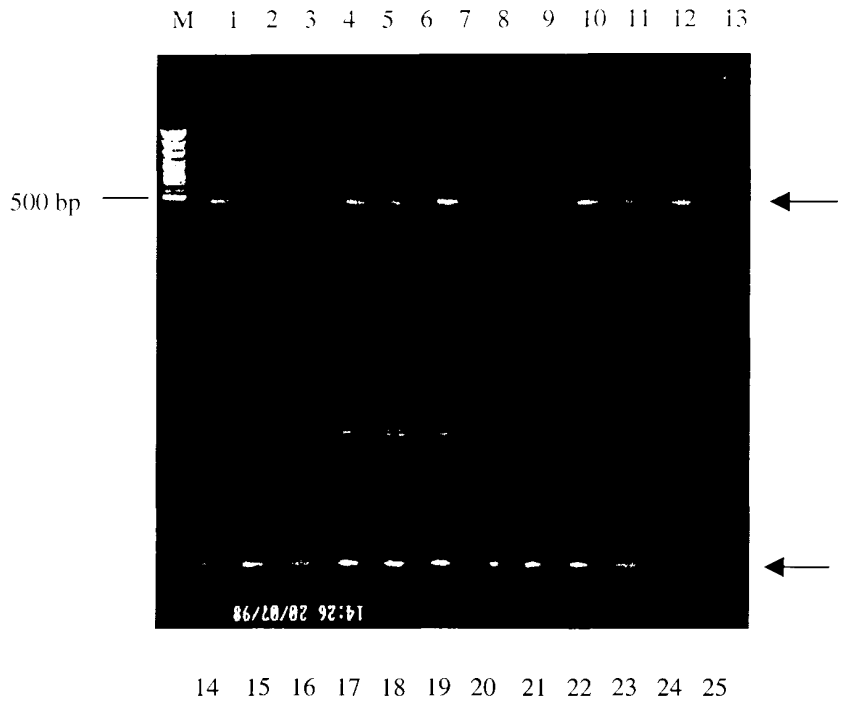


Figure 7.3. PCR amplification of *pilE* class II gene from various species and isolates of commensal *Neisseria*. Amplified products of approximately 500 bp in 1% agarose gel. (1, 2: positive control *N. meningitidis* Fam18; 24, 25:negative control)

A dendrogram showing relatedness of the Neisserial pilins was constructed using the neighbour-joining method (using Phylip Distance Methods), by consideration of only synonymous sites within the sequences (Figure 7.5). The class II meningococcal pilins are clearly separated from the gonococcal and class I meningococcal pilins and clustered with pilin encoded by commensal strains. By this method of analysis, class II pilins display closest homology to amino acid sequences derived from *N. subflava bv flava* and *N. lactamica*.

Table 7.1. Phenotypic analysis of commensal *Neisseria* spp. by reactivity with the pilus specific monoclonal antibody SM14 and elucidation of surface structures by electron microscopy

Strain	Species	Electron microscopy		SM14 Reactivity
		Pili visible	Blebbing	
C501	*N. lactamica*	+	+	+
C517	*N. lactamica*	+	+	+
C623	*N. lactamica*	+	+	-
C628	*N. lactamica*	+	+	+
C908	*N. lactamica*	+	+	Not detected
C911	*N. lactamica*	+	+	+
C980	*N. lactamica*	+	+	-
F302	*N. lactamica*	-	+	-
F303	*N. lactamica*	+	+	Not detected
F304	*N. lactamica*	-	+	+
F305	*N. lactamica*	+	+	+
F306	*N. lactamica*	+	+	+
F307	*N. lactamica*	-	+	+
F308	*N. lactamica*	-	+	-
F309	*N. lactamica*	-	+	+
F310	*N. lactamica*	+	+	-
F311	*N. lactamica*	+	+	-
F312	*N. lactamica*	-	+	+
F313	*N. lactamica*	+	+	+
F314	*N. lactamica*	+	+	Not detected
F316	*N. lactamica*	+	+	-
C245	*N. pharyngis*	+	+	+
C365	*N. pharyngis*	+	+	+
C440	*N. subflava* bv *flava*	+	+	+
C843	*N. subflava* bv *flava*	-	+	+
C351	*N. sicca*	+	+	+
C394	*N. sicca*	+	+	+
C619	*N. sicca*	+	+	+

Figure 7.4. Alignment of class I (C311) and class II (Fam18) meningococcal pilin sequences with the derived amino acid sequences from several commensal *Neisseria* species. Yellow shading indicates identity and boxed area indicate 90 % homology between the different pilin types as measured by the GES scale. Numbers indicate amino acids positions within the polypeptide sequence.

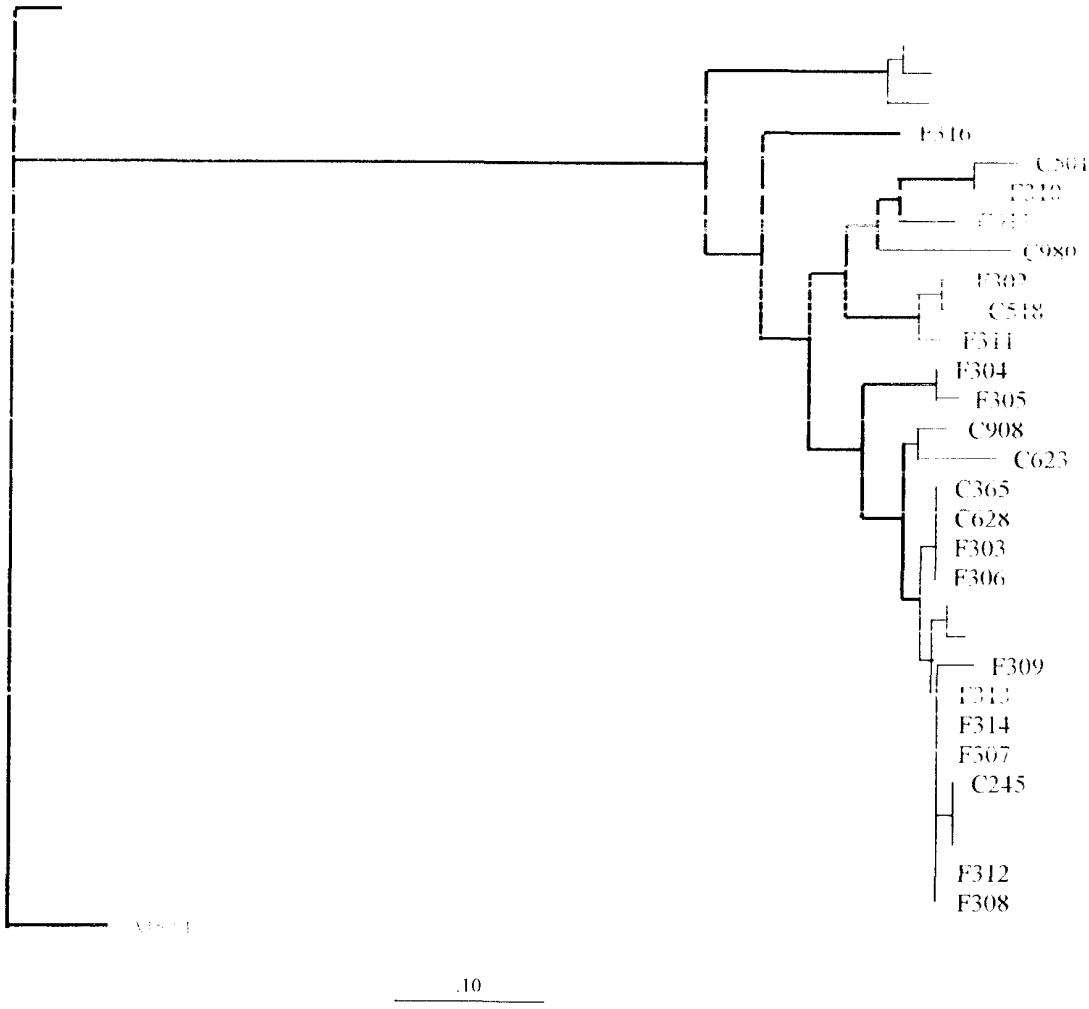


Figure 7.5. Dendrogram constructed by the 'neighbour-joining' method using PilE sequences from strains of *N. gonorrhoeae* (pink) and *N. meningitidis* (red) Class I C311 / Class II Fam18 and C114, and deduced peptide sequences from pilin coding loci of *N. lactamica* (black), *N. subflava* bv *flava* (green), *N. sicca* (yellow), and *N. pharyngis* (blue). In construction of the dendrogram only synonymous sites were considered. Scale : amino acid substitution per position.

7.3. Phenotypic analysis of commensal *Neisseria* possessing *pilE* sequences

7.3.1. Immunoblotting

Expression of pilin in the commensal *Neisseria* strains was detected using monoclonal antibody SM14 (kindly provided by Virji, M.) which binds to both classes of meningococcal pilin. Binding of SM14 was identified using an alkaline phosphatase-conjugated anti-mouse antibody and substrate 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrasolium (BCIP/NBT).

Despite encoding *pilE* sequences, not all commensal strains were found to express detectable levels of SM14-reactive pilin subunits (Table 7.1.).

7.3.2. Electron microscopy

Surface structures of *Neisseria* commensals were visualised by electron microscopy in order to investigate polymerisation of pilin subunits into filamentous pili. In the majority of strains pili and membrane blebbing characteristic of *Neisseria* species were clearly visible (Table 7.1.).

7.4. Detection of the *porA* gene by PCR

The diagnostic PCR test confirmed the presence of *porA* in all of the investigated meningococcal strains (Figure 7.6.). Failure to detect a PCR product was found in commensal *Neisseria* strains, except in one *Neisseria* species. This organism had been identified as *N. subflava* bv *flava*, but on subsequent culture it displayed some properties of both *N. subflava* bv *flava* and *N. meningitidis*. It did not give the typical yellow colonies seen with *N. subflava* bv *flava* although it did utilise sucrose, unlike meningococci. It did not agglutinate with meningococcal grouping sera.

7.5. Sequence of *N. subflava* bv *flava porA* gene

PCR product from the strain *N. subflava* bv *flava* found to be positive by PCR was purified and sequenced. The sequence showed great homology to that of

Figure 7.6. PCR amplification of *porA* gene from clinical isolates of *N. meningitidis* (2-5 and 10-12) and various species and isolates of commensal *Neisseria* (6-7). Amplified products of approximately 1200 bp in 1 % agarose gel. *N. meningitidis* (1)- positive control; 8-9 – negative control

the meningococcal *porA* gene with 174 out of 1082 bases (16 %) mismatched. The greatest variation was again in the two variable regions. The deduced amino acid sequence of the first variable region was similar to that described for non-subtypable meningococci (McGuinness, B.T. *et al.*, 1993). Amino acid deletions were also seen in the semivariable regions, as with the previous sequence.

7.6. PCR amplification of *iga* gene

N. meningitidis and some commensal *Neisseria* strains were checked by PCR amplification. To identify the IgA protease in *N. meningitidis* strains the oligonucleotides (forward 5' GCA TTG GTC AGA GAC G 3' and reverse 5' ATA ATC TTC GAG ACG GC 3') were used as primers for PCR amplification and were derived from the published sequence of the *N. meningitidis* HF13 (accession number X82474) *iga* gene (Lomholt, H *et al.*, 1995). The primers produce a product corresponding to position 517-891 bp of the published sequence. The

appearance of the ~370 bp band was a positive amplification and was found only in the amplification mixture of the *N. meningitidis* strains (Figure 7.7.).

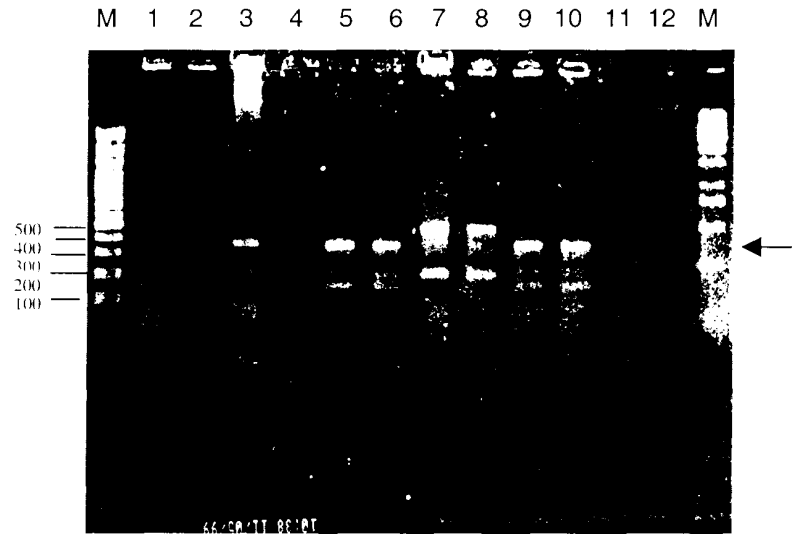


Figure 7.7. PCR amplification of *iga* gene from *N. meningitidis* (C311) - 5, 6 - (F360)- 9, 10 - (F362) - 3 - and various species and isolates of commensal *Neisseria*- 1, 2, 4, 7, 8. Amplified products of approximately 370 bp in 1 % agarose gel. Negative control - 11, 12

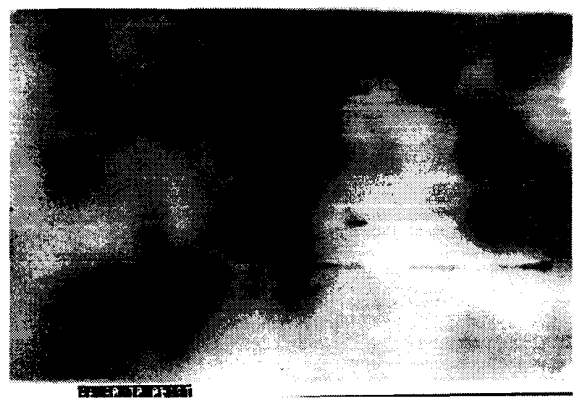
7.7. Slot blot and Southern blot hybridisation

DNA slot blot hybridisation provides a rapid method to determine whether a microorganism carries a particular DNA sequence of interest. *N. meningitidis* strains showed sequence homology with the *iga* PCR product. Weaker hybridisation signal was found with some *N. lactamica* and *N. cinerea* strains (Figure 7.8.).

Southern blot hybridisation was used to further investigate homologies of the meningococcal *iga* gene within several strains of *N. lactamica* and *N. cinerea*. *Hinc*II and *Apo*I restriction endonuclease digests of chromosomal DNA of *N. meningitis*, *N. lactamica*, and *N. cinerea* were electrophoretically separated and denatured. The DNA was then transferred to a positively charged membrane by capillary transfer. The membranes were hybridised with DIG-labeled PCR product. DIG-labeled probes hybridised to an ~1800 bp *Apo*I fragment of *N.*

meningitidis, *N. lactamica* and *N. cinerea* DNAs. In contrast *Hinc*II digested DNA from the different strains did not show uniform hybridisation patterns. *N. meningitidis* 4.5 kb, 2.5 kb, 1.0 kb, 300 bp, and 200 bp DNA fragments were detected with a 370 bp meningococcal probe, whereas commensal *Neisseria* 1 kb, 500 bp and 300 bp DNA fragments were weakerly detected (Figure 7.9.).

These hybridisation data show that there are putative homologies within the *N. meningitidis* chromosome and that of several *N. lactamica* and *N. cinerea* strains. The uniform *Apo*I restriction endonuclease pattern within this region is indicative of conserved nature for putative homologies.



F360	N.m.	N.c.	F316	N.l.	C619
C518	F311	F310	F304	F303	F375
C351	F305	F308	C394	C843	F305
F313	E.c.	C567	C245	F302	F309
F312	F307	C365	F306	C623	F314
C628	C450	C440	F381	F379	F376
F362					

Figure 7.8. Slot blot hybridisation with DIG-labeled meningococcal *iga* gene oligonucleotide amplificated by PCR. The numbers within the table refer to list of *Neisseria* strains. (See the table pp. 27.-28. in this thesis.) N.m: *N. meningitidis* (positive control), N.c.: *N. cinerea*, N.l.: *N. lactamica*, E.c.: *E. coli* (negative control)

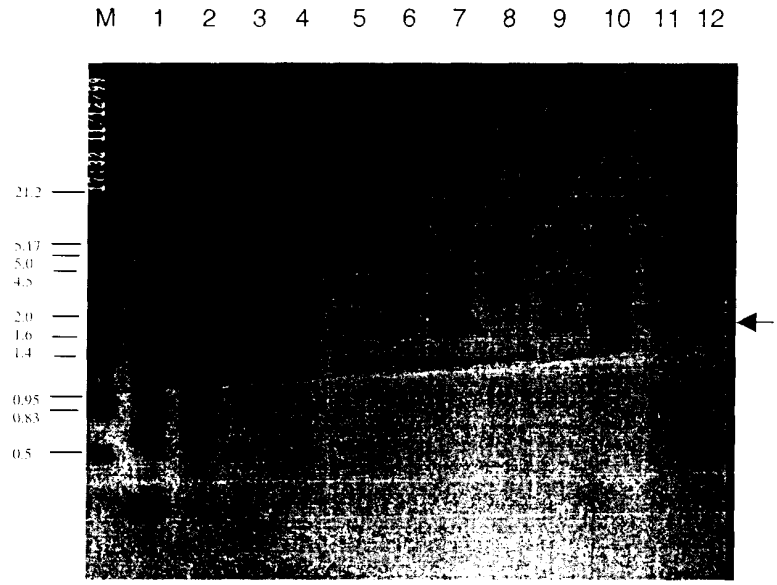


Figure 7.9. Southern blot hybridisation with DIG-labeled meningococcal *iga* gene oligonucleotide amplificated by PCR. *Apo*I restriction endonuclease digests of chromosomal DNA of *N. meningitis* (1, 2) , *N. cinerea* (3, 4, 5), and *N. lactamica* (6-11), *E. coli* – negative control - (12) were used in the hybridisation.

7.8. Discussion and conclusion

Various commensal *Neisseria* species were shown by electron microscopy to express pili, some of which reacted with SM14, a monoclonal antibody directed against meningococcal pilin. Amplification of pilin coding sequences from commensal strains has allowed further comparisons between the known types of neisserial pili. Alignment of deduced amino acid sequence of the commensal pilins with distinct types of meningococcal pilin reveals high levels of identity between commensal and class II pilE subunits (Figure 7.4.).

Clustering of the class II meningococcal and commensal pilins in dendrograms produced from synonymous residues (Figure 7.5.) also conveys similarities between these pilins, which appear to be distinct from gonococcal and class I meningococcal pilins. Laboratory based transformation of meningococci expressing class I pili with class II *pilE* sequences has so far proved unsuccessful Any interspecies recombination at *pilE* loci would therefore appear to occur at

low frequency, possibly due to sequence divergence between class I and class II coding sequences reducing the efficiency of recombination.

Considering these data, it is likely that interspecies transfer of pilin encoding sequences occur though with a very low frequency. Class II meningococcal pilin sequences would be acquired through transformation of *N. meningitidis* with *pilE* sequences from commensals co-residing in the nasopharynx.

Class 1 outer membrane protein is the antigen used for subtyping meningococci (Frasch, C.E. *et al.*, 1985) and is also a valuable epidemiological marker (McGuinnes, B.T. *et al.*, 1991), as well as a potential vaccine component (Zollinger, W.D. and E. Moran 1991). Deletion or substitution of just one amino acid in variable region 2 is associated with an increased resistance to bactericidal antibodies (McGuinness, B.T. *et al.*, 1991, Rosenqvist, E. *et al.*, 1993). Class 1 protein can thus influence virulence of the bacterium.

PCR amplification of *porA* gene may be useful for typing disease and carrier strains (Woods, J.P. *et al.*, 1994, Newcombe J. *et al.*, 1997). The presence of the *porA* gene suggests the organism is a meningococcus.

The sequence of the product from a *N. subflava* bv *flava* isolate, showed great homology to the published porA sequence, and changes in the semivariable regions were similar to those found in the other product sequenced. The sequence in variable region 1 was also similar to that found in non-subtypable meningococci (McGuinness, B.T. *et al.*, 1993). This organism thus possesses the *porA* gene. The organism may be a strain of *N. subflava* bv *flava* which has acquired the gene by interspecies variation. Alternatively it may be a non-capsulated meningococcus, which has acquired the ability to utilise sucrose. The organism was isolated from a contact of a child who had septic arthritis due to a group B meningococcus. It is most likely that it is a meningococcus.

Meningococcal and gonococcal strains produce IgA protease, which contribute to virulence of bacteria. The *iga* gene sequence was detected by PCR amplification only in meningococci similarly to *pilE* (class I) and *porA* sequences. Slot blot and Southern blot hybridisation data show that there are putative homologies within meningococcal chromosome and that of several commensal

Neisseria strains (*N. lactamica* and *N. cinerea*). The uniform pattern in Southern blot hybridisation is indicative of conserved nature for putative homologies. Further investigation is necessary to analyse these sequences and prove the homology.

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Appendix A. 1. Culture media

1.1. Liquid media

Minimal media

M9 medium (5x) per liter

30 g Na_2HPO_4;
15 g KH_2PO_4;
5 g NH_4Cl;
2.5 g NaCl;
15 mg $CaCl_2$;
Autoclave.

Supplement per liter if required:

10 ml 20 % carbon source (sugar or glycerol) (sterile);
L amino acids to 40 µg/ml (sterile); or
DL amino acids to 80 µg/ml (sterile);
Antibiotic.

M63 medium (5x) per liter

10 g $(NH_4)_2SO_4$;
68 g KH_2PO_4;
2.5 mg $FeSO_4 \cdot 7H_2O$;
Adjust the pH to 7.0 with KOH.
Autoclave.

Supplement per liter:

1 ml 1 M $MgSO_4$ (sterile);
10 ml 20 % carbon source (sugar or glycerol) (sterile);

and, if required:

0.1 ml 0.5 % vitamin B1 (thiamine) (sterile);
5 ml 20 % Casamino Acids (sterile); or
L amino acids to 40 µg/ml (sterile); or
DL amino acids to 80 µg/ml (sterile);
Antibiotic.

LB (Luria -Bertani medium)

10 g Bacto tryptone;
5 g Bacto yeast extract ;
10 g NaCl;

Adjust the volume of solution to 1000 ml with dH_2O and the pH to 7.0 (with 5 N NaOH). Autoclave.

Tryptone broth

10 g Tryptone;
5 g NaCl;

Adjust the volume of solution to 1000 ml with dH_2O and the pH to 7.0 (with 5 N NaOH). Autoclave.

Nutrient broth

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid or Difco).

BHI (Brain-heart infusion broth)

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid or Difco).

TSB (Tryptone soya broth)

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid or Difco).

SOB

20 g Bacto tryptone;
5 g Bacto yeast extract;
0.5 g NaCl;
950 ml dH_2O;
10 ml 250 mM KCl;

Adjust the pH to 7.0 (with NaOH). Adjust the volume of solution to 1000 ml with dH_2O. Autoclave.

SOC

SOB medium;
20 mM sterile glucose.

1.2. Solid media

Nutrient agar

Nutrient agar is nutrient broth solidified by addition of agar (1.2 %).

BHI (Brain-heart infusion) agar

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid or Difco).

TSA (Tryptone soya agar)

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid or Difco).

LB agar (Luria Bertani agar)

10 g tryptone;
5 g yeast extract;
5 g NaCl;
1 ml 1N NaOH;
15 g agar;

Adjust the volume of solution to 1000 ml with dH_2O. Autoclave.

EMB (Eosin methylene blue) agar

This is reconstituted from the dehydrated form according to the manufacturer's instructions (Oxoid).

Blood agar

39 g Columbia blood agar base;

Adjust the volume of solution to 1000 ml with dH_2O. Autoclave.

5 % sheep or ox blood

Cool to 50 °C, add the blood and pour plates.

1.3. Semisolid media

Semisolid agar

LB medium;
0.2 % agar. Autoclave.

2. Buffers and solutions

TAE (Tris acetate EDTA) 50X

242 g Tris base;
57.1 ml glacial acetic acid;
100 ml 0.5 M EDTA (pH 8.0)

Adjust the volume of solution to 1000 ml with dH_2O.

TBE (Tris borate EDTA) 5X

54 g Tris base
27.5 g boric acid
20 ml 0.5 M EDTA

Adjust the volume of solution to 1000 ml with dH_2O.

Alkaline Lysis Buffers for Minipreparations of Plasmid DNA

Solution I

50 mM glucose;
25 mM Tris-HCl (pH 8.0);
10 mM EDTA (pH 8.0);

Solution II

0.2 N NaOH (freshly diluted from 10 N stock)
1 % SDS (from 10 % stock)

Solution III

60 ml 5 M potassium acetate;
11.5 ml glacial acetic acid;
28.5 ml dH_2O. Final pH 4.8

TE (Tris-EDTA buffer)

10 mM Tris-HCl (pH 8.0);
1 mM EDTA (pH 8.0). Autoclave.

PBS (Phosphate-buffered saline)

8 g NaCl;
1.21 g K_2HPO_4;
0.34 g KH_2PO_4;

Adjust the volume of solution to 1000 ml with dH_2O and the pH to 7.3. Autoclave.

DPBS (Dulbecco's phosphate-buffered saline)

Solution A

8 g NaCl;
0.2 g KCl;
1.15 g Na_2HPO_4;
0.2 g KH_2PO_4;

1000 ml dH_2O. Adjust the pH to 7.3. Autoclave.

Solution B

2.0 g $CaCl_2$;
2.0 g $MgCl_2 \cdot 6H_2O$;
100 ml dH_2O. Filtered through 0,45µm membrane.

Complete Dulbecco PBS:

Add 0.5 ml solution B to 100 ml Dulbecco solution A to make the complete salt solution. Adjust the pH to 7.4.

Denaturation solution

0.5 M NaOH
1.5 M NaCl

Neutralising solution

1.0 M Tris-HCl
1.5 M NaCl
pH 8.0

20 x SSC (Sodium chloride, Tri-sodium citrate) solution

0.3 M Tri-sodium citrate
2.0 M NaCl
pH 7.0

Washing solution 1 x

1.0 x SSC
0.1 % SDS Sodium dodecyl sulphate

Washing solution 0.5 x

0.5 x SSC
0.1 % SDS Sodium dodecyl sulphate

Appendix B

Rapid method for preparing genomic DNA

Bacterial genomic DNA was isolated using a modification of the method of O'Relly *et al.* (O'Relly, et al., 1986).

1. Agar plates containing the appropriate growth medium were seeded to produce bacterial lawns.
2. Cells from one plate were harvested in 1.5 ml TE buffer in Eppendorf tube and cells were pelleted.
3. Following removal of the supernatant, 1 ml of lysis mixture (Tris-HCl 10mM, pH:8.0; EDTA 10mM; NaCl 100mM; SDS 2% [w/v]; DTT 39mM; proteinase K 50 $\mu g.ml^{-1}$) was added and mixture vortexed. This was then incubated at 37 ^{o}C for 30 min.
4. The lysate was extracted with phenol-$CHCl_3$ twice.
5. After ethanol precipitation, DNA was resuspended in 150 µl TE buffer. 10 µl RNase A solution (10 $mg.ml^{-1}$) was added and mixture was incubated at 37 ^{o}C for 1h.

Isolation of plasmid DNA

Minipreparations of plasmid DNA were performed as described by Sambrook *et al.* (Sambrook, J. *et al.*, 1989).

1. 2 ml LB broth culture containing the appropriate antibiotics was grown overnight at 37°C with shaking.
2. Cells were pelleted and were resuspended in 100 µl of ice cold Solution I (see Appendix A) by vigorous vortexing
3. 200 µl of freshly prepared Solution II (see Appendix A) was added to suspension and was mixed.

4. 150 µl of ice cold Solution III (see Appendix A) was added and the mixture vortexed. This was then stored on ice for 3-5 min.
5. The lysate was cleared by centrifugation at 12000 g for 5 min. and the supernatant was transferred into a fresh tube.
6. The lysate was extracted with an equal volume of phenol:chloroform twice.
7. The double-stranded DNA was precipitated with 2 volumes of ethanol at room temperature.
8. DNA was collected by centrifugation at 12000 g for 5 min. and redissolved in 50 µl of TE buffer

Agarose gel electrophoresis

DNA was analysed on 0.6-2.0 % (w/v) agarose gels in TBE buffer, containing ethidium bromide (0.1 $\mu g.ml^{-1}$). Agarose gels were subjected to electrophoresis for 1-3 h at a constant voltage of 100 V. DNA was visualised on a 302 nm UV transilluminator and photographed.

Recovery of DNA fragments from agarose gels

Purification of discrete DNA fragments or PCR product was performed by QIAquick gel extraction kit (Qiagen Ltd) according to the manufacturer's instructions.

Transformation

Transformation of *E. coli* was performed according to the method of Brown *et al.* (Brown et al., 1979).

1. An overnight starter culture was diluted 1:100 in fresh prewarmed nutrient broth and incubated at 37 °C with shaking.

2. Incubation was continued until an A_{660} of approximately 0.2 was reached. Cells were then harvested and washed once with 1/5 of original volume of ice-cold, 10mM $CaCl_2$.
3. Cells pellets were resuspended in 1/20 of the original volume of ice-cold 75mM $CaCl_2$.
4. 200 µl competent cells were added to 10-500 ng DNA solution in an Eppendorf tube and incubated for 45 min on ice.
5. The transformation mix was then heat pulsed at 42 °C for 2 min. and diluted with 0.5 ml prewarmed SOB.
6. Following incubation at 37 °C for 1 h. cells were diluted and plated on selective media to yield discrete colonies.

DNA digestion with restriction endonuclease

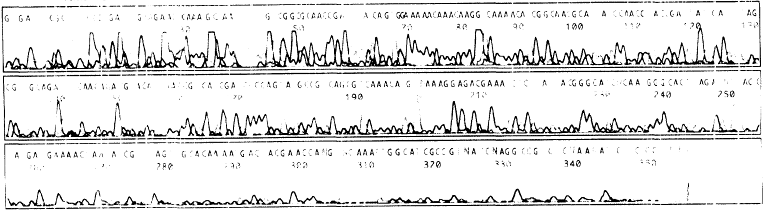
Digest were typically carried out in final volumes of 20 µl, but volumes were altered as necessary. After the addition of DNA, 1/10 of the final volume of the required 10 x restriction buffer (Sambrook, J. *et al.*, 1989) was added. The desired volume was made up with sterile distilled water. Following addition of restriction endonuclease(s) (2 U/µg DNA), incubation was carried out for 2 h, at the optimum temperature for the enzyme. If the DNA was to be analysed by agarose gel electrophoresis 1/10 of the final volume of loading buffer was added prior to sample loading. If DNA was intended for use in further manipulations, the enzyme was inactivated by phenol extraction and ethanol precipitation.

ABI PRISM

Model 3?? | 0?1360
Version ?.3 | ?ga??1
ABI50 | 1360
Version ?.2 | Lane ?

Signal G 113 A 231 T 137 C 59
DT4%Ac{A Set-AnyPrimer}
SEQ MATRIX #1726
Points 1450 to 5000 Pk 1 Loc. 1450

Thu May 20 1999 9:29 AM
Wed May 19 1999 4:39 PM
Spacing 10.10(10.10)



ABI PRISM

Model 3[illegible]3 | 03-360
Version [illegible].3 | igcpro2
ABISC | 1680
Version [illegible].2 | Lane 3

Signal G:145 A:287 T:222 C:85
DT4%Ac{A Set-AnyPrimer}
SEQ MATRIX #1726
Points 1450 to 5000 Pk 1 Loc: 1450

Thu. May 20. 1999 9:29 AM
Wed. May 19 1999 4:39 PM
Spacing 10.02{10.02}