STUDIES ON VIRULENCE GENE REGULATION IN UROPATHOGENIC *ESCHERICHIA COLI*

Ph.D. Thesis

by

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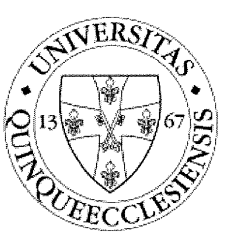

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Acknowledgements

I would like to express my thanks to my supervisor, Professor Levente Emődy for his help in all respects during the course of this study. I also thank Professor Jörg Hacker, Gabriele Blum-Oehler and Ulrich Dobrindt for their help and guidance. I am grateful to Rózsa Lajkó, Mrs. Márta Tárnai, Barbara Plaschke, and Anja Waldschmidt for their technical assistance.

I owe a debt of gratitude to my parents and Maria for their support. Furthermore, I thank to all the members of the Department of Medical Microbiology (University of Pécs) and Institute for Molecular Biology of Infectious Diseases (University of Würzburg) for their advises and for providing a friendly working atmosphere.

I would also like to thank the Bayerische Forschungsstiftung for their financial support.

List of abbreviations

aa	amino acid
ABU	asymptomatic bacteriuria
AFA	afimbrial adhesin
bp	base pair
BSA	bovine serum albumin
CFU	colony forming unit
DAEC	diffusely adherent *Escherichia coli*
EAggEC	enteroaggregative *Escherichia* coli
EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
EtOH	ethanol
HPI	"high pathogenicity island"
HRPO	horseradish peroxidase
IL	interleukine
kDa	kilodalton
LPS	lipopolysaccharide
MENEC	*Escherichia coli* responsible for meningitis
NBM	new-born meningitis
OD	optical density
ORF	open reading frame
PAI	pathogenicity island
PCR	polymerase chain reaction
RNAP	RNA polymerase
SEM	standard error of mean
SEPEC	*Escherichia coli* responsible for septicaemia
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
WT	wild type (strain)

1. INTRODUCTION

1.1. Urinary tract infections

Urinary tract infection (UTI) is one of the most common infectious diseases in industrialized countries. As many as 50% of women report having had at least one UTI in their lifetimes (15). In the USA, UTIs cause 7 million patient visits per year with total costs exceeding one billion dollars (9). UTI implies involvement of either the bladder (cystitis) or the kidneys and their collecting systems (pyelonephritis), or both. Although UTI may remain localised to the bladder, lower UTI (cystitis) always carries the potential of spread to the kidney. Furthermore, it is often difficult to clinically distinguish UTI that is confined to the bladder from that which also affects the kidney(s). Moreover, bacterial colonisation of the urinary tract may be completely free of clinical symptoms, in that case the term „asymptomatic bacteriuria" (ABU) is used.

1.1.1. Pathomechanism

Acute pyelonephritis is caused by bacterial infection, while chronic pyelonephritis is a more complex disorder. In the chronic form, bacterial infection plays a dominant role as well, but other factors such as vesicoureteral reflux and obstruction are also critically involved in the pathogenesis.

There are two forms by which pathogens can reach the kidneys. In the less common way, the causative agent reaches the urinary tract through the bloodstream. Bacteria (or rarely fungi) can cause *haematogenous infection* from distant sites in the course of septicaemia, infective endocarditis, or trauma. In the majority of cases (more than 95%), however, UTI is an *ascending infection,* where bacteria are derived from the patient's own faecal flora.

Certain predisposing conditions may increase the risk of UTI. *Urinary obstruction* (congenital or acquired) leads to abnormal urine flow with potential urine stasis. In that case, less virulent bacteria may also cause infection. *Vesicoureteral reflux* allows urine (and bacteria) to be actively propelled up one or both ureters, finally leading to pyelonephritis. *Females* have an 8 times higher risk of acquiring UTI. This has been variously ascribed to anatomical endowments, hormonal changes affecting adherence of bacteria, and urethral trauma during sexual intercourse, or a combination of all these factors. Moreover, *pregnancy* further increases the risk probably due to hormonal changes as well as by an obstructional mechanism. *Catheterisation* may carry bacteria into the bladder from the urethra.

Furthermore, long-term catheterisation provides an attachment site for bacteria within the urinary tract. *Diabetes mellitus, immunosuppression, and immunodeficiency* are accompanied with general susceptibility to any infection, hence these conditions increase the risk of UTI non-specifically.

The first step in the pathogenesis of ascending UTI appears to be the colonisation of the distal urethra and vagina by enterobacteria (154). From the urethra pathogens may gain entrance into the bladder. Under normal conditions, organisms introduced into the urinary tract are cleared by the continual flushing of urine (153). Other factors such as acidic pH, osmolarity, salts, urea, and organic acids present in urine can reduce bacterial survival. In addition, a number of substances including low molecular weight sugars, secretory IgA, and uromucoid can act as anti-adherence factors inhibiting bacterial attachment (121). When the natural defence mechanisms are overwhelmed by virulent bacteria, adhesion and colonisation may occur evolving into UTI. The factors by which bacteria can sidestep these defence mechanisms are discussed later (under 1.2.2.).

Since the urinary tract is typically a sterile environment, colonisation of the epithelium results in a number of cellular responses. Different bacterial stimuli such as LPS and type 1 pili activate epithelial cells (121). Upon activation, they secrete cytokines like IL-6 and IL-8. These potent neutrophil chemotactic molecules together with the induced adhesion molecule ICAM-1 elicit neutrophil migration into the urothelium (1;2). Through a network of secreted cytokines, leukocytes launch an immunological cascade with a possible elimination of the pathogen. Simultaneously, exfoliations of epithelial cells, to which large number of bacteria are adhered, facilitate bacterial clearance from the urinary tract (121). Apparently, the infectious process in the urinary tract can be considered as a continuous interplay between innate defence mechanisms against bacteria armoured by virulence factors.

1.1.2. Causative agents

In the majority of UTIs the causative agent is originated from the patient's own faecal flora. Gram-negative bacilli or enterococci are responsible for more than 95% of all UTIs. The prevalence of various bacterial species, however, is different in case of ambulant - and hospitalised patients. The occurrence of different bacteria isolated from UTIs is presented in Table 1. *Escherichia coli* is by far the most common etiological agent of urinary tract infections, accountable for 70-95% of community acquired UTIs and about 50% of all nosocomial UTIs (72;137;167).

Table 1. Prevalence of bacterial species isolated from urinary tract infections (137)

Bacterium species	Ambulant patients	Hospitalised patients
Escherichia coli	89.2	52.7
Proteus mirabilis	3.2	12.7
Klebsiella pneumoniae	2.4	9.3
Enterococci	2.0	7.3
Enterobacter aerogenes	0.8	4.0
Pseudomonas aeruginosa	0.4	6.0
Serratica marcescens	0	3.3
Staphylococci	1.6	1.4
Other species	0.4	3.3

1.2. *Escherichia coli*

Termed as *Bacterium coli commune*, the species was isolated and described by Theodor Escherich from the faeces of a normal infant in 1885 (51). It is a Gram-negative enterobacterium that constitutes about 0.06% of the normal human intestinal flora. The ubiquitous organism is a commensal intestinal bacterium widely distributed among animals as well. *E. coli* colonises the infant bowel within hours of life (107) and usually remains a harmless member of the commensal flora. With the acquisition of virulence traits, however, some strains may become pathogenic associated with numerous types of intestinal and systemic infections in humans or animals (148).

1.2.1. Pathogroups

Beside commensal variants of the same species, some *Escherichia coli* strains are able to cause a wide variety of diseases. *E. coli* has evolved the ability to cause disease in several body systems involving different mechanisms of pathogenesis. *E. coli* strains causing intestinal infections (i.e., diarrheagenic *E. coli*) can be grouped into at least six pathogroups: enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). The salient clinical features of infection and the pathogenic mechanisms of strains in these categories of diarrheagenic *E. coli* were recently reviewed by Nataro and Kaper (143).

Other strains are responsible for extraintestinal infections, such as urinary tract infections (UPEC), newborn meningitis (MENEC) and septicaemia (SEPEC). Characteristics of UPEC strains are discussed in detail later in this chapter. *E. coli* is responsible for a third of cases of neonatal meningitis with an incidence of 0.1 per 1,000 live births. (40). Case fatality rates are still very high ranging from 25 to 40%. Furthermore, the occurrence of long term neurologic sequelae in nonfatal cases is 33 to 50% of neonates with *E. coli* meningitis (40). Virulence properties of MENEC strains are partially identical to those of SEPEC, as development of meningitis requires preliminary bacteriaemia. Prominent virulence factors associated with strains causing septicaemia and newborn meningitis include K1 capsular polysaccharide, aerobactin, and Sfa_{II} fimbriae (89).

Wound infections and abscesses due to *E. coli* most commonly follow surgical operations in the course of which the alimentary tract is entered. Furthermore, *E. coli* can produce several types of diseases in animals.

The spectrum of diseases caused by *E. coli* is due to the acquisition of specific virulence genes harboured on plasmids, bacteriophages, or within distinct chromosomal DNA segments termed pathogenicity islands that are absent from the genomes of commensal strains. Prior to the identification of specific virulence factors, serotypic analysis was the predominant means by which pathogenic strains were differentiated. Kauffmann proposed a scheme according to which *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (17). A specific combination of these antigens defines the serotype of an isolate. *E. coli* of a specific serotype can reproducibly be associated with certain clinical syndromes. However, these surface antigens themselves do not confer pathogenicity. Rather, specific clonal lineages (i.e., certain serotypes) have served as „hosts" for an ordered acquisition of virulence genes (93). Hence, serotypes serve as readily identifiable markers that correlate with specific virulent clones of *E. coli.* Nevertheless, serotyping is tedious, expensive, and has only limited sensitivity and specificity. Thus, detection of pathogenic *E. coli* has focused increasingly on the identification of characteristics which themselves determine virulence. This may include *in vitro* phenotypic assays which correlate with the presence of specific virulence traits (e.g., haemolysis, haemagglutination) or detection of the genes encoding these virulence factors (28).

1.2.2. Virulence factors of uropathogenic *E. coli*

Uropathogenic isolates of *E. coli* possess virulence factors that differentiate them from commensal strains and those strains causing intestinal infections (79). These traits are

absolute requirements for survival and pathogenicity within the urinary tract of the otherwise healthy host. Virulence factors of UPEC include fimbriae and afimbrial adhesins, toxins, siderophores and other iron uptake mechanisms, and certain O and K antigens.

1.2.2.1. Adhesins

Adherence factors enable UPEC to adhere to uroepithelial cells (35). Adhesins include fimbriae (termed also as pili) and afimbrial adhesins. Fimbriae of UPEC are usually thin, rod-shaped, fibre-liked structures, which in most cases are heteropolymeric structures rather than simple multimers of a single structural subunit. In case of P-fimbriae, type 1 fimbriae, S fimbriae and F1C fimbriae, the fimbria appears to be a composite structure consisting of a rod-shaped structure of 6-7 nm in diameter comprising over a thousand structural (major) subunits and minor subunits located at the tip of the pilus. The adhesin subunit is situated at the very tip of the fimbria often connected with so-called adapter subunits. While the adhesin subunit mediates specific binding to carbohydrate moieties on the surface of eukaryotic cells, the function of the other minor subunits is not yet clear. They have been proposed to play a role in adherence onto mammalian extracellular matrix proteins (88). Composite structure of fimbriae implies that the synthesis, export, correct folding and ordered assembly of these subunits during biogenesis occurs in a co-ordinated manner (152). On the other hand, curli fibers that are also regularly expressed by UPEC, are composed of one single subunit, which can be polymerised to reach a size exceeding that of the whole bacterium. Binding by this fimbria seems to be non-specific. Furthermore it is not expressed at human body temperature, rather at 30°C suggesting a pathogenic role in the very early phase of infection (e.g., adherence to periurethral skin surface) by UPEC (130). In contrast to fimbriae, afimbrial adhesins lack the fibre-like appendages formed by the major subunits. In this case, the adhesins are located directly on the bacterial surface. Their eukaryotic ligand could be, nevertheless, identical to that of fimbrial adhesins, such as in case of the Dr adhesin family, which involves Dr fimbriae and afimbrial adhesins AFA-I and AFA-III.

Although common or type 1 pili are also carried by commensal *E. coli* strains, it is one of the most important adhesion factors of UPEC. It not only mediates adhesion to mannose oligosaccharides on bladder epithelial cells (27), but may be also involved in bacterial invasion (111). Recently, type 1 fimbriae were shown to be involved in urothelial apoptosis, a characteristic phenomenon of UTI (84).

P, S, and F1C fimbriae are more exclusively associated with extraintestinal isolates of *E. coli*. Tip subunits of these adhesins also recognise carbohydrate moieties: Galα(1-4)Gal, α-sialyl-2,3-β-galactose, and GalNAcβ(1-4)Galβ, respectively.

Dr family adhesins, including Dr fimbriae and afimbrial adhesins AFA-I and AFA-III, bind the Dr^a blood group antigen present on decay accelerating factor and may facilitate ascending colonisation of the urinary tract (127). A recent study indicated that mutation in the *dra* region encoding Dr fimbriae prevented tubulointerstitial nephritis in an *in vivo* model (53). Interestingly, similar animal experiments using mutants affected in single determinants involved in the biosynthesis of P- or S- fimbriae indicated that their mutational inactivation does not significantly alters virulence of the corresponding strain (118). This fact may reveal that although fimbriae are important contributory factors to the infectious potential, they are not necessarily sufficient by themselves to cause infection and disease (152).

1.2.2.2. Toxins

Toxins are prominent virulence factors of many pathogens. There are 3 toxins proposed to play a role in urovirulence: α-haemolysin, cytotoxic necrotizing factor-1 (CNF1), and cytolethal distending toxin (CDT). α-haemolysin is discussed in detail in the next chapter.

1.2.2.2.1. Cytotoxic necrotizing factor and cytolethal distending toxin

CNF1 belongs to a group of bacterial toxins that modify Rho, a subfamily of small GTP-binding proteins that are regulators of the actin cytoskeleton (3). The two variants, CNF1 and CNF2 share 99% amino acid similarity, however, only CNF1 is associated with extraintestinal *E. coli* infections in humans, most notably urinary tract infections. The gene for CNF1 is chromosomally encoded as part of pathogenicity islands in uropathogenic *E. coli* (25). The toxin is synthesised as a hydrophilic polypeptide of approximately 115 kDa that remains primarily cytoplasmic because of the lack of a signal sequence. Recent structure and function analysis of CNF1 indicates that the toxin has distinct binding and enzymatic domains (101). Eukaryotic cells intoxicated with CNF1 exhibit membrane ruffling, the formation of focal adhesions and actin stress fibers, and DNA replication in the absence of cell division, a phenomenon that results in enlarged multinucleated cells. The drastic changes apparent in CNF1-treated cells are a result of the toxin's capacity to modify Rho. Epidemiological data support the role of CNF1 as a virulence factor in human extraintestinal infections (6). Furthermore, evidence of the toxin's pathogenic role was shown recently in animal models (134).

Cytolethal distending toxins (CDTs) are secreted proteins produced by a number of unrelated pathogenic microbes including uropathogenic *E. coli* (14). CDTs are characterised by their capacity to inhibit cellular proliferation by inducing an irreversible cell cycle block at the G_2/M transition (34). CDT is composed of three polypeptides, CdtA, CdtB, and CdtC, having molecular masses of approximately 30, 32, and 20 kDa, respectively. Although the role of the individual proteins in cellular toxicity is not firmly established, genetic and biochemical evidence suggests that all three polypeptides are required for CDT activity (44). Recently, it was demonstrated that the CdtB polypeptide bears a striking pattern-specific homology to mammalian type I DNase enzymes. Mutational analysis of CdtB indicated a significant reduction in CdtB-related DNase and cell cycle arrest activities (45), however, CdtB itself was insufficient to promote cell cycle arrest. A mechanism of CDT action involving nuclear targeting and chromosomal damage would represent a unique mode of action for a microbial product. Direct role of the toxin in uroinfection, however, remains to be proven.

1.2.2.2.2. α-haemolysin

1.2.2.2.2.1. The RTX toxin family

The *E. coli* α-haemolysin is the best-characterised representative of the RTX toxin family, which is disseminated among pathogenic bacteria. These pore-forming toxins include the bifunctional adenylate cyclase-haemolysin of *Bordatella pertussis* (52), the haemolysins of *Proteus vulgaris* (90), *Morganella morganii (90)*, *Moraxella bovis* (56), the leukotoxins of *Pasteurella haemolytica* (165) and *Actinobacillus* spp. (48), and the so-called enterohaemolysin of *E. coli* O157 (146). Beside a 31-73 % sequence identity to *E. coli* α-haemolysin, these toxins share postranslational maturation, type I secretion process, and C-terminal calcium binding domain. The latter consists of glycine-rich nonapeptide repeats, which has led to the RTX (repeat in toxin) family nomenclature. Notable recent addition to this toxin family may be the RTX determinant carried by epidemic strains of *Vibrio cholarae* (103). Although RtxA lacks the nonapeptide repeats, its gene organisation strongly suggests it is a member of the RTX family. Furthermore, RtxA possesses an 18-residue glycine- and aspartate-rich repeat, which may also form a Ca-binding β-roll motif, essential for the activity of RTX toxins (see later).

1.2.2.2.2.2. Synthesis, maturation, and secretion

The synthesis, maturation, and secretion of α-haemolysin is determined by the *hlyCABD* operon (91). The four genes are co-transcribed from the same promoter. Transcription of the

distal genes required for the export of the toxin are, however, uncoupled from that of *hlyC* and *hlyA* by a Rho-independent terminator motif, expressional consequences of which will be discussed later. Secretion requires and additional outer membrane protein (TolC), which is encoded by a separate gene (175). Transcriptional organisation of genes related to α-haemolysin is shown in Figure 1.

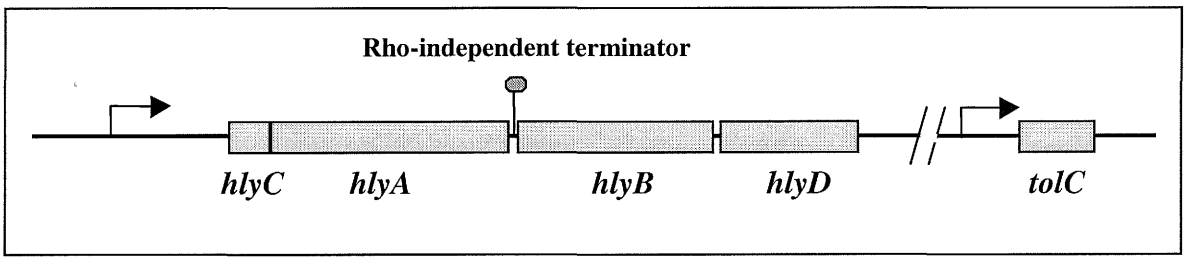

Fig. 1. Transcriptional organisation of genes involved in the synthesis maturation and secretion of α-haemolysin molecules. *hlyA* encodes the protoxin, which is activated by the gene product of *hlyC*. *hlyB* and *hlyD* together with *tolC* encode the secretion apparatus (see text).

The α-haemolysin protoxin consists of 1024 amino acids and is encoded by gene *hlyA*. The pro-HlyA is maturated to the active form in the cytosol by the gene product of *hlyC*. It was shown that this maturation is a fatty acylation of two internal lysine residues: K564 (KI) and K690 (KII). The homodimer HlyC associated with acyl-acyl carrier protein (acyl-ACP) recognises two independent domains (FAI and FAII), each of which spans one of the target lysine residues (158) (Fig. 2.). Both recognition domains require up to 70 amino acids for full wild type acylation. Considering that both regions (FAI and FAII) have the same function, there is little (21%) identity between their sequences. The only consistent identity is a glycin proceeding the lysine residues to be acylated. Furthermore, the affinity of FAI for HlyC is four times greater then that of FAII, nevertheless both KI and KII need to be acylated for haemolytic activity (159).

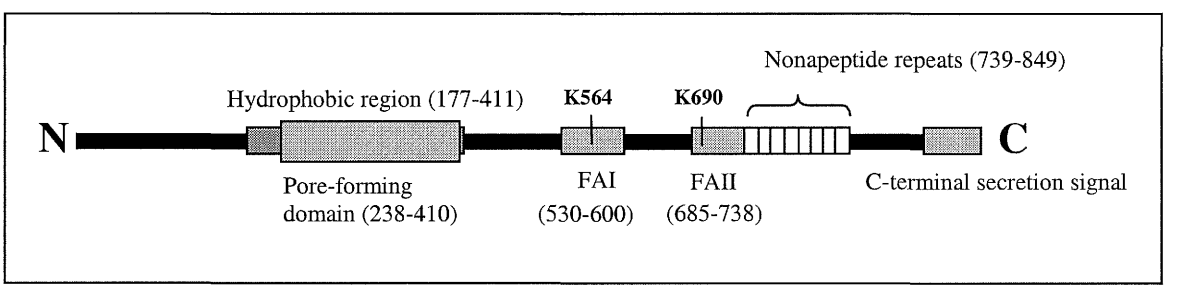

Fig. 2. Schematic representation of important regions within the HlyA protoxin molecule. Numbers denote amino acid positions numbered from the N terminus. K564 and K690 represent lysine residues to be acylated.

Export of the acylated mature toxin is achieved by a typical type I secretion system (157). The HlyA secretory apparatus comprises HlyB, HlyD and TolC. HlyB is an inner membrane ATPase, which couples ATP hydrolysis to HlyA export. The secretion signal on HlyA is located on the C-terminal and consists of 24 to 80 amino acids (78;92). The recognition peptide on HlyA probably interacts with HlyB, which is supported by the finding that mutations within the C-terminal of HlyA are partially compensated by suppressor mutations in HlyB (150). The function of HlyD is less clear, but probably it serves as a bridge to the outer membrane (148). TolC is a trimeric porin-like structure, which is believed to form a periplasmic bridge to the energised components of the secretory complex (93).

1.2.2.2.2.3. Insertion into membranes and cytotoxicity

Once secreted, the toxin binds Ca^{2+}, which is an absolute requirement for cytotoxic activity (27). Calcium ions bind to the glycine-rich repeats located on the C-terminal of HlyA (28;107) (Fig. 2.). After binding Ca^{2+}, these form an unusual structure called parallel β-barrel or β-superhelix (17). Binding of calcium can only occur outside the bacteria, since intracellular level of free calcium is tightly regulated to a level too low for HlyA activation (51).

Following activation by HlyC-dependent acylation (intracellularly) and calcium binding (extracellularly) the toxin seems to have a two-stage interaction with eukaryotic membranes. First, there is a reversible adsorption, which is followed by an irreversible insertion (14). Interestingly, neither Ca^{2+}-binding nor acylation is essential for insertion into phospholipid vesicles yet both are absolute necessary for toxin activity (76). After integration into the membrane, the toxin can not be extracted without the use of a detergent (21). A highly conserved region of HlyA is essential for insertion and lysis. This domain was identified by integration of photoactively labelled recombinant peptides into phospholipid vesicles (76). The data provided by Hyland *et al.* are strongly complementary to those from a parallel spectroscopic study (144). The suggested region is located between amino acids 238-410, which spans the only hydrophobic region (aa 177-411) in the otherwise hydrophilic HlyA protein (Fig. 2.). A predicted α-helical region lies within this sequence, which is highly conserved within the RTX toxin family. Ludwig *et al.* proposed a model, according to which the aa 238-411 region consists of 3 helical domains, which are the principal regions that insert into membranes (108). Mutations causing deletions or altering the hydrophobicity of this region reduce or abolish the haemolytic activity of the toxin (109).

According to a more recent model proposed by Soloaga *et al.*, α-haemolysin contains 10 amphipathic helixes by which the toxin is inserted into membranes (156). The latter model suggests that α-haemolysin occupies only one of the membrane phospholipid monolayers, i.e., it is not a transmembrane protein. This model would need a new explanation for the cytotoxic action of RTX toxins, as non-transmembrane arrangement would be not compatible with the concept of pore-forming toxins.

How α-haemolysin creates lesions in eukaryotic membranes remains to be revealed. The original hypothesis was that HlyA forms a transmembrane protein structure with an aqueous channel conceptually similar to the structure and function of other known pore-forming haemolysins such as the *Staphylococcus aureus* α-toxin (21). Alternative proposals are that HlyA disrupts membranes by either a detergent-like activity (131) or a monolayer-specific mechanism (156).

1.2.2.2.2.4. Target cell specificity and cellular response

α-haemolysin has a wide spectrum of cytocidal activity, i.e., it exhibits little target cell specificity. The activated toxin „attacks" erythrocytes, granulocytes (50), monocytes (23), endothelial cells (166), and renal epithelial cells (82). HlyA can even insert into synthetic planar lipid membranes, suggesting a non-specific binding mechanism (115). The absence of a specific receptor is further supported by observations reporting no saturation of HlyA binding (74). With a certain contradiction, glycophorin was proposed to act as a receptor for α-haemolysin as it increased sensitivity toward HlyA when incorporated into pure phosphatidylcholine liposomes (38). Presumably, activated α-haemolysin would insert into (and consequently destroy) all eukaryotic membranes having closer contact with. Due to its rapid aggregation and subsequent poor solubility, however, α-haemolysin does not enter a soluble, cell-free state. This fact may reflect an evolutionary pressure to avoid systemic intoxication that would kill the host (155). Consequently, *in vivo* target cell specificity is probably rather determined by the binding potential of bacteria to different cells, which may provide the intimate contact essential for toxin „targeting".

Haemolytic *E. coli* strains subvert host cell functions such as signal transduction pathways, cytoskeleton rearrangement, vacuolar trafficking, and cytokine production. At sublytic concentration, HlyA is a potent trigger of generation of inositol triphosphate and diacylglycerol stimulating the respiratory burst and the secretion of vesicular constituents (22). Release of IL-1β (but not of TNFα) from leukocytes and that of leukotriene B4 and

nitric oxide from endothelial cells is stimulated by HlyA (23;87;166). It is possible that some of the biological effects so far assigned to the HlyA toxin actually reflect co-operative responses to both toxin and LPS as HlyA can not be free from LPS molecules while maintaining full activity. Nevertheless, in some cases, results certainly specific for HlyA can be shown. Higher IL-1β levels without elevation of serum TNFα levels elicited by HlyA is distinct from the pattern typical of LPS (114). The benefit to *E. coli* as a result of the induction of these host responses is not obvious. A consequence of the release of mediators may be to induce inflammation, disrupt epithelial cell junctions, and favour translocation.
Members of the RTX toxin family were proposed to induce apoptosis (178). Host cell death by apoptosis would occur without leakage of cellular components and without inflammation, hence it would mean an alternative pathway to death.

1.2.2.2.2.5. Regulation of expression

Regulation of α-haemolysin-expression is multifactorial. Low temperature, high osmolarity and anaerobiosis repress transcriptional rate (120). Effects of these environmental factors are mediated by Hha, a representative of a new class of transcriptional regulators in different *E. coli* strains (119). Hha represses expression of the *hly* operon through changes in DNA topology by forming a complex with global regulatory protein H-NS (126).
In some cases, the influence of various extracellular iron concentrations on secreted α-haemolysin was shown (96). These results are, however, contradictory, hence the role of iron in the regulation of haemolytic activity needs to be investigated in more detail.
Transcription of the operon is uncoupled by a Rho independent terminator sequence located between *hlyA* and *hlyB* (Fig. 1.). Presence of this motif results in two alternative (a shorter *hlyCA* and a longer *hlyCABD*) transcripts. An antiterminational mechanism displayed by RfaH, however, favours formation of the full-length transcript, hence suppressing operon polarity. Mutational inactivation of gene *rfaH* results in a dramatic decrease in the amount of exported HlyA, while intracellular levels are not considerably affected (99).
$tRNA_5^{leu}$ encoded by the minor leucine tRNA gene *leuX* was also shown to affect transcription of the haemolysin operon, and its influence was independent from those elicited by Hha or RfaH (42). Furthermore, it was speculated that beside these characterised regulatory mechanisms some other factor(s) may exist that influence expression of α-haemolysin (42).

1.2.2.3. O-, K-antigens and serum resistance

Diseases caused by *E. coli* are associated with relatively few serological groups, even though a very large number exists in nature. Uropathogenic strains often belong to serogroups O1, O2, O4, O6, O9, O16, O18, and O75 implying important role of certain clonal lineages in the evolution of uropathogens. Furthermore, clinical isolates from UTI are frequently encapsulated. In a study reported by Falkenhagen *et al.*, 253 *E. coli* strains isolated from urinary tract infections were investigated (145), 86% of which expressed capsular (K-) antigen. Among the 26 different K antigens determined, K1 and K5 were the most common, found in 32% and 33% of the cases, respectively. High prevalence of these two capsular serogroups in pathogenic isolates is not astonishing, since both capsular oligosaccharides mimic human antigens, thus preventing effective immune response against bacteria expressing them.

Resistance to complement-mediated serum activity is an important virulence factor in *E. coli* isolated from extraintestinal infections. Resistance to serum killing is multifactorial and has been associated with several surface components of *E. coli* among which capsule and lipopolysaccharide are thought to be the most important (79). In the above mentioned study, percentage of serum-resistant strains was between 11% and 63% in the different O serogroups, the highest frequency was found in O6 (63%) and O2 strains (43%). Among all serum-resistant strains carrying 13 different K antigens, K1 and K5 were the most common ones, with a percentage of 62% altogether. According to another report, some O antigens by themselves might be protective against the killing effect of serum (76).

1.2.2.4. Iron acquisition and haemin receptors

The availability of iron, an essential nutrient for bacterial growth, is severely limited in mammalian hosts. In secretory fluids and in blood, eukaryotic carrier glycoproteins, such as lactoferrin and transferrin complex the poorly soluble Fe^{3+} ions. Nevertheless, most of iron is located intracellularly, primarily bound to ferritin and chelated in haem. In order to be able to compete with the host for iron, pathogenic bacteria have developed different mechanisms to acquire this essential growth factor. Low molecular weight chelators (siderophores) are secreted by several pathogens. These molecules „liberate“ Fe^{3+} from host carriers and - by recognising specific receptors located in the outer membrane - transport it into the bacterial cell (59). Alternatively, many pathogenic bacteria can directly utilise iron-containing host compounds through specific receptors (32;37;55;59). Several Gram-negative pathogens have

been reported to be able to use haem and its protein complexes as iron sources. *Haemophilus influenzae* type b (36), *Yersinia enterocolitica* (163), *Yersinia pestis* (73;169), *Vibrio cholerae* (67;68;129), Neisseriae (97;164), *Shigella dysenteriae* (116) possess outer membrane proteins involved in haem utilisation.

In *Escherichia coli* O157:H7 the gene *chuA* which codes for a 69 kDa outer membrane protein responsible for haem uptake was recently identified (170). The *chuA* sequence shows very high homology to that of the formerly described *shuA* gene of *S. dysenteriae* type 1 (116). The gene is part of a larger locus, termed haem transport locus, which appears to be widely distributed among pathogenic *E. coli* strains including UPEC (181). The locus contains 8 open reading frames and is inserted within the region located at 78.7 minute of the *E. coli* K-12 chromosome (see Fig. 14.).

The ability to use haem/haemoglobin might be especially advantageous to pathogenic bacteria. Contribution of haemin receptor molecule ChuA to virulence was shown recently using an isogenic *chuA* mutant of uropathogenic *E. coli* strain CFT073 (171). Uropathogens often secrete cytotoxins, which beside initiating tissue invasion gain access to the intracellular haem reservoir. Cytotoxin production coupled with the capability to utilise haem/haemoglobin could serve as an effective iron acquisition strategy during the progression of infection.

1.2.2.5. Pathogenicity islands

Specific genes that encode virulence factors are present in the genome of pathogenic members of a species but are absent in non-pathogenic variants. On the basis of early observations, virulence associated genes were thought to be solely extrachromosomal elements which were horizontally transferable. In the 1980s, however, blocks of chromosomal regions were identified, which carry virulence genes and the presence of which are associated exclusively with virulent strains of a species. These regions were proven to be spontaneously deletable indicating the possibility of horizontal gene transfer (64;106), and were termed pathogenicity islands (PAIs) (26). By now, several PAIs have been identified both in Gram-negative and Gram-positive bacteria (63). From the accumulated data, some common features of PAI became manifest (61):

- PAIs carry genes encoding at least one, but more often several virulence factors. These include adhesins, invasins, iron-uptake systems, toxins, type III and type IV secretion systems, etc.
- PAIs are present in virulent strains but absent from non-virulent members of the same species.

- PAIs often consist of DNA whose G+C content is different from that of the „core genome". Different codon usage in genes can be also characteristic.
- PAIs are often flanked by short direct repeats.
- PAIs are associated with tRNA genes. tRNA genes often resemble to attachment sites for bacteriophages. Since PAIs often carry phage integrases, tRNA genes may serve as target sites for bacteriophage-derived elements located on PAIs.
- In addition to phage integrases, PAIs often carry other mobility factors such as transposases and insertion elements.
- PAIs often contain regulatory elements, which control expression of virulence factors.
- PAIs are unstable genetic regions. Deletions may occur by the direct repeats located on both ends or via IS elements and homologous sequences.

Although PAIs share common features, they represent a rather heterogeneous group regarding size, compositions and encoded functions. They often carry unrelated genes, cryptic genes, pseudogenes and even „junk DNA". PAIs seem to have their own evolution through acquisition and deletion of certain regions leading to a very complex structure. Having been horizontally transferable DNA regions, they contribute to the evolution of bacterial pathogens (61).

1.2.3. Virulence gene regulation

Pathogenic bacteria synthesise virulence factors, which enable them to survive and colonise in the host. Constitutive expression of virulence determinants, however, would be needless and energetically exhausting for bacteria. Moreover, presence of some virulence factors could even be disadvantageous at certain points of the infectious process. For effective pathogenesis, bacteria sense their environment and regulate the expression of genes encoding virulence factors.

The regulation of pathogenicity is complex. Usually, large operons encoding virulence determinants (such as those encoding fimbriae) contain their own specific transcriptional regulators that either activate or repress transcription. In addition, certain global regulators and other regulatory elements sensing environmental stimuli may affect expression of virulence genes. Apparently, regulatory proteins involved in virulence gene regulation often form a correlative, complex network (66).

1.2.3.1. Regulatory mechanisms

Presence of virulence factors must be regulated in a way that they are only expressed at a certain stage and anatomical site of infection. *In vivo*, bacteria first need to sense that they have entered into a host. Temperature seems to be an appropriate signal, since several virulence factors such as adhesins, haemolysin and other secreted proteins are specifically expressed at the normal host body temperature (66). Iron availability is also an indicator of the host environment since iron is sequestered in body fluids by specific proteins such as transferrin or lactoferrin. Hence, low iron concentration induces the expression of virulence factors like siderophores, haemolysins and other toxins. Other environmental factors such as pH, osmolarity, O_2 tension and availability of carbon sources and amino acids may serve as a fine indicator of the precise tissue location. Although bacterial response to environmental factors has been investigated profoundly, the regulatory mediators eliciting the proper alteration on the transcriptional rate of the corresponding genes are not yet fully known.

Much attention has been focused lately on two-component regulatory systems. These systems consist of an outer membrane protein which monitor certain environmental parameters, and the actual regulatory protein that mediates the adaptive response, usually by a change in gene expression (47). The sensor component undergoes autophosphorylation by detecting the external signal. The phosphate residue is then transferred to the regulator protein, which becomes activated upon phosphorylation. In *E. coli*, some group I capsules are regulated by the RcsABC system which shows homology to the two-component histidine kinase signalling system (54).

Global regulators can control the expression of several genes encoding both virulence traits and housekeeping proteins. These regulatory molecules provide an energetically advantageous way through simultaneous control of genes whose products are somehow related to each other such as in case of virulence factors. For example, expressions of several virulence factors are normally repressed by the histone like protein H-NS. Environmental changes indicating the entry into the host, however, repress H-NS mediated transcriptional „silencing". This results in increased expression of several virulence factors simultaneously. Directly or indirectly, H-NS regulates the expression of P-, S-, type 1- and curli fimbriae and is also involved in the regulation of α-haemolysin production (7).

Other global regulators, such as CRP (cAMP receptor protein), Lrp (leucine responsive protein) RpoS (alternative sigma factor), and Fur (ferric uptake regulator) have been also reported to influence expression of a large number of genes in *E. coli*, several of which are

related to virulence. Some of these regulators recognise specific nucleotide sequences in the vicinity of the promoter regions they influence. Their effects are confined to those genes possessing these specific binding sites. A well-characterised example for this is the regulatory effect elicited by the Fur protein, which plays a general role as a sensor of iron availability in the cell. The apoprotein binds Fe^{2+} (if present), and the cofactor-bound protein binds to so-called Fur boxes in the operator regions of iron-regulated operons. The bound complex represses transcription of operons possessing Fur boxes. In the absence of iron, however, the Fur protein separates from the operator region, thus derepressing transcription. This system regulates many genes involved in iron uptake (siderophores, iron-siderophore complex receptors) as well as toxin genes *hly* and others (105). Additional regulatory proteins may be superimposed on the Fur repressor to provide the fine-tuning necessary for the precise regulation of individual virulence genes in response to iron and other environmental signals.

Virulence factor expression is also influenced by population density. Quorum sensing is a phenomenon by which bacteria sense and respond to their own population density. The most common form of quorum sensing is mediated by the production and subsequent perception of an autoinducer. By sensing the density of the secreted autoinducer, bacteria estimate if their population is sufficiently present to initiate the appropriate reaction (180). In Gram-negative bacteria, quorum sensing is often performed by the LuxR family of transcriptional regulators, which affect phenotypes as diverse as conjugation, bioluminescence and expression of virulence genes.

An interesting aspect of virulence gene regulation is the expressional control through rare tRNAs. When a gene contains large number of unusual codons recognised by specific minor tRNAs, translation requires efficient transcription of the corresponding tRNA gene. *E coli* strain 536 carries two spontaneously deletable PAIs, deletions of which result in a truncation of tRNA genes *leuX* and *selC* that serve as integration sites of PAI I_{536} and PAI II_{536}, respectively. Loss of functional $tRNA_5^{leu}$ (encoded by *leuX*) affects expression of several virulence factors such as type 1 fimbriae, haemolysin, flagella and serum resistance phenotype (135). *Trans*-complementation of the tRNA locus leads to a restoration of these properties. Virulence genes often show unusual codon usage, e.g., gene *fimB* required for type 1 fimbrial expression contains 5 of the triplet TTG to be recognised by $tRNA_5^{leu}$. Similarly, loss of the selenocysteine-specific tRNA (encoded by *selC*) influences the ability of strain 536 to grow under anaerobic conditions as the enzyme formate dehidrogenase (FDH) involved in mixed acid fermentation contains the rare amino acid, selenocysteine.

Not only deletions of specific regulatory loci, but introduction of certain genes can also attenuate virulence. Pathogenic bacteria lack large DNA regions, which are present in non-pathogenic strains. These deletions have been termed black holes. Introduction of certain genes carried on these regions into pathogenic variants may decrease their virulence (112).

1.2.3.2. RfaH as a virulence regulator

The RfaH protein acts as a transcriptional regulator in *Escherichia coli, Salmonella enterica* serovar Typhimurium, and possibly in other gram-negative bacteria (11). During the last two decades, several operons that are dependent on RfaH for full expression have been identified in various strains. These include *rfa, rfb, hly, tra, chu, cps, kps*, whose altered expressions in the absence of RfaH result in decreased amount of LPS core (104), O-antigen (177), α-haemolysin (13;99), F-factor (142), group I- (133), group II- (162) and group III (8) capsules, respectively.

The exact mechanism by which RfaH enhances expression of these components is not yet fully understood. According to the present view RfaH-regulation takes place at the level of transcription antitermination, hence suppressing operon polarity. It reduces termination of transcripts in a manner analogous to the N and Q proteins of bacteriophage λ (20). According to this model, modification of RNA polymerase by a host protein occurs while transcribing specific sites, which can be distant from the termination signal (136). Indeed, the effect of RfaH is highly dependent on a *cis*-acting region termed JUMPStart sequence. Recently, association of RfaH and a 8 bp motif located within the JUMPStart sequence (the *ops* element) was described (12).

Interestingly, all determinants affected by RfaH encode for components that are secreted or anchored in the outer membrane. Furthermore, they all are somehow related to bacterial virulence: they provide shelter against host defence mechanisms (capsules and intact LPS) or serve as a cytotoxin (α-haemolysin) contributing to dissemination and supplying bacteria with essential nutrients (e. g. iron containing intracellular substances) during infection. Alternatively, F-factor encoding the sex-pilus is of great importance in „bacterial fertility", hence accelerating pathogenic microbial evolution.

1.2.4. Uropathogenic *Escherichia coli* strain 536

The model organism of this study, *E. coli* strain 536 (O6:K15:H31) is one of the few well-characterised uropathogenic *E. coli* strains. It was isolated from a patient suffering from acute pyelonephritis (18). UPEC 536 produces numerous virulence factors including two distinct α-

haemolysins, several fimbriae (P-related, Sfa_I, type-1, curli), siderophore systems (enterobactin, yersiniabactin), a type II capsule (K15), and it exhibits serum resistance (60). Several of the genes encoding these virulence traits are located on pathogenicity islands. UPEC strain 536 carries at least four pathogenicity islands on its chromosome. PAI I_{536} and PAI II_{536} containing *hly* and *prf* genes are flanked by direct repeats, along which PAIs can be deleted by homologous recombination. PAI III_{536} and PAI IV_{536} encode the S fimbrial adhesin and the yersiniabactin iron uptake system, respectively. PAI IV_{536} appears to be identical to the "high pathogenicity island" (HPI) of yersiniae (147). While HPI is flanked by direct repeats in yersiniae, only one of the repeats is present in UPEC strain 536, whereupon deletion of HPI has not yet been detected in this strain. Similarly, PAI III_{536} has not been shown to be deletable.

All four PAIs are integrated within tRNA genes: *selC*, *leuX*, *thrW*, and *asnT*, respectively. Furthermore, the presence of an additional PAI inserted within tRNA gene *pheV* was proposed recently (77). Deletion of PAI I_{536} results in the truncation of *selC* encoding selenocysteine tRNA. Similarly, deletion of PAI II_{536} causes inactivation of the minor tRNA gene *leuX*. Consequences of the truncated tRNA genes on virulence and growth abilities are discussed above.

Urovirulence of *E. coli* strain 536 was proven in several animal models before (62). Virulence of mutants lacking either PAI I_{536} or PAI II_{536} or both was investigated and reported (85;122). Similarly, pathogenic consequences of the sole loss of tRNA genes that serve as integration sites for PAIs were thoroughly examined (41;135).

2. AIMS

2.1. Investigations on the expression of α-haemolysin

Different plasmid or chromosomally borne *hly* determinants encoding the cytotoxin α-haemolysin share high sequence homologies within the coding region of the operon, while 5' flanking regions are highly dissimilar. Consequently, haemolytic activities deriving from various *hly* operons appear to be different. Moreover, expression of α-haemolysin seems to be under the influence of several regulatory factors, and yet some other regulators are speculated to exist. To get more insight into the regulatory mechanisms of *hly* determinants, we aimed:

- To compare haemolytic activities deriving from the individual *hly* determinants of strain 536 using deletion mutants, which lack one or both pathogenicity islands carrying *hly* operons.
- To determine the nucleotide sequence of both haemolysin determinants of strain 536 by
    - Construction of a cosmid gene bank
    - Selection of haemolytic clones containing individual *hly* determinants
    - Sequencing of both *hly* operons by the 'primer walking' method
- To clone both *hly* determinants of strain 536 containing different length of untranslated leader sequence. Furthermore, to produce recombinant haemolysin operons containing various parts of *hlyI* fused to *hlyII* sequences.
- To determine the amount of intracellular and secreted HlyA molecules as well as to monitor *in vitro* and *in vivo* haemolytic activity deriving from these clones, which could result in the identification of specific DNA regions that are responsible for different haemolytic properties originated from the two distinct *hly* determinants of UPEC strain 536.

2.2. Studies on RfaH-mediated virulence gene regulation in strain 536

Regulatory protein RfaH is one of the most important factors, that influence haemolytic activity. The novel haemin receptor molecule, ChuA was speculated to be functionally dependent on active α-haemolysin. We aimed to investigate whether expression of ChuA is coregulated by RfaH in *E. coli* strain 536 as follows:

- To compare ChuA protein levels expressed by wild type strain 536 and its isogenic *rfaH* mutant using a specific antiserum
- To prove that decreased level of ChuA in the *rfaH* mutant is a consequence of lower transcriptional rate and not an indirect effect of the altered cell surface
- To sequence gene $chuA_{536}$ and identify the so-called JUMPStart sequence, which is a conserved motif shared by RfaH-affected operons

Furthermore we aimed:

- To prove that RfaH acts as a global virulence regulator in UPEC strain 536, by comparing the function, structure and amount of several pathogenicity factors, such as capsule, LPS, siderophores, α-haemolysin, flagella, and different fimbriae in strain 536 and its *rfaH* mutant.
- To give evidence that loss of regulatory protein RfaH, in fact, attenuates virulence in different mouse models.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Table 2. and Table 3., respectively.

Table 2. Strains used in this study (continued on next page)

E. COLI STRAIN	CHARACTERISTICS	SOURCE/REFERENCE
536	Wild type uropathogenic strain, O6:K15:H31, Sm^R	(18)
536-114	Deletion mutant lacking PAI I_{536}	(26)
536-225	Deletion mutant lacking PAI II_{536}	(26)
536-21	Deletion mutant lacking both PAI I_{536} and PAI II_{536}	(26)
536*rfaH*::*cat*	Gene *rfaH* inactivated by a *cat* cassette, Sm^R, Cm^R	(123)
HB101	K-12 laboratory strain, F^-, *ara*-14, *gal*K2, *hsd*S20 (*hsr*$^-$, *hsm*$^-$), *rec*A13, *sup*E44, *lacZ*4, *leu*B6, *pro*A2, *thi*-1, *rsp*L20 (Sm^R), *xyl*-5, *mtl*-1, λ^-	(31)
XL-1 Blue MR	K-12 laboratory strain, *recA*1, *endA*1, *gyrA*96, *thi*1, *hsd*R17, (r_k^-, m_k^-), *supE*44, *relA*1, *lac*-, λ^-, [F´, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15, Tn10(Tc^R)]	Stratagene
DH5α	K-12 laboratory strain, 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/*lacZ*ΔM15	Bethesda Research Laboratories
J96	Uropathogenic strain, O4:K6	(75)
AD110	Uropathogenic strain, O6:K2	(172)
764	Uropathogenic strain, O18:K5:H5/11	(132)
2980	Uropathogenic strain, O18:K5:H5/11	(132)
RZ430	Uropathogenic strain, O6:K$^-$:H31	(185)
RZ451	Uropathogenic strain, O6:K18/22:H31	(185)
RZ532	Uropathogenic strain, O6:K$^+$:H31	(185)
RZ439	Uropathogenic strain, O6:K5	(184)
RZ441	Uropathogenic strain, O6:K5	(184)
RS218	NBM, O18:K1:H7	(151)
IHE3034	NBM, O18:K1:H7/9	(89)
IHE3036	NBM, O18:K1:H7/9	(89)
EDL933	EHEC, O157:H7	(128)
9167/91	EHEC, O157:H7	(39)

6578/93	EHEC, O157:H7	(140)
5159/91	EHEC, O157:H7	(140)
86-24	EHEC, O157:H7	(43)
E32511	EHEC, O157:H7	(69)
SF493/89	EHEC, O157:H$^-$	(81)
3574/92	EHEC, O157:H$^-$	(39)
3978/91	EHEC, O157:H$^-$	(139)
5291/92	EHEC, O157:H$^-$	(139)
2907/97	EHEC, O55:H6	(123)
5720/96	EHEC, O26:H$^-$	(183)
3697/97	EHEC, O26:H$^-$	(145)
5714/96	EHEC, O103:H2	(145)
ED147	EHEC, O26:H11	(138)
ED142	EHEC, O111:H$^-$	(138)
78/92	EHEC, O111:H$^-$	(168)
95004730	EHEC, O111:H$^-$	RP[1]
E2348/69	EPEC, O127:H6	(102)
179/2	EPEC, O55:H6	(123)
156A	EPEC, O55:H7	(29)
182A	EPEC, O55:H7	(29)
37-4	EPEC, O55:H$^-$	(102)
76-5	EIEC, O143:HND	(123)
12860	EIEC, O124:HND	(123)
EDL1284	EIEC, serotype not determined	(123)
C9221a	ETEC, O6:K15:H16	(123)
DPA065	EAEC, O119:HND	AG[2]
5477/94	EAEC, O86:H7	(123)
7484/94	EAEC, O86:H18	(123)
DDC4441	EAEC, O128:HND	(123)
17-2	EAEC, O3:H2	(173)
5464/95	EAEC, O3:H2	(123)

[1] RP: From the strain collection of Robert Pringle (Victoria Infectious Diseases Reference Laboratory, Australia)

[2] AG: From the strain collection of Anna Giammanco (Dipartimento di Igiene e Microbiologia, University of Palermo, Italy)

Table 3. Plasmids used in this study

PLASMID	VECTOR	INSERT	REFERENCE
	PGEM® T-Easy	Ap^R, *ori*f1, *lacZ*	Promega
pSMK1	PGEM® T-Easy	*rfaH* cloned in pGEM® T-Easy, Ap^R	(123)
	SuperCos1	Cosmid vector	Stratagene
pCos3b33	SuperCos1	Cosmid clone of *E. coli* 536 containing the coli haemin uptake (*chu*) locus	(123)
pCos4b22	SuperCos1	Cosmid clone containing part of PAI I_{536} with the whole *hlyI* determinant	This study
pCos2g43	Supercos1	Cosmid clone containing part of PAI II_{536} with the whole *hlyII* determinant	This study
	pUC18	Ap^R, *ori*ColE1, *lacZ*	(182)
pGNH100	pUC18	3' end of *hlyC* and *hlyABD* of *hlyI*	This study
pGNH101	pUC18	*hlyCABD* of *hlyI*	This study
pGNH102	pUC18	*hlyCABD* of *hlyI* and 500 bp of upstream region	This study
pGNH103	pUC18	*hlyCABD* of *hlyI* and 1000 bp of upstream region	This study
pGNH104	pUC18	*hlyCABD* of *hlyI* and 1500 bp of upstream region	This study
pGNH106	pUC18	*hlyCABD* of *hlyI* and 1800 bp of upstream region	This study
pGNH204	pUC18	*hlyCABD* of *hlyII* and 1500 bp of upstream region	This study
pGNH124B	pUC18	*hlyC* and 1500 bp of upstream region originated from *hlyII* fused together with *hlyABD* of *hlyI*	This study
pGNH214B	pUC18	*hlyC* and 1500 bp of upstream region originated from *hlyI* fused together with *hlyABD* of *hlyII*	This study
	pACYC184	*ori*P15A, Cm^R, Tc^R	(33)
pGNH35	pACYC184	ORF upstream of *hlyI*, Cm^R	This study
pGNH45	pUC18	ORF upstream of *hlyI*, Ap^R	This study

3.2. Primers

Oligonucleotides used in this study are listed in Table 4.

3.3. Media, culture conditions, and antibiotics

Preparation of culture media, buffers, and solutions was performed as described by Sambrook *et al.* (141). Bacteria were grown routinely at 37 °C in Luria-Bertani broth (LB) or LB solidified with 1.5 % agar (Biolab, Hungary). For haemolytic activity tests, supernatants were obtained from cultures grown in 2xTY medium. For the determination of siderophores,

bacteria were grown in M9 medium (141). In iron-restricted studies 0.1-0.4 mM of the iron chelator 2,2' dipyridyl (Sigma) was added to the media. When appropriate, media were supplemented with the following concentrations of antibiotics: ampicillin (Ap) 100 μg/ml, chloramphenicol (Cm) 30 μg/ml, streptomycin (Sm) 50 μg/ml, tetracycline (Tc) 15 μg/ml.

Table 4. Primers used in this study

NAME	LENGTH	SEQUENCE (5' → 3')	BINDING SITE
shuA-F	20	GTC ACG TCC GCA ATT TAC CT	*shuA*, *chuA* gene
shuA-R	19	CCG TTA CGA CCA TCC TGT G	*shuA*, *chuA* gene, reverse
shu1	21	GGT ATT TAT GGT TCA GTG ATG	upstream of *shu/chu* locus, inwards
shu2	19	GTCTGA CTG ATA ATG TCT C	5' end of *shu/chu* locus, outwards
shu3	18	ATG ACC GAC GTT CAT CAG	3' end of *shu/chu* locus, outwards
shu4	21	TTT TCT CAC TCA AAT TGA ACG	downstream of *shu/chu* locus, inwards
chuApr-1	24	GTC AGT GGG GTA AAA AGA AAC GGC	leader sequence of *chuA* gene,
chuApr-2	28	GGG GGG ATA GCC ATA AAC ACA GGA TGG T	leader sequence of *chuA* gene
chuA-1	24	CAT TGC TCT GGC GTA AAT CAC CCC	*chuA* gene, reverse
chu-2	26	GAT TAG GCG CGA GCG CGT TTG GGC GA	*chuA* gene
chu-4	27	CGA CTG GTT AAA TGC AGA TGC AAA AAT	*chuA* gene
chu-5	26	GTC GCT TCT ATA CCA ACT ATT GGG TG	*chuA* gene
chuArev-3	27	CCC TGC GAT GTC ACG CAC GTA CTT TCC	downstream of *chuA* gene, reverse
hlyAson-1	28	GTC TGC AAA GCA ATC CGC TGC AAA TAA A	*hlyA* gene
hlyAson-2	25	CTG TGT CCA CGA GTT GGT TGA TTA G	*hlyA* gene, reverse
hly1upstr-1	32	TAT TCT AGA TGA AGC AAG GTG CAG GAA ATA AA	leader sequence of *hlyI*
hly1upstr-2	32	TTA TCT AGA GGA AAT GGA GTG CCG TCT GTT CT	leader sequence of *hlyI*
hly1upstr-3	34	TTA TCT AGA ATG TGG CGA AAC TGA TAA TCA AAT T	leader sequence of *hlyI*
hly1upstr-4	32	TTA TCT AGA GAT GGT GAT CCA CAT GAA TTT GA	leader sequence of *hlyI*
hly1upstr-5	33	TTA TCT AGA GCC TGT TTT TGT CAA TGG TGG TAC	leader sequence of *hlyI*
hly1upstr-6	34	TAT TCT AGA CTG GTC TTA TTA CGC TGA TTT GCC T	leader sequence of *hlyI*
hly2upstr-4	32	TTA TCT AGA GGG TAC TGG GAA GAC CAG GGT TA	leader sequence of *hlyII*
hlyrev-KpnI	34	ATA GGT ACC TTA ACG CTC ATG TAA ACT TTC TGT T	downstream of both *hlyI* and *hlyII*, reverse
hlyG-rev	34	TAT GGA TCC TAC CTG ATG ATA TGG GGC AAC CTG A	leader sequence of *hlyI*, reverse
M13uni	17	GTA AAA CGA CGG CCA GT	pUC18 sequencing primer
M13rev	17	CAG GAA ACA GCT ATG AC	pUC18 sequencing primer
SuperCos-F	20	CGG CCG CAA TTA ACC CTC AC	SuperCos1 sequencing primer
SuperCos-R	23	GCG GCC GCA TAA TAC GAC TCA CT	SuperCos1 sequencing primer

3.4. Sera

Sera against HlyA and HemR were provided by Ivaylo Gentschev (Institute for Microbiology, University of Würzburg) and Jürgen Heesemann (Institute for Hygiene and Medical

Microbiology, University of Munich). HemR is the haemin receptor of *Yersinia enterocolitica*, which shows high sequence homology to ChuA (the haemin receptor of *E. coli*). The serum against HemR, in fact, appears to be cross-reactive with ChuA. Sera against Prf, Sfa_I and flagella of strain 536 were raised and supplied by A. Salam Khan (Institute for Molecular Biology of Infectious Diseases, University of Würzburg).

3.5. Reagents and kits used for standard molecular biology methods

Primers were obtained from commercial sources (Sigma Oligosys or MWG GmbH). For routine PCR reactions the REDTaq ReadyMix (Sigma) or PCR SuperMix (Gibco) was used. PCR-generated fragments to be cloned were amplified either by KlenTaq LA Polymerase (Sigma) or Expand Long Template PCR (Roche) systems. Ligations were performed by using the high concentration T4 ligase supplied by New England Biolabs. Routine plasmid preparations were performed by the alkaline lysis method (Sambrook) followed by phenol-chloroform extraction. For high purity plasmid preparations the Qiagen Plasmid Purification Systems were used. Agarose was supplied by Gibco. DNA extraction from agarose gels was achieved using QIAquick Gel Extraction kit (Quiagen) or GenElute Agarose Spin Columns (Sigma). Restriction endonucleases were provided by MBI Fermentas and Amersham Pharmacia. For isolation of RNA the RNeasy Mini Kit (Quiagen) was used. Rnasin Ribonuclease Inhibitor (Promega) was applied while working with RNA.

3.6. Methods

3.6.1. Cosmid library construction and selection of clones

Chromosomal DNA was isolated using Nucleobond® AX columns (Macherey-Nagel) and partially digested with *Sau*3AI. The fragments were cloned into the cosmid vector SuperCos 1 (Stratagene) as described by the manufacturer. Recombinant cosmid clones were packed in lambda phage particles by using Gigapack III XL packaging mixture (Stratagene). Cosmid containing particles were used to transduce *E. coli* XL-1 Blue MR cells. Approximately 1500 clones were picked and conserved in glycerol stock cultures. Cosmid clones carrying *chuA* determinants were identified by colony- and Southern blot hybridisation as described by Stratagene using PCR-generated DNA fragments labelled with an ECL™ direct labelling kit (Amersham-Pharmacia). Haemolytic clones were selected on blood agar, and were verified by PCR using specific primer pairs.

3.6.2. Sequencing

Nucleotide sequence was determined from cosmid clones of strain 536 by the dideoxynucleotide chain termination method (143) using an ABI Prism 310 automatic sequencer. Assembling of the overlapping sequences was performed by the AutoAssembler 2.1. program, and was analysed by the Basic Local Alignment Search Tool (BLAST) program (5).

3.6.3. Cloning procedures

Haemolysin determinants were amplified by long distance PCR (KlenTaq LA Polymerase, Sigma) using primers containing the recognition sites for the restriction endonucleases *Kpn*I or *Xba*I. After digestion and purification, the fragments were ligated into pUC18 vector digested with *Kpn*I and *Xba*I. pGNH124B and pGNH214B were constructed from PGNH104 and pGNH204 by ligation of the 5' ends (leader sequence together with the 5' end of *hlyC*) from one haemolysin determinant with the 3' ends from the other corresponding operon (containing the 3' end of *hlyC* and *hlyABD*). Fusion was achieved at the single *BamH*I site within *hlyC*. All plasmids were introduced into competent DH5α cells, which exhibited strong haemolytic activity upon transformation. Clones were verified by plasmid isolation followed by restriction mapping. Sequencing was performed using the M13 sequencing primer pairs (Fermentas) to ensure proper ligation of inserts into pUC18.

The ORF located upstream of *hlyI* (bps 1657-779) was amplified by PCR as described above using primers hly1upstr-5 and hlyG-rev. Sticky ends were produced through digestion with *Xba*I and *BamH*I. The purified PCR product was ligated into the corresponding sites of digested pACYC184 vector. The obtained plasmid was termed pGNH35, which was then introduced into DH5α cells harbouring compatible haemolytic plasmids. To achieve higher copy number, the same PCR product was cloned into the corresponding sites of vector pUC18 and the resulting plasmid was termed pGNH45.

3.6.4. Transformation

Competent cells were produced by the $CaCl_2$ method. Briefly, 50 ml of LB medium was inoculated with 1 ml of DH5α cells grown overnight. The culture was shaken at 37°C until 0.3-0.4 value at OD_{600}. Bacteria were pelleted and washed once with ice cold 0.1 M $CaCl_2$. After centrifugation, bacteria were resuspended in 1.25 ml of 0.1 M $CaCl_2$ and were incubated on ice for 30-45 minutes. Then, bacterial suspension was mixed with 0.52 ml of concentrated glycerol. 150 µl aliquots of competent cells were stored at -80°C.

Transformation was performed following standard protocols described by Sambrook *et al.* (141). 20 µl of the ligation mixture was added to one cap (150 µl) of competent cells. The mixture was incubated on ice for 30 minutes. Introduction of the plasmids into competent cells was accomplished through a 3-minutes incubation at 43°C. After bacteria were incubated for 5 minutes on ice, they were given 1 ml of LB medium. To allow recovery and expression of antibiotic resistance, transformed bacteria were incubated at 37°C for 1 hour, and then they were plated onto agar plates containing the selective antibiotic.

3.6.5. Assay of haemolytic activity

Haemolytic activities of culture supernatants were determined using a formerly described method (65) with minor modifications. Briefly, erythrocytes were obtained from a healthy individual and were washed three times with 150mM NaCl, 20mM $CaCl_2$. 1 % suspensions of erythrocytes were incubated for 40 min at 42°C with 1:10- or 1:100-diluted cell-free supernatants of bacterial cultures grown in 2xTY medium. After short centrifugation, released haemoglobin was measured photometrically (A_{543}). Degree of haemolysis was quantified as percentage of total haemolysis obtained by Triton X-100 (Sigma).

3.6.6. Western blot analysis

Whole-cell extracts obtained from an LB culture were separated by SDS-PAGE as described by Laemmli (94). Alternatively, secreted α-haemolysin was precipitated overnight from culture supernatants by the addition of 10 % trichloro-acetic acid (TCA). Protein samples were blotted onto nitrocellulose membrane using a Mini Trans-Blot cell (Bio-Rad). The blocked membranes were treated with specific antisera and secondary antibody (anti-rabbit IgG – horseradish peroxidase conjugate; DakoA/S, Denmark) diluted suitably in TBST containing 2% skimmed milk. The blot was developed using ECL detection reagents (NEN Life Science, Boston, MA) followed by luminography.

3.6.7. RNA isolation and Northern blots

Total RNA was isolated from bacteria harvested from iron-low medium using RNeasy Mini Kit (Quiagen). RNasin Ribonuclease Inhibitor (Promega) was applied from the first step of the isolation procedure. Northern blot analysis was performed as described (8). 10 µg of isolated RNA per lane was separated on a 1.2% agarose-formaldehyde gel and was transferred to a Biodyne B Transfer Membrane (Pall Ltd., England) overnight by capillary blotting. The DNA probe specific for the 3' end of *chuA* was generated by PCR using primers chu-5 and shuA-

rev and was labelled with the ECL™ direct labelling system (Amersham-Pharmacia). Hybridisation was carried out overnight at 42°C as described by the manufacturer. Before luminography, the filter was washed twice for 15 min in 0.5 x SSC, 0.4% SDS (50°C), and then twice for 5 min in 2 x SSC (20°C).

3.6.8. Southern hybridisation

Chromosomal DNA was isolated as described before (57). Fragments digested with *BglI* were separated by electrophoresis through a 0.8% agarose gel. DNA was transferred to Biodyne B Transfer Membrane (Pall Ltd., Portsmouth, England) using a vacuum blotter (Amersham-Pharmacia). The generation, labelling and detection of the *chuA*-specific probe as well as the hybridisation procedure were performed as described above for Northern blot analysis.

The oligonucleotides used for hybridisation were: probe-2 (originated from strain 536); 5'-TGA ATT ATC AGA AAT ATT CGG CAA TTT TAC GGG ATA TAT ACG CTA ATA GCT TCC CGT GGT GAT ATC TAA TCA-3' and probe-3 (originated from strain EDL933); 5'-CGA GTT ATC AGG CAA TTT CAT GGG ATA TAA ACG C-3'. The probes were labelled with digoxigenin using the DIG Oligonucleotide 3'-End Labelling Kit (Roche). Prehybridisation and hybridisation were carried out at 30°C in high SDS concentration hybridisation buffer for 4 h and overnight, respectively. The filters were washed twice for 10 minutes at room temperature in 2xSSC, 0.1% SDS. Hybridised nucleotides were detected using DIG Luminescent Detection Kit (Roche) following the standard protocol provided by the manufacturer.

3.6.9. LPS analysis

Bacteria grown on solid LB were suspended in distilled water and boiled for 30 min. Suspensions were sonicated in a Realsonic Cleaner equipment for 10 min. LPS was purified by the procedure of Hitchcock *et al.* (70). SDS-PAGE was performed on a 12.5% gel according to the method of Laemmli (94). Gels were fixed overnight in a solution of 7% acetic acid and 25% 2-propanol and were silver stained as described by Nelson *et al.* (124).

3.6.10. Production of serum against K15 capsule

Capsule-specific serum was produced following the principles described by Kiesewalter and Seltmann (83). Bacteria (*E. coli* strain 536) were incubated in 50% EtOH overnight at 4°C to destroy protein antigens. After washing, bacteria were diluted to $2x10^8$/ml in PBS. Rabbits were immunised with 0.2, 0.5, 1, and twice 2 ml of bacterial suspensions in 4-day-intervals. Four days following the last inoculation, test sera were taken and tested by tube agglutination.

Afterwards, rabbits were sacrificed, sera were collected and diluted to 1:10 in sterile saline. Removal of non-specific antibodies was achieved by absorption with living and boiled cells of the uropathogenic *E. coli* strain RZ532 (O6:K^+:H31) (83). 0.5 g of bacteria were suspended in each ml of diluted serum, and the suspension was incubated for 1h at 37°C and overnight at 4°C. Next day, bacteria were harvested, resuspended in saline and cooked for 1 h. Following centrifugation, the pellet was resuspended in the same serum and suspensions were incubated again for 1 h at 37°C and overnight at 4°C. Through this procedure, sera became free of any immunoreactive antibodies to *E. coli* 536 except those against K15-antigen, as proven by ELISA.

3.6.11. ELISA

96-well plates (C.E.B., France) were coated overnight with 0.2 ml bacterial suspensions (10^9 CFU/ml) in coating buffer at 4°C. The following day, plates were washed with PBS + 0.05 % Tween 20 (washing buffer), and then blocked with PBS containing 2% BSA (Sigma) for 1 h at 37°C. K15-specific serum was diluted in PBS + 0.5% BSA and incubated with the antigen-coated plates for 90 min. Serial dilutions were conducted across the plates. After three washes, plates were probed with commercial anti-rabbit immunoglobulin conjugated with HRPO (Dako A/S, Denmark). The ELISA substrate was *ortho*-phenylenediamine (Sigma) dissolved in citric acid buffer containing H_2O_2. The OD was measured at 490 nm on a conventional ELISA plate reader. Duplicates were used for each strain and dilution, and assays were repeated twice.

3.6.12. Detection of siderophores

The amount of secreted siderophores was determined using the CAS (Chrome Azurol S) assay (149) with minor modifications. Bacteria were grown overnight in M9 medium. To induce siderophore production, the iron chelator 2,2' dipyridyl was added at a final concentration of 0.1-0.4 mM. The cultures were incubated for an additional 3 h at 37°C. Then, bacterium-free supernatants were mixed with an equal volume of CAS assay solution (149). After reaching equilibrium (1-2 h) the absorbance was measured at 640 nm.

3.6.13. Serum bactericidal test

Bacteria grown in LB medium were washed with saline and diluted to 10^6 CFU/ml. 100 µl aliquots of bacterial suspensions were mixed with an equal volume of normal human serum and incubated at 37°C for 4 hours in microtiter plates. Samples were taken at 0, 0.5, 1, 2, 3,

and 4h timepoints. Viable cell count was determined by plating onto LB plates and incubating overnight at 37°C. Assays were performed with normal- and heat inactivated (56°C for 30 min) serum. Triplicates were used for each strain and assays were performed 3 times.

3.6.14. Animal experiments

Animal experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory animals (25) in a laboratory authorised by the Hungarian rule (Decree No. XXVII. 1998) and by the subsequent regulation (Gov. Order No. 243/1998).

3.6.14.1. Lung toxicity assay

The lung toxicity test serves as an ideal model for the assessment of *in vivo* haemolysin production. The assay was performed as described by Hacker *et al.* (62). Briefly, 3-week-old CFLP mice (Gödöllő, Hungary) weighing 10-12 g were infected intranasally under superficial ether anaesthesia with 0.05 ml (~$3x10^9$ CFU/ml) bacteria grown overnight in LB medium. 20 mice were applied for each strain. The animals were observed for 24 h and death rates were recorded.

3.6.14.2. Ascending urinary tract infection model in mice

Intravesical infections of 2-3-day-old suckling CFLP mice (Gödöllő, Hungary) were performed as described by Allison *et al.* (4). Bacteria were grown overnight at 37 °C in LB, harvested by centrifugation, washed once, and normalised to the required inoculum density (10^7 CFUs/ml) in PBS by adjusting the suspension to the appropriate OD_{600} value, which had been verified by viable count. 25 µl of this bacterial suspension containing 0.05% Pontamin Sky Blue dye (Searle Pharmaceuticals, High Wycombe, UK) was introduced into the bladder directly through the abdominal wall. The stain, which had no toxic or antibacterial effects served as an indicator for successful inoculation (i.e., the stain became localised only to the bladder, which was visible through the hairless skin). In order to exclude the possibility of vesicoureteral reflux caused by the inoculum, in an additional experiment, mice were sacrificed immediately after inoculation. Neither dye nor bacteria were detectable in the kidneys verifying this experiment to be a suitable model of ascending urinary tract infection. 6-14 infant mice were injected simultaneously with each strain, and assays were repeated 4 times. Mice that survived infection were sacrificed 21 days post-infection. The bladder and both kidneys were removed in sterile conditions, homogenised in PBS, and aliquots were

plated onto agar plates containing a selective antibiotic. Additionally, bacterial counts were determined from the blood obtained by puncturing the heart.

3.6.14.3. Co-infection experiments in mice

Mice were infected with a bacterial suspension containing $2x10^4$ CFUs/ml of the wild type strain 536 mixed with either $2x10^5$ CFUs/ml or $2x10^6$ CFUs/ml of its isogenic *rfaH* mutant (providing 1:10 or 1:100 concentration ratios, respectively). The procedure of injecting the mixture into the bladder was the same as described above. Urine samples were taken daily for 20 days. Urine was diluted in saline and was plated onto LB agar plates containing either streptomycin alone or in combination with chloramphenicol. Since both wild type strain 536 and its *rfaH* mutant were resistant to streptomycin, but only the mutant possessed chloramphenicol resistance, CFUs of both strains could be established this way. Faeces of dams were checked and found to be negative for streptomycin-resistant aerobic bacteria indicating that no faecal contamination of the samples was possible. Furthermore, randomly taken colonies were identified by slide agglutination using specific antisera. On the 21st day post-infection, mice were sacrificed and colony counts were determined from the blood, the bladder and the kidneys as described above.

3.7. Nucleotide sequence accession number

The nucleotide sequence of *chuA* originated from the uropathogenic *E. coli* strain 536 has been deposited in the GenBank under accession number AF280396.

3.8. Statistical analysis

All experiments were repeated at least twice. Results were statistically analysed using the Student *t* test or alternatively, the X^2 probe when indicated. Graphs usually represent means and standard error of means (error bars) of values. In order to avoid confusing complexity of figures, in some cases, however, results of one experiment representative of several similar assays are shown.

4. RESULTS

4.1. Haemolytic activity originated from *hlyI* and *hlyII* is different

4.1.1. Haemolytic activity of PAI mutants

Strain 536 carries two distinct *hly* determinants both of which encoding active α-haemolysin molecules. *hlyI* and *hlyII* are located on pathogenicity islands; PAI I_{536} and PAI II_{536}, respectively. Determining haemolytic activity originated from mutants lacking PAI I_{536} or PAI II_{536} revealed that loss of PAI II_{536} did not significantly change the strain's haemolytic properties, while that of PAI I_{536} caused a 97% decrease in haemolytic activity (Fig. 3.). Naturally, loss of both pathogenicity islands resulted in a non-haemolytic phenotype. These observations suggested that expressions of *hlyI* and *hlyII* differed to a great extent, which was proven by Northern blots as well (122). Nevertheless, pathogenicity islands, are large DNA loci that often carry genes encoding regulatory proteins. Hence, beside the loss of *hly* determinants, deletion of PAI I_{536} or PAI II_{536} may result in a simultaneous loss of regulatory element(s) affecting haemolytic activity deriving from the remaining *hly* determinant.

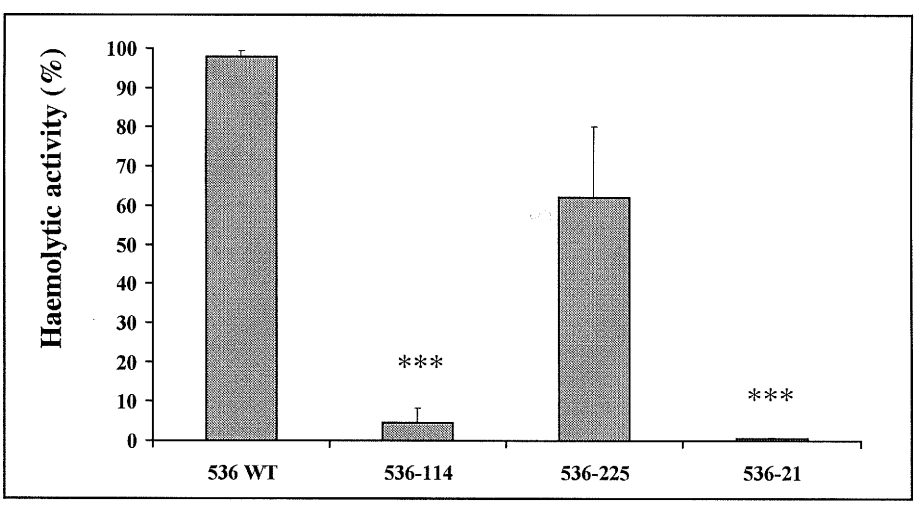

Fig. 3. Haemolytic activity of strain 536 and its PAI deletion mutants. Means and SEM of values originated from three independent assays are shown. Asterisks indicate significant differences (*: p<0.001).**

4.1.2. Sequence analysis of *hlyI* and *hlyII*

To gain a more accurate insight into haemolytic activity deriving from the individual *hly* determinants of strain 536, sequencing of both operons was performed. Beside the approximate 7.3 kb-long coding regions, about 2 kbs of the 5' flanking regions were sequenced. Nucleotide sequence of the four genes showed high similarity to the

corresponding genes originated from the other *hly* determinant of strain 536 (Table 5.) and to other *hly* sequences found in the genebank (data not shown). The untranslated 5' leader sequences, however, did not show any similarity to each other suggesting the presence of dissimilar promoter regions.

The upstream region of *hlyI* resembled to a high extent (96% identity) to the corresponding region originated from strain J96. A 142 bp-long region located between bp 636-495 upstream of the start codon of gene *hlyC* in strain J96, however, appeared to be deleted in the *hlyI* determinant of strain 536. While this region was flanked by 5-bp direct repeats (TGGAG) in strain J96, only one copy was present in strain 536 indicating a minideletion mediated by the repeats.

On the other hand, upstream region of *hlyII* exhibited only moderate and short homologies to sequences found in databases. The region between bp 683-569 upstream of the first start codon showed 86% (100/115) identity to the *hlyR* sequence, which was reported to enhance expression of plasmid encoded α-haemolysin molecules (174). This 115 bp long region contained the so-called *ops* element required for RfaH mediated regulatory activity. The sequence located 5' direction from this region (bp 1041-749 upstream of ATG) appeared to be 96% homologous to sequences identified in *E. coli* strain C5 (MENEC) by representational difference analysis technique (30).

Table 5. Nucleotide and amino acid sequence similarities between genes of *hlyI* and *hlyII* and the encoded proteins

	Length (bp or aa)	Identity	% identity
hlyC	513	502/513	97.9
hlyA	3075	3006/3075	97.8
hlyB	2124	2101/2124	98.9
hlyD	1437	1428/1437	99.4
HlyC	170	169/170	99.4
HlyA	1024	1011/1024	98.7
HlyB	707	707/707	100
HlyD	478	476/478	99.6

4.1.3. Haemolytic properties deriving from cloned *hly* determinants

In possession of the nucleotide sequences, cloning of *hly* determinants originated from strain 536 was performed using a PCR-based method. Different clones of *hlyI* containing various

length of leader sequences were constructed (see Fig. 8.). On the other hand, *hlyII* was cloned with approx. 1500 bps of 5' flanking region. To ensure that different expression of the two *hly* determinants of strain 536 is a consequence of distinct promoter regions, recombinant molecules were constructed, in which leader sequence of one operon was fused to coding region of the other operon and vice versa giving rise to plasmids pGNH124 and pGNH214. All determinants were cloned into pUC18 and plasmids were transformed into the same carrier strain (DH5α). Restriction patterns of these haemolytic plasmids are shown in Fig. 4.

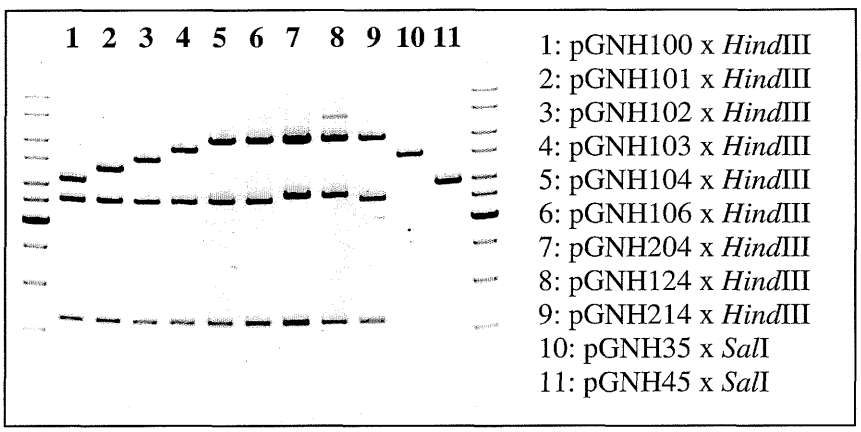

Fig. 4. Restriction pattern of recombinant plasmids related to *hly* determinants of strain 536

The amount of intracellular and secreted HlyA molecules deriving from the cloned *hlyI* (pGNH104) and *hlyII* (pGNH204) determinants as well as fused *hly* determinants (pGNH124 and pGNH214) were determined by Western blots. The results are shown in Fig. 5. Expression of *hlyI* appeared to be significantly higher than that of *hlyII*. Fusion of *hlyI*-originated 5' flanking region to *hlyII* determinants (pGNH124) resulted in an enhanced expression. On the contrary, *hlyII*-originated upstream region caused a decreased expression of HlyA encoded by *hlyI* (pGNH214).

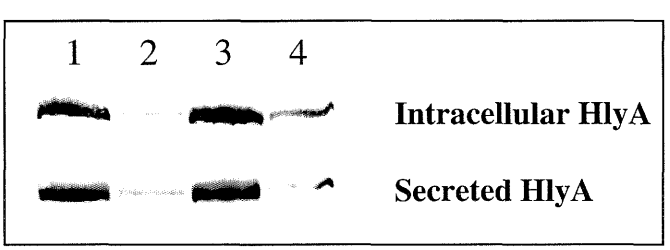

Fig. 5. Amounts of intracellular and secreted HlyA molecules originated from equal number of bacterial cells carrying haemolytic plasmids: pGNH104 (1), pGNH204 (2), pGNH124 (3), and pGNH214 (4).

Functional haemolytic activity originated from these plasmids were determined in a liquid assay. The results presented in Fig. 6. suggested that *in vitro* haemolytic activity was not primarily dependent on the amount of toxin molecules secreted rather on the origin of HlyA, i.e., whether HlyA was encoded by *hlyI* or *hlyII*.

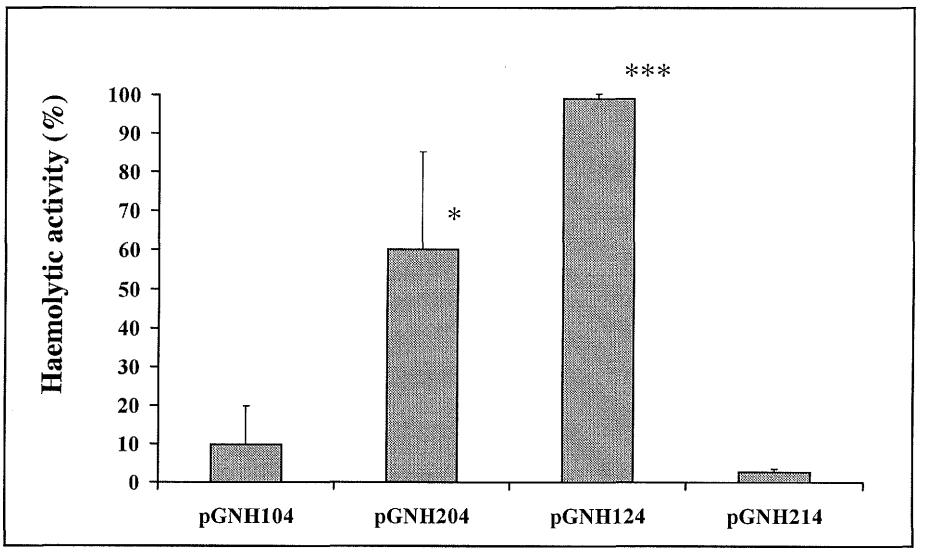

Fig. 6. Haemolytic activity deriving from cloned *hly* determinants. Graph shows means and SEM values resulted from five independent experiments. Asterisks indicate significant differences in comparison to pGNH104 (*:p<0.05, ***:p<0.001).

In vivo haemolytic activity was assessed in the mouse lung toxicity assay. In this model, death rate was shown to be elicited primarily by secreted α-haemolysin. The results summarised in Fig. 7. were in good correlation with the *in vitro* haemolytic activity shown above.

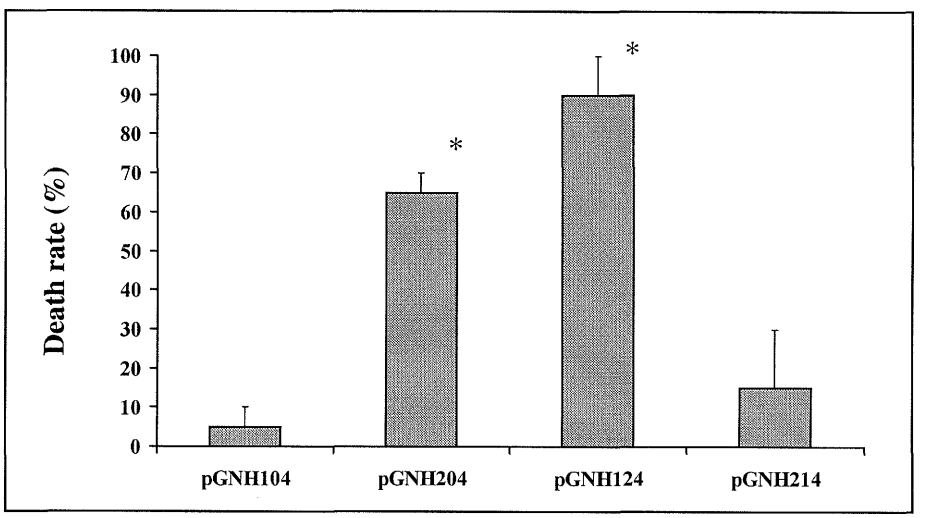

Fig. 7. Death rate elicited by cloned *hly* determinants in the mouse lung toxicity assay. In each group, 20 mice were used in two independent experiments. Asterisks indicate significant differences in comparison to pGNH104 (*:p<0.05).

4.2. Identification of a *cis*-acting regulatory element upstream of *hlyI*

Haemolytic properties of cloned *hlyI* determinants were compared using clones that carried different lengths of leader sequence. Schematic representation of the cloned *hlyI*-originated DNA regions is shown in Fig. 8. Restriction pattern of recombinant plasmids carrying the inserts represented in the figure below is shown in Fig. 4.

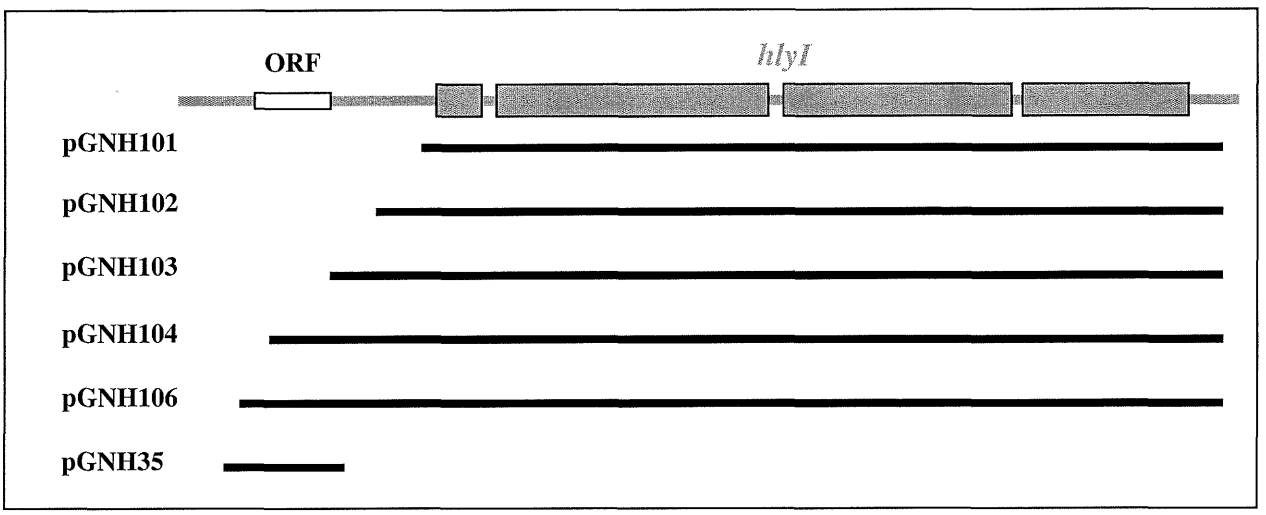

Fig. 8. Schematic representation of cloned *hlyI*-originated sequences

The amounts of intracellular and secreted HlyA originated from the recombinant *hly* determinants were visualised by Western blots, the results of which are shown in Fig. 9.

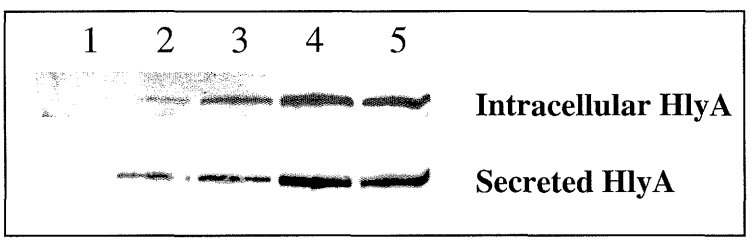

Fig. 9. Amounts of intracellular and secreted HlyA molecules originated from equal number of bacterial cells harbouring haemolytic plasmids: pGNH101 (1), pGNH102 (2), pGNH103 (3), pGNH104 (4) and pGNH106 (5)

Plasmids possessing 1500 bps (pGNH104) or more (pGNH106) of the leader sequence produced considerably more of both intracellular and secreted HlyA than *hlyI* determinants cloned with shorter 5' flanking region (pGNH102 and pGNH103). The promoterless *hlyI* (pGNH101) did not express HlyA.

In vitro haemolytic activity elicited by these plasmids is presented in Fig. 10. Surprisingly, those plasmids expressing higher amount of HlyA appeared to be less active in the liquid haemolytic test.

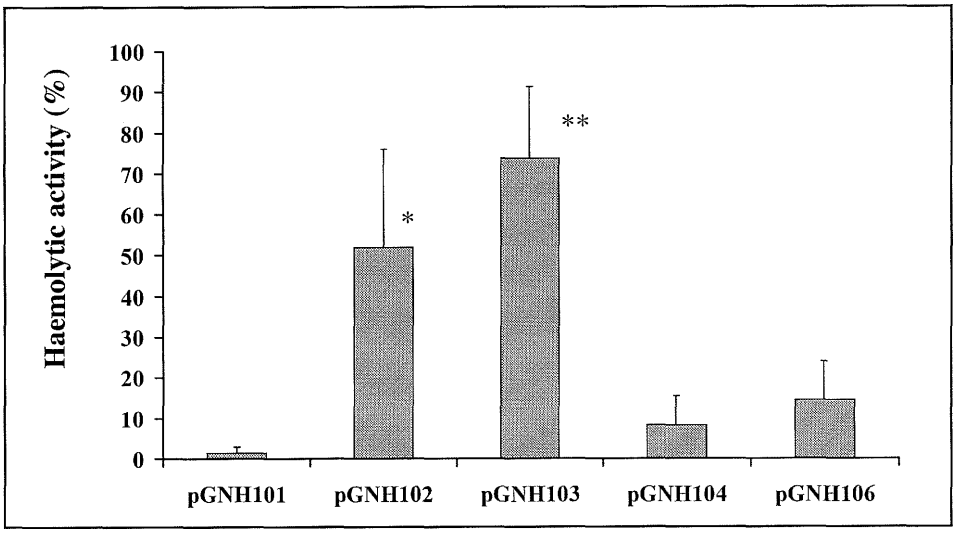

Fig. 10. Haemolytic activity deriving from cloned *hly* determinants. Graph shows means and SEM values resulted from four independent experiments. Asterisks indicate significant differences in comparison to pGNH104 (*: p<0.05, **: p<0.01)

Fig. 11. represents lethality rates of mice due to *in vivo* haemolysis assessed by the lung toxicity assay. These results were again in tune with the haemolytic activity determined *in vitro*.

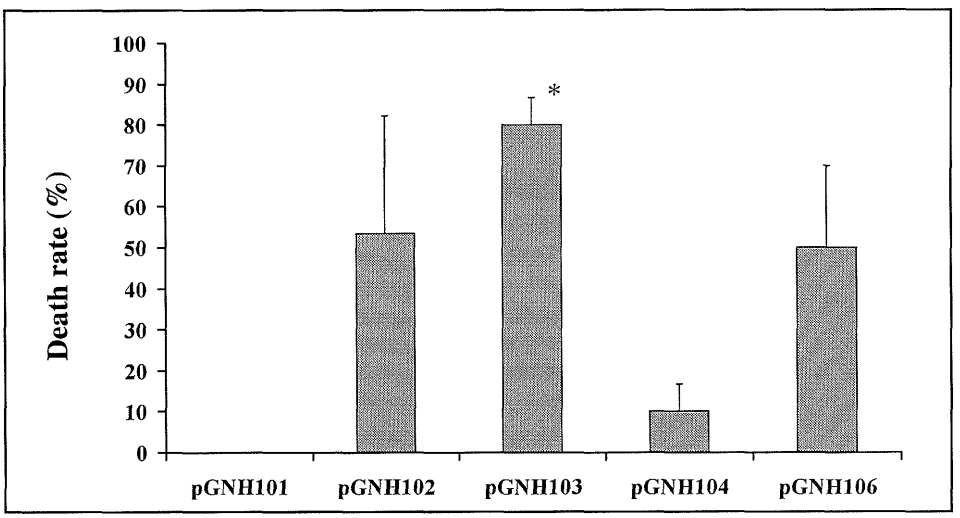

Fig. 11. Death rate elicited by cloned *hly* determinants in the mouse lung toxicity assay. In each group, 30 mice were used in three independent experiments. Asterisk indicates significant difference in comparison to pGNH104 (*: p<0.05)

To exclude the possibility that altered haemolytic properties of clones possessing longer 5' flanking regions derive from the gene product of the ORF located at bps 1657-779 upstream of the first start codon, the region bps 1999-727 was cloned into pACYC184 giving rise to plasmid pGNH35 (see Fig. 8.). Introduction of this compatible plasmid into strains harbouring either pGNH103 or pGNH214, however, did not modify *in vitro* haemolytic activity of these

bacteria (data not shown). Moreover, no detectable protein encoded by the cloned ORF derived from recombinant plasmids pGNH35 (the ORF cloned into pACYC184) or pGNH45 (the same ORF cloned into pUC18) (Fig. 12.).

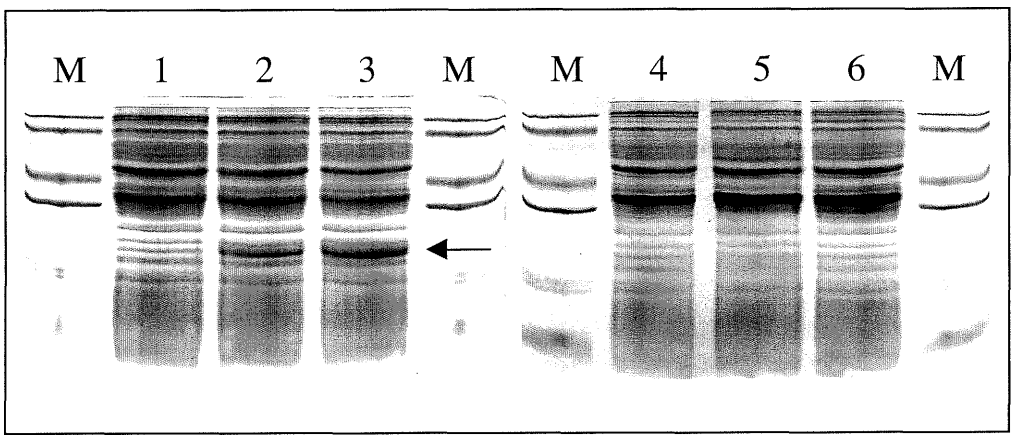

Fig. 12. Protein profiles of total bacterial lysates of DH5α harbouring no plasmid (lane 1 and 4), pACYC184 (2), pGNH35 (3), pUC18 (5), or pGNH45 (6). Bacteria were collected in mid-log phase of growth. 'M' denotes protein molecular weight marker (LMW, Amersham-Pharmacia). The arrow points toward the gene product of *cat* allowing chloramphenicol resistance.

4.3. Haemolytic activity is affected by RfaH

Regulatory protein RfaH was reported to influence expression of α-haemolysin determinants of different origin (13;99). To test whether haemolytic activity originated from strain 536 is affected by the loss of protein RfaH, *in vitro* haemolysis deriving from culture supernatants of wild type strain 536, the mutant 536*rfaH*::*cat*, and its *trans*-complemented variant were compared (Fig. 13.). The wild type strain exhibited strong haemolytic activity during exponential phase of growth reaching a plateau at mid-exponential phase. (Fig. 13.B shows growth curves of these strains.) Haemolysis elicited by the *rfaH* mutant was hardly detectable at any time. Introduction of the cloned *rfaH* gene (pSMK1) into the mutant restored high haemolytic activity.

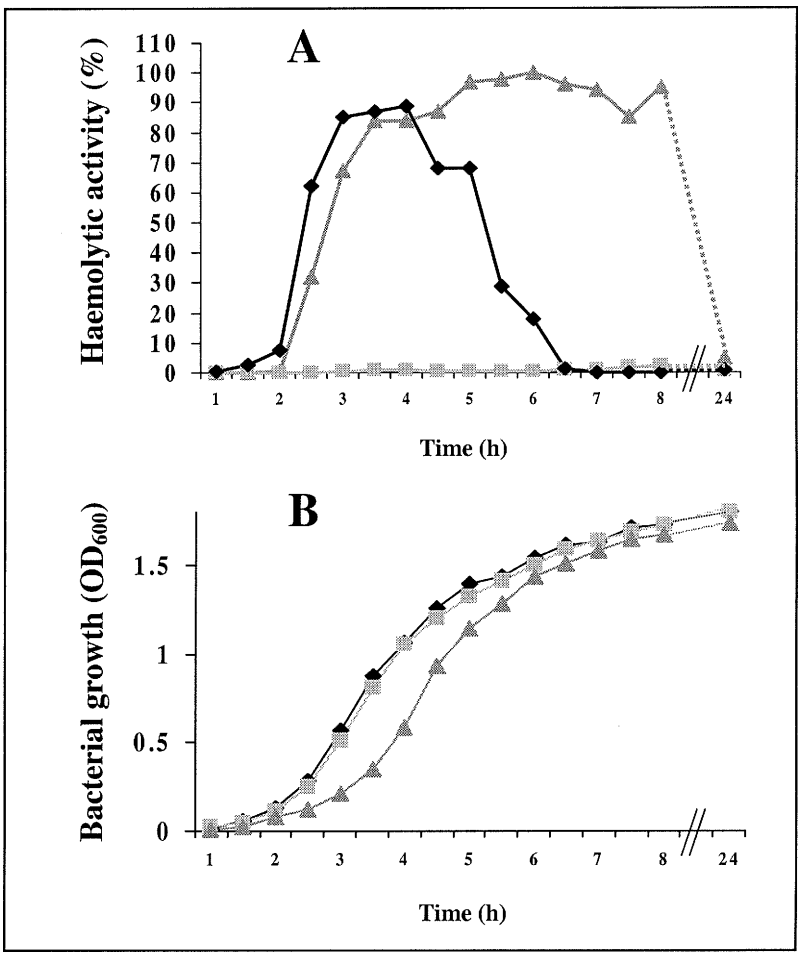

Fig. 13. (A) Haemolytic activity derived from *E. coli* strain 536 (◆), its isogenic mutant 536*rfaH*::cat (■), and *trans*-complemented strain 536*rfaH*::cat (pSMK1) (▲). Values represent percentage relative to total haemolysis. (B) Growth curves of strain 536 (◆), 536*rfaH*::cat (■), and 536*rfaH*::cat (pSMK1) (▲).

4.4. RfaH regulates the expression of haemin receptor ChuA

4.4.1. The ChuA receptor level is decreased in the *rfaH* mutant of *E. coli* strain 536

Pathogenic strains of *E. coli* often carry the so-called coli haemin uptake (*chu*) locus (181), which is inserted into a distinct site of the chromosome (Fig. 14.). To test whether *E. coli* strain 536 carries the *chu* locus, PCR reactions were performed using oligonucleotides outlined in Fig. 14.

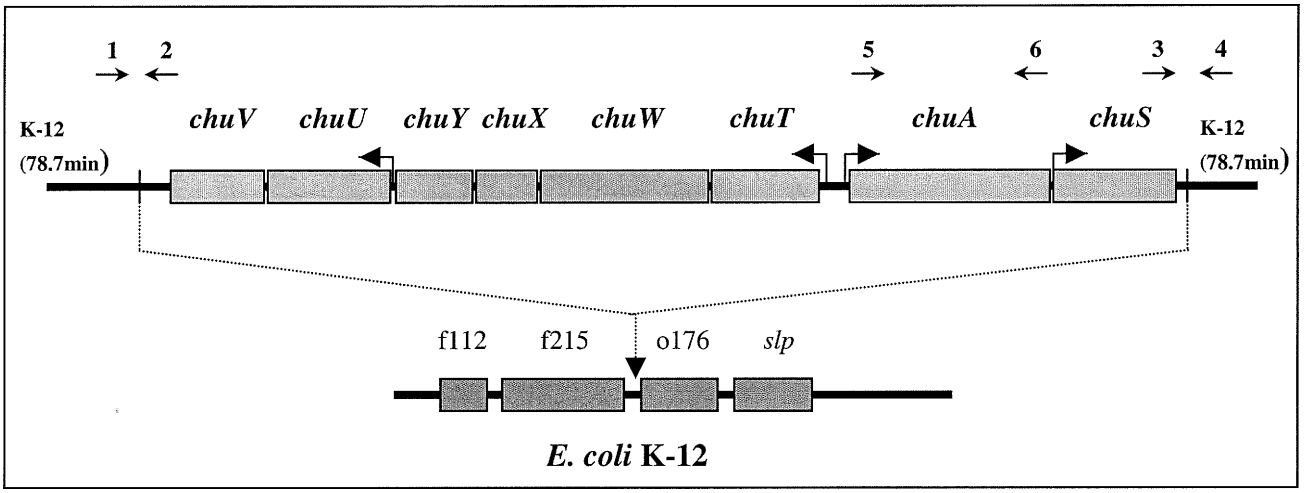

Fig. 14. Genetic organisation and integration site of the *chu* locus. Boxes represent individual genes. Large arrows denote promoters. Small arrows labelled with numbers symbolise the following primers used for PCR reactions; 1: shu1, 2: shu2, 3: shu3, 4: shu4, 5: shuA-F, 6: shuA-R.

The results of the PCR reactions are shown in Fig. 15. Positive reactions using primer pairs shu1+shu2 and shu3+shu4 suggest the presence and usual integration of the *chu* locus within the chromosome of strain 536. Furthermore, the existence of the gene encoding the haemin receptor (*chuA*) was verified by using specific primers shuA-F and shuA-R. In correspondence with former reports, K-12 strain HB101 does not carry the *chu* locus.

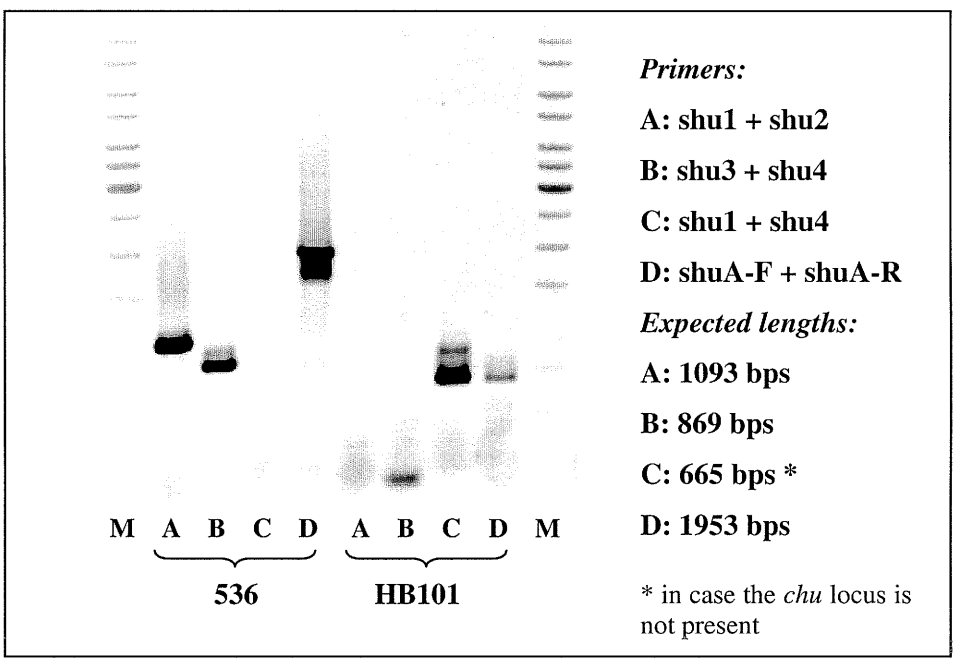

Fig. 15. PCR reactions using specific primer pairs for verifying the presence of the *chu* locus and the chuA gene (see text). M denotes the Gene Ruler 1 kb DNA ladder (MBI Fermentas).

To determine the amount of the haem binding outer membrane protein ChuA in *E. coli* 536, Western blot analysis was performed using a HemR-specific antiserum (Fig. 16.). In order to

verify that expression of the detected protein was iron dependent, we used total cellular lysates from bacteria grown in normal, as well as in iron-depleted medium. The 70 kDa-protein reacting with the antiserum was only detectable if bacteria were grown in low-iron medium. To gain information on the regulation of ChuA, a mutant in which gene *rfaH* was disrupted by a *cat* cassette was used for further studies. It was obvious that the quantity of protein ChuA was strongly reduced in the *rfaH* strain compared to the wild type. Complementation of the mutant strain with the cloned *rfaH* gene (pSMK1) restored higher protein levels.

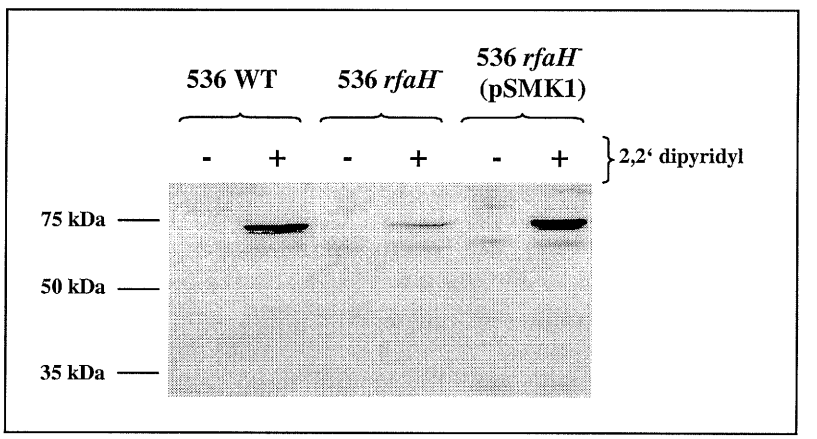

Fig. 16. Western blot analysis of whole cell extracts of *E. coli* strain 536 and its derivatives using a HemR-specific antiserum. The strains were grown in the presence (+) or absence (-) of the iron chelator 2'2' dipyridyl. In the *rfaH* mutant strain, gene *rfaH* was disrupted by a *cat* cassette.

4.4.2. Effect of RfaH on the transcription of gene *chuA*

To investigate whether altered ChuA expression in strain 536*rfaH*::*cat* was a consequence of decreased *chuA*-transcription, Northern blot analysis of total cellular RNA was performed. The *chuA*-specific DNA probe hybridised with a 2.2-2.3 kb mRNA, which correlates with the *chuA* transcript (Fig. 17.). The inactivation of gene *rfaH* resulted in reduced levels of *chuA* mRNA, however the length of the transcript was not altered. Overexpression of RfaH (in the mutant strain carrying pSMK1) manifested in an increased transcription of *chuA* compared to the wild type. These data clearly indicate that RfaH is involved in transcriptional regulation of the gene *chuA*.

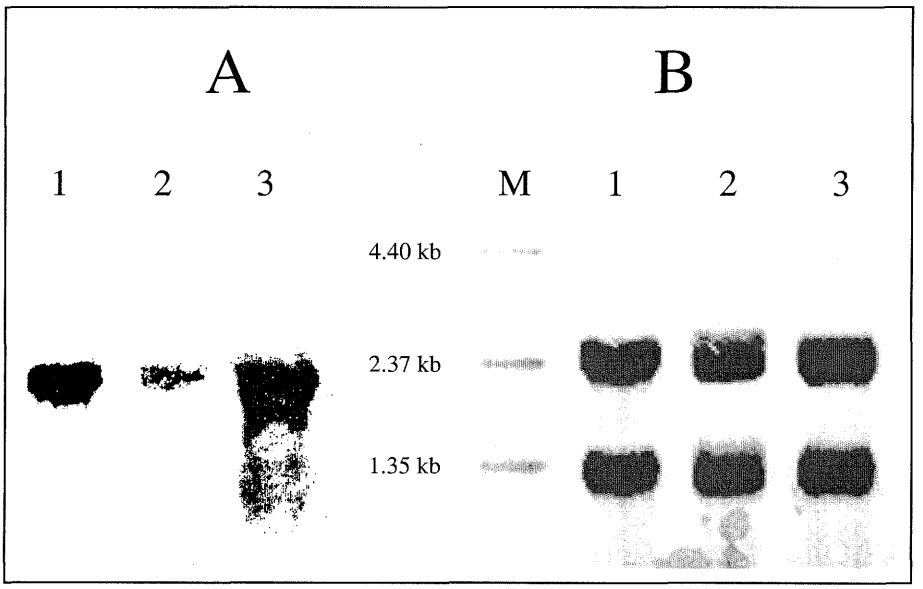

Fig. 17. Influence of RfaH on the *chuA* transcription of *E. coli* 536 and derivatives. A. Hybridisation of an ECL-labelled *chuA*-specific probe to total RNA isolated from strain 536 (1), 536*rfaH*::*cat* (2), 536*rfaH*::*cat* (pSMK1) (3). B. 23S and 16S rRNA stained with 0.3% methylene-blue after transfer of separated total RNA to a nylon membrane (internal control).

4.4.3. Sequence analysis of gene *chuA* from *E. coli* strain 536

It was previously shown, that genes regulated by the RfaH protein often carry so-called JUMPStart sequences. In order to determine, whether such a motif is located in the *chuA* gene of strain 536, sequencing of this gene along with its 5' flanking region was performed. Within the *chuA* gene, a region was identified that highly resembled to the reported JUMPStart sequences. Comparison of this motif to JUMPStart sequences originated from other *E. coli* operons known to be regulated by RfaH is shown in Fig. 18. The 39 bp-region found in gene *chuA* was located 1158 bp downstream from the start codon. It contained an *ops*-like motif: with the exception that the first G of the element is substituted by a C, the other 7 bases of this motif matched perfectly as well as the conserved 5' C which is located downstream of the *ops* element. In the 3' region of the 39 bp-long JUMPStart sequence, a relatively conserved direct repeat could be identified with the relevant spacing also found in other JUMPStart sequences. Coding region of *chuA* showed a very high homology (>97%) to the corresponding sequences derived from *E. coli* O157:H7 (170) and *S. dysenteriae* (116). The potential promoter region was located about 300 bp upstream of the start codon, which was overlapped by a putative Fur-box. The presence of this motif, neighbouring the promoter, explained the observed effect of iron availability on ChuA levels.

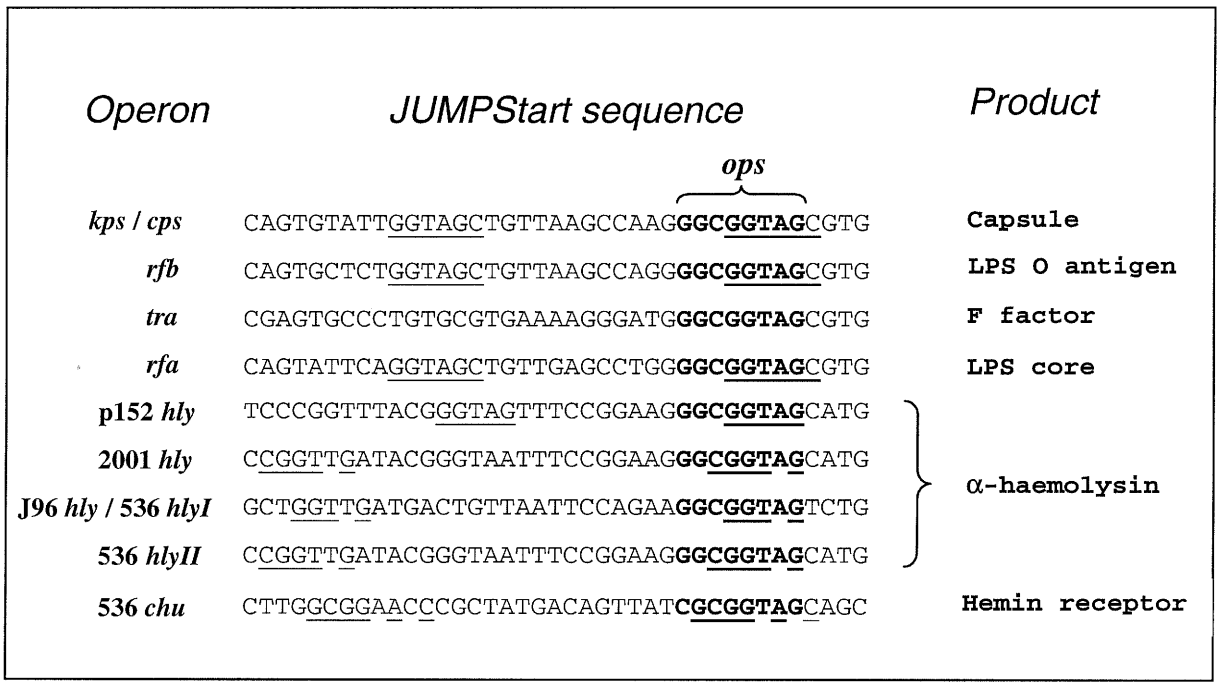

Fig. 18. Comparison of JUMPStart sequences originated from different *E. coli* operons. Bold letters denote the *ops* element, underlined bases represent the imperfect repeats found within the JUMPStart sequences. Accession numbers or references of the sequences: *cps:* AF104912, *kps*: X53819, *rfb*: U09876, *tra*: U01159, *rfa*: M86935, p152 *hly*: M14107 and X07565, 2001 *hly*: (125), J96 *hly*: M10133, 536 *hlyI* and *hlyII*: this study, 536 *chu*: AF280396, EDL933 *chu*: U67920

In contrast to the high homology between the coding regions of the different *chuA/shuA* determinants, the upstream region of *chuA* derived from strain 536 showed less, partially no similarity to the corresponding regions originated from *E. coli* O157:H7 and *S. dysenteriae.* A 74 bp region located between the putative promoter and the start codon of the *E. coli* 536-specific determinant was replaced by a totally different 73 bp motif in *S. dysenteriae* and *E. coli* O157:H7. In the uropathogenic strain, this region was flanked by 6 bp direct repeats, that might had served as a site for recombination. In *E. coli* O157:H7 this region is bordered by similar, nevertheless, imperfect repeats (Fig. 19.).

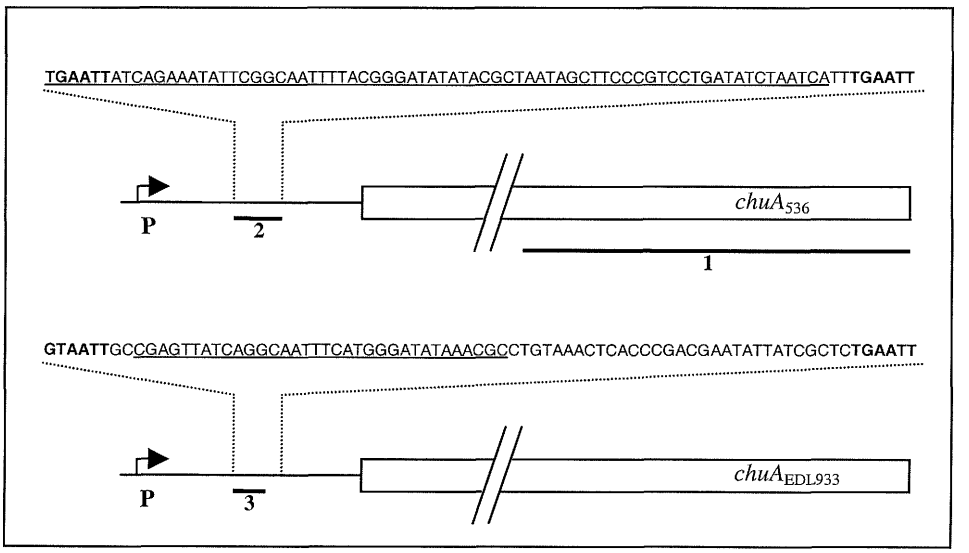

Fig. 19. Genetic map of the *chuA* gene originated from uropathogenic *E. coli* strain 536 (A) and enterohaemorrhagic *E. coli* strain EDL933 (B). The *chuA* coding regions are indicated by boxes, the 5` flanking regions by single lines. The arrows labelled with P denote the promoters. The sequences of the upstream element specific for strain 536 or strain EDL933 are given. Bases in bold represent the direct repeats flanking the dissimilar regions. The numbers and thick lines denote the probes used for Southern hybridisation (see text). The sequences of oligonucleotides used as probes are underlined.

4.4.4. Identification and distribution of two distinct variants of gene *chuA*

In order to investigate the distribution of *chuA* as well as that of the two different 5' flanking sequences, three probes (Fig. 19.) were used to hybridise chromosomal DNA originated from several *E. coli* strains representing different pathogroups. The results of the hybridisation experiments are summarised in Table 6. The probe specific for the 3' end of the *chuA* gene (probe-1) hybridised with numerous intestinal and all extraintestinal pathogenic *E. coli* strains indicating the presence of the *chuA* determinant in the genome of these isolates. However, the *chuA*-specific probe hybridised to two distinct bands: to a larger DNA fragment (~12 kb) in case of the extraintestinal and some of the EPEC strains, whereas in EHEC O157, EIEC and some other EPEC strains the *chuA* probe hybridised to a smaller fragment (~11 kb). In correspondence with former investigations, none of the tested nonO157-EHEC, EAggEC and ETEC representatives carried *chuA*. The oligonucleotide specific for the *chuA*-upstream region (probe-2) of strain 536 hybridised to all strains that carried *chuA* on the 12 kb fragment, while that originated from the O157:H7 strain EDL933 (probe-3) hybridised to the 11 kb fragment, suggesting that two distinct variants of the *chuA* determinant exist, which show differences in their flanking sequences. The existence of these two variants could be of importance for the evaluation of distributional order of the locus.

Table 6. Distribution of *chuA* and the two distinct *chuA*-upstream regions among pathogenic *E. coli* strains

STRAIN	SEROTYPE	PATHOGROUP	Probe-1*†	Probe-2*	Probe-3*
HB101		**K-12**	-	-	-
536	O6:K15:H31	**UPEC**	12	+	-
J96	O4:K6	**UPEC**	12	+	-
AD110	O6:K2	**UPEC**	12	+	-
764	O18:K5:H5/11	**UPEC**	12	+	-
2980	O18:K5:H5/11	**UPEC**	12	+	-
RZ439	O6:K5	**UPEC**	12	+	-
RZ441	O6:K5	**UPEC**	12	+	-
RS218	O18:K1:H7	**NBM**	12	+	-
IHE3034	O18:K1:H7/9	**NBM**	12	+	-
IHE3036	O18:K1:H7/9	**NBM**	12	+	-
EDL933	O157:H7	**EHEC**	11	-	+
9167/91	O157:H7	**EHEC**	11	-	+
5159/91	O157:H7	**EHEC**	11	-	+
86-24	O157:H7	**EHEC**	11	-	+
E32511	O157:H7	**EHEC**	11	-	+
6578/93	O157:H7	**EHEC**	11	-	+
SF493/89	O157:H$^-$	**EHEC**	11	-	+
3574/92	O157:H$^-$	**EHEC**	11	-	+
3978/91	O157:H$^-$	**EHEC**	11	-	+
5291/92	O157:H$^-$	**EHEC**	11	-	+
2907/97	O55:H6	**EHEC**	11	-	+
5720/96	O26:H$^-$	**EHEC**	-	-	-
3697/97	O26:H$^-$	**EHEC**	-	-	-
ED147	O26:H11	**EHEC**	-	-	-
5714/96	O103:H2	**EHEC**	-	-	-
ED142	O111:H$^-$	**EHEC**	-	-	-
78/92	O111:H$^-$	**EHEC**	-	-	-
95004730	O111:H$^-$	**EHEC**	-	-	-
E2348/69	O127:H6	**EPEC**	12	+	-
179/2	O55:H6	**EPEC**	12	+	-
156A	O55:H7	**EPEC**	-	-	-
182A	O55:H7	**EPEC**	11	-	+
37-4	O55:H$^-$	**EPEC**	-	-	-
76-5	O143:HND	**EIEC**	11	-	+
12860	O124:HND	**EIEC**	-	-	-
EDL1284	Not determined	**EIEC**	11	-	+
C9221a	O6:K15:H16	**ETEC**	-	-	-
DPA065	O119:HND	**EAggEC**	-	-	-
5477/94	O86:H7	**EAggEC**	-	-	-
7484/94	O86:H18	**EAggEC**	-	-	-
DDC4441	O128:HND	**EAggEC**	-	-	-
17-2	O3:H2	**EAggEC**	-	-	-
5464/95	O3:H2	**EAggEC**	-	-	-

* The probes used are described in Fig. 19.

† Numbers indicate the size of fragments hybridised with the probe in kilobases after digestion of chromosomal DNA with *BglI*.

4.5. RfaH influences the expression of additional virulence determinants

RfaH has been reported to influence the expression of different operons in various *E. coli* strains. In order to investigate whether several virulence factors are coregulated by RfaH we determined the LPS-structure, the amount of K-antigen, the expressions of flagella and various fimbriae, and siderophore mediated iron binding in strain 536 and its derivatives. The regulatory influence of RfaH on the expression of α-haemolysin and haemin receptor ChuA in this strain is discussed above.

The **LPS structure** of strains 536 (Fig. 20, lane A), 536*rfaH*::*cat* (Fig. 20, lane B) and 536*rfaH*::*cat* (pSMK1) (Fig. 20, lane C) were compared by SDS-PAGE. The presence of a functionally active *rfaH* gene (lanes A and C) resulted in intact LPS structures, whereas the mutant strain (lane B) showed an 'R' phenotype.

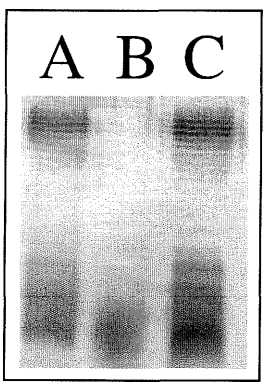

Fig. 20. LPS structures obtained from wild type strain 536 (A), mutant 536*rfaH*::*cat* (B), and its *trans*-complemented variant 536*rfaH*::*cat* (pSMK1) (C). Equal quantities of purified LPS were silver stained following separation by SDS-PAGE.

To evaluate the **amount of K15 capsule** expressed by strain 536 and its derivatives, we performed ELISA using a K15-specific serum. The results are shown in Fig. 21.

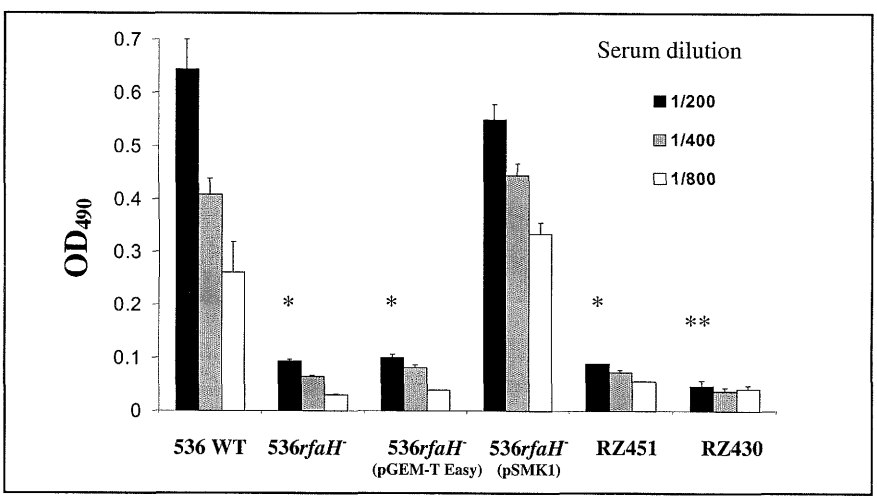

Fig. 21. Immunoreactivity of *E. coli* strain 536 (O6:K15:H31) and its variants to different concentrations of a K15-specific serum. *E. coli* strains RZ430 (O6:K⁻:H31) and RZ451 (O6:K18/22:H31) were used as negative controls. The graph reflects means and SEM (error bars) of values originated from two similar assays. Significant differences in comparison to 536 WT are indicated with asterisk(s) (*: p<0.05, **: p<0.01).

In comparison to the wild type strain, the *rfaH* mutant showed decreased immunoreactivity to this serum. Introduction of pSMK1 into the mutant strain, but not that of the control vector pGEM® T-Easy, restituted higher immunoreactivity to the K15-antiserum. In order to verify that these variations were due to the different expression of the capsule (i.e., the serum was specific for K15-antigen), we used additional O6:H31 uropathogenic *E. coli* strains possessing either K18/22 or no capsular antigens as negative controls. Indeed, these strains showed low immunoreactivity to the serum used.

Expressions of **Sfa_I- and P related (Prf) fimbriae** as well as **H31 flagella** were determined at different phases of growth by Western blots.

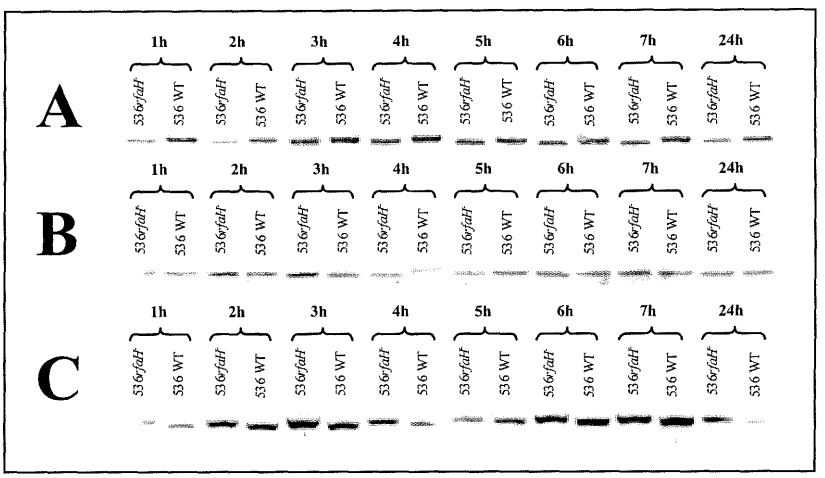

Fig. 22. Expression of H31 flagella (A), P related fimbria (Prf) (B) and S fimbrial adhesin (Sfa_I) (C) in strain 536 and its *rfaH* mutant. Equal number of bacteria were taken at different time points from a culture grown at 37 °C. Proteins were separated by SDS-PAGE and visualised by Western blot using specific antisera.

As shown in Fig. 22., expressions of Sfa_I- and Prf do not seem to be influenced by RfaH in strain 536. Similarly, no major differences could be shown in H31 flagellar expression between 536 and its *rfaH* mutant, except that maximal expression of the flagella appears to be delayed in the mutant in comparison to the wild type strain.

Siderophore mediated iron utilisation was determined by a highly sensitive chemical assay. In this test, we could assess the total siderophore mediated iron binding, which could be activated by 2,2' dipyridyl in a dose dependent manner (Fig. 23.). However, we could show no significant difference between the iron binding capacities derived from strain 536 and its *rfaH* mutant (Fig. 23.).

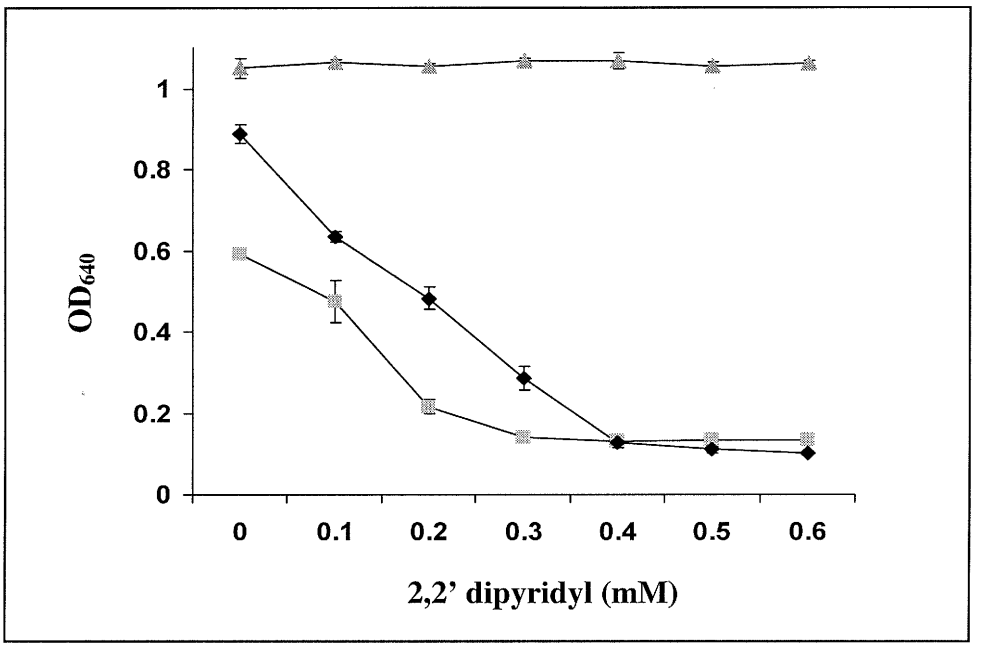

Fig. 23. Siderophore mediated iron binding derived from culture supernatants of *E. coli* strain 536 (◆), 536*rfaH::cat* (▩), and control medium (▲). Bacteria grown in M9 medium (0.5 at OD_{600}) were further incubated in M9 containing different concentrations of the iron chelator 2,2' dipyridyl for 3 h at 37 °C. The amount of secreted siderophores in culture supernatants was determined by the CAS assay (see text).

4.6. An intact RfaH protein is required for serum resistance

Serum resistance of uropathogenic *E. coli* strain 536 and its derivatives is shown in Fig. 24.

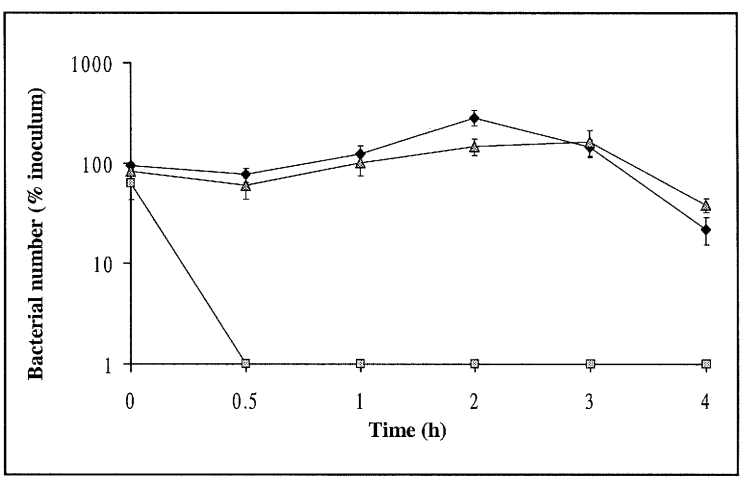

Fig. 24. Serum resistance of *E. coli* strain 536 (◆), its isogenic mutant 536*rfaH::cat* (▩), and *trans*-complemented strain 536*rfaH::cat* (pSMK1) (▲). Bacteria were incubated in 50 % serum obtained from a healthy individual. The graph represents means ± SEM of values originated from three individual assays.

The wild type strain was able to survive in 50% normal human serum for at least 4 hours, whereas its isogenic *rfaH* mutant resisted not more than 30 minutes. *Trans*-complementation of the mutant strain with the *rfaH* gene restored resistance to the bactericidal effect of human serum. All three strains could, however, survive for 4 hours in a similar experiment when heat inactivated serum was used (data not shown).

4.7. The *rfaH* mutant of *E. coli* 536 is attenuated in virulence

4.7.1. The infant mouse model of ascending urinary tract infection

E. coli 536 is a virulent uropathogenic strain isolated from a patient suffering from acute pyelonephritis. The strain's urovirulence has already been proven in mouse uropathogenicity models before (62). To assess whether RfaH plays any role in the uropathogenicity of the strain, we infected infant mice intravesically with the wild type strain, its isogenic *rfaH* mutant as well as with the mutant carrying either pGEM® T-Easy or pSMK1 (gene *rfaH* cloned into pGEM® T-Easy). The results are summarised in Table 7. While all mice injected with strain 536 died within 3-4 days, 82% of the animals receiving 536*rfaH::cat* survived for as long as 3 weeks without any sign of severe infection. Introduction of pGEM® T-Easy (vector control) into the *rfaH* mutant did not influence the lethality rate. On the contrary, supplementation of the mutant with pSMK1 partially restored virulence. All mice surviving for 3 weeks were sacrificed and the bacterial counts were determined from the blood, the bladder, and the kidneys. The number of bacteria - if any - was found to be very low (data not shown), indicating no or moderate bacterial colonisation in these mice.

Table 7. Lethality rate of infant mice infected intravesically

Bacteria injected[a]	Number of mice infected[b]	Lethality rate (%)
536	38	100
536*rfaH::cat*	39	18
536*rfaH::cat* (pGEM® T-Easy)	41	15[c]
536*rfaH::cat* (pSMK1)	35	63[d]

[a] Mice were infected intravesically with 25 µl of bacterial suspension (10^7 CFU/ml) directly through the abdominal wall.
[b] 6-14 mice were infected simultaneously and four independent experiments were performed.
[c] Significantly different in comparison to lethality rate elicited by the wild type strain (X^2 probe, $p<0.001$).
[d] Significantly different in comparison to lethality rate elicited by strain 536*rfaH::cat* (X^2 probe, $p<0.001$).

4.7.2. Determination of LD_{50}

To provide a model in which bacterial colonisation could be assessed, the infectious dose needed to be optimised in order to avoid high lethality, on the other hand still supply sufficient number of bacteria for effective colonisation. In the light of these, the LD_{50} value (the dose which kills 50% of the experimental animals) seemed to be an appropriate infectious dose. From preliminary results, the LD_{50} value of strain 536 in the infant mouse model of ascending urinary tract infection could be estimated between 10^4 to 10^5 CFU/ml. To state more precisely, we infected mice with inocula containing different concentrations of bacteria between the above values. The results are shown in Fig. 25. From this graph the LD_{50} value could be settled to be approximately $2x10^4$ CFU/ml.

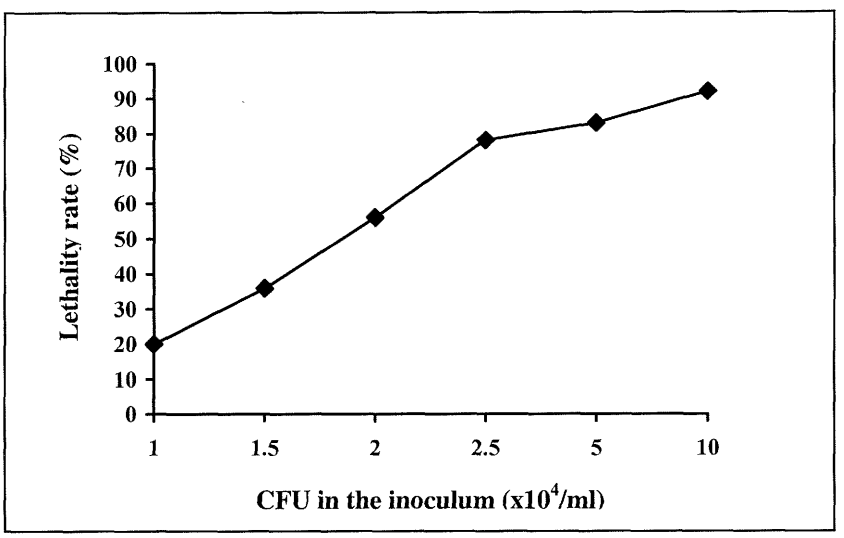

Fig. 25. Determination of LD_{50} value of strain 536 in the infant mouse model of ascending urinary tract infection. 5-13 mice were infected with 25 µl of inoculum containing different concentrations of bacteria.

4.7.3. Co-infection experiments

Wild type strain 536 and its isogenic *rfaH* mutant were used to coinfect infant mice intravesically. The inoculum contained standard concentration of the wild type strain ($2x10^4$ CFUs/ml) which was complemented with either $2x10^5$ CFUs/ml or $2x10^6$ CFUs/ml of its *rfaH* mutant, leading to bacterial ratios of 1:10 (exp. 1) or 1:100 (exp. 2), respectively. In spite of the relatively low cell counts in the inoculum, several of the infected mice died within a few days. Mortality rates were 25% (3/12) and 77% (17/22), in exp. 1 and exp. 2, respectively. From the surviving animals urine samples were taken daily for 20 days and viable counts of strains 536 and 536*rfaH*::*cat* were determined. The results obtained are shown in Fig. 26.

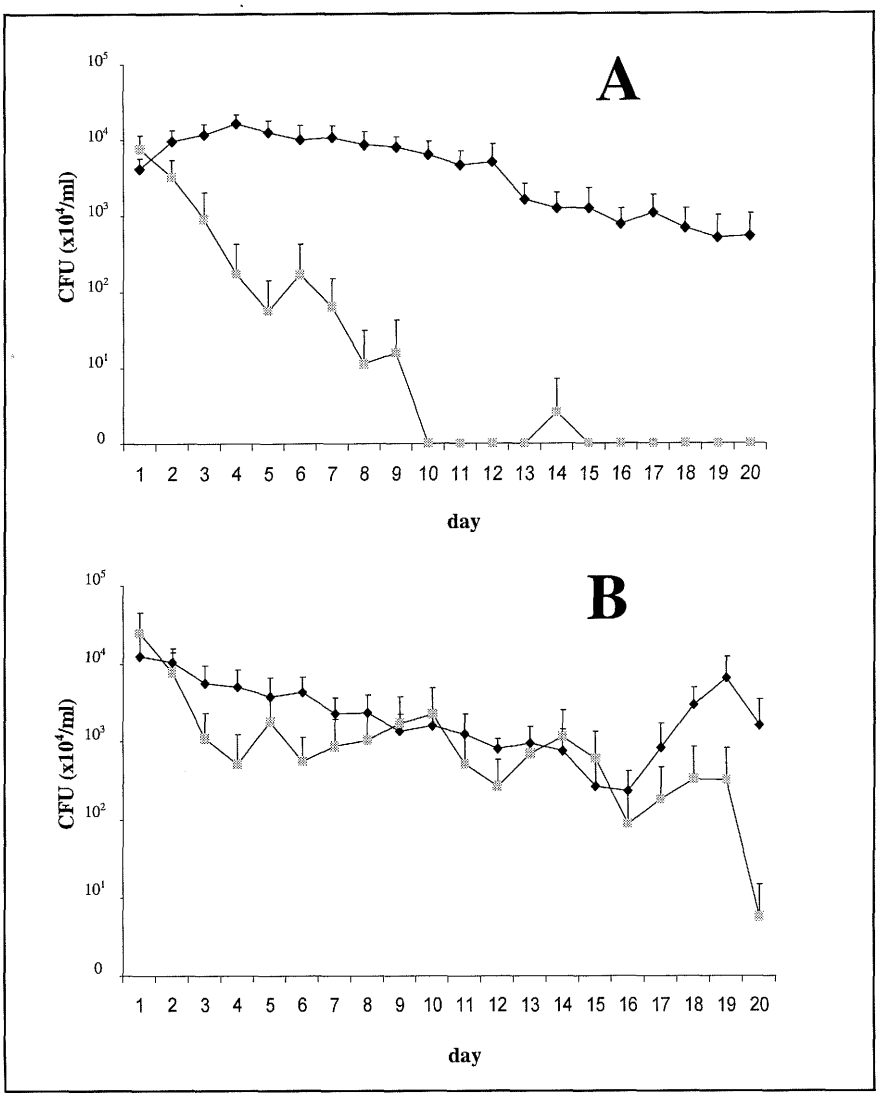

Fig. 26. Number of *E. coli* 536 (◆) and 536*rfaH::cat* (▒) cells eliminated with the urine following intravesical coinjection of bacteria into mice. The inoculum contained 5x10^2 CFUs of wild type strain 536 complemented with either 5x10^3 CFUs (A), or 5x10^4 CFUs (B) of its isogenic *rfaH* mutant. Graphs represent means ± SEM of values originated from 9 (A), and 5 (B) animals.

Wild type strain 536 was permanently present in the urine of infected mice throughout the study-period. Although its concentration was constantly decreasing, it still exceeded 10^7 CFUs/ml on day 20 post-inoculation in both experiments. On the contrary, mutant strain 536*rfaH::cat* disappeared from the urinary tract within 10 days even when inoculated at a 10-times higher concentration (exp. 1) (Fig. 26.A). Higher doses of the mutant strain in the inoculum (exp. 2) resulted in tedious elimination, however, after 3 weeks the urine was virtually free of the mutant strain (Fig. 26.B).

On the 21st day post-infection, mice were sacrificed and colony counts from the blood, the bladder, and the kidneys were determined. The results are shown in Fig. 27. The blood never contained any bacterium (not shown). However, the bladder and the kidneys comprised high number of the wild type strain 536 in all mice. In contrast, strain 536*rfaH*::*cat* was never present in the kidneys, and was only detectable in the urine in exp. 2. Even in this case, bacterial counts of strain 536 exceeded that of its *rfaH* mutant by a factor of more than 10^5.

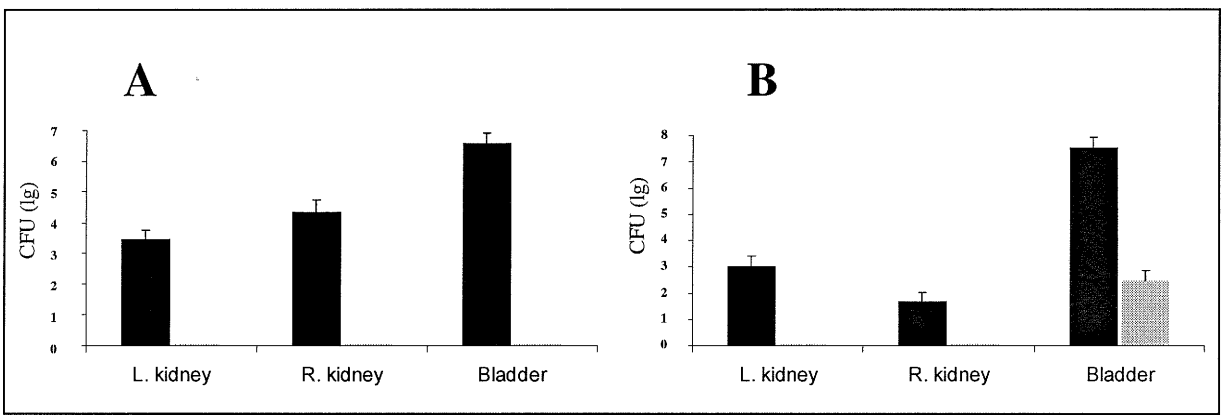

Fig. 27. Viable counts in different organs on 21st day post-infection. Mice were inoculated intravesically with a mixture of $5x10^2$ CFUs of wild type strain 536 complemented with either $5x10^3$ CFUs (A), or $5x10^4$ CFUs (B) of its isogenic *rfaH* mutant. Means ± SEM of values originated from 9 (A), and 5 (B) mice are shown. Dark bars represent CFUs of wild type strain 536, bright bars those of mutant strain 536*rfaH*::*cat*. L.: left. R.: right.

4.7.4. Acute lung toxicity in mice

The lung toxicity assay provides a model, in which acute „toxicity" of bacteria can be assessed (62). The results obtained with *E. coli* 536 and its derivatives in this assay are summarised in Table 8.

Table 8. Lethality rate of mice infected intranasally

Bacteria instilled[a]	Number of mice infected[b]	Lethality rate (%)
536	20	100
536*rfaH*::*cat*	20	10[c]
536*rfaH*::*cat* (pGEM® T-Easy)	20	0[c]
536*rfaH*::*cat* (pSMK1)	20	65[d]

[a] Mice were infected intranasally under superficial ether anaesthesia with 50 µl ($3x10^9$ CFU/ml) bacteria.
[b] 2x10 mice were infected for each strain in two independent experiments.
[c] Significantly different in comparison to lethality rate elicited by the wild type strain (X^2 probe, $p<0.001$).
[d] Significantly different in comparison to lethality rate elicited by strain 536*rfaH*::*cat* (X^2 probe, $p<0.001$).

The 100% lethality rate elicited by the wild type strain was reduced to 10% in case of its isogenic *rfaH* mutant. Supplementation of the mutant with an intact *rfaH* gene on pSMK1 increased death rate to 65%. On the other hand, introduction of the control vector (pGEM® T-Easy) into the mutant strain had no positive effect on virulence.

5. DISCUSSION

In order to be able to survive and colonise in the host, pathogenic bacteria produce traits that are termed virulence factors indicating their close correlation with pathogenic isolates. An important factor in effective pathogenesis within the urinary tract is the production of cytotoxins. With regard to uropathogenic *E. coli*, the potential pathogenic role of only few toxins was proposed from which α-haemolysin is far the most thoroughly investigated. As many as 40-50% of all uropathogenic isolates produce the toxin, the rate of which further increases among highly virulent strains causing acute pyelonephritis (24). α-haemolysin has been proven to contribute to virulence of *E. coli* strains causing extraintestinal infections (46;113;179). The activated toxin HlyA inserts into membranes of host cells and causes lysis through pore formation. By destroying eukaryotic cells, the toxin not only provides opportunity for deeper invasion, but in addition supplies bacteria with nutrients liberated from host cells (e.g., iron containing substances).

The genetic determinants of α-haemolysin have been studied extensively. The genes responsible for the synthesis, activation and secretion of the toxin are grouped into one large operon. However, effective export of the toxin requires an additional outer membrane protein, which is encoded by a separate gene. Furthermore, effective transcription requires several regulatory elements encoded at diverse sites of the chromosome. Interestingly, the *hlyCABD* operon is carried on large conjugative plasmids in strains causing infections in different animals, whereas human isolates always carry *hlyCABD* on the chromosome. Furthermore, in case of human uropathogens, these determinants are located on pathogenicity islands (60). Interestingly, at least in some cases, the genes encoding α-haemolysin are closely associated with those encoding another prominent toxin of uropathogenic *E. coli*, the cytotoxic necrotizing factor (25).

In spite of the relatively large amount of available sequence information on α-haemolysin, factors directing expression of the operon are still not completely understood. Nucleotide sequences of the four contiguous genes are well conserved, on the contrary, 5' flanking regions are highly dissimilar (86). Consequently, haemolytic activities deriving from various *hly* operons appear to be different (58). Our sequence analysis on the two *hly* determinants originated from *E. coli* strain 536 confirm these observations, i.e., the upstream regions of the two operons show no homology to each other. The leader sequence of *hlyI*, nevertheless,

shows very high homology to the corresponding region of strain J96. The 5' flanking region of *hlyII*, on the other side, shows less explicit similarity to the α-haemolysin determinant originated from haemolytic plasmid pHly152 and to that of MENEC strain C5.

Cloning of both *hly* determinants revealed that the amounts of both intracellular and secreted α-haemolysin molecules are significantly higher in case of *hlyI* in comparison to *hlyII,* which agrees with mRNA levels reported elsewhere (122). On the contrary, *in vitro* haemolytic activity deriving from *hlyI* appears to be considerably decreased when compared to *hlyII.* Results of the *in vivo* haemolytic test (that can be assessed in the mouse lung toxicity assay) are parallel to the *in vitro* results, i.e., cloned *hlyI* caused a lower mortality in this assay compared to the corresponding clone originated from *hlyII.* To gain more insight into expressional regulation of the two *hly* operons, fusion haemolysin determinants were constructed, in which upstream region together with *hlyC* deriving from one operon was fused to *hlyABD* originated from the other determinant. The results obtained from these clones revealed that placing the strong promoter region of *hlyI* in front of structural genes of *hlyII*-origin results in an elevated level of toxin expression, whereas fusion of *hlyII*-upstream region with genes of *hlyI* yields a lower expressional rate. However, haemolytic activity of the corresponding HlyA molecules are not significantly altered.

From these results two findings could be speculated: Firstly, different haemolytic activity originated from the two *hly* determinants of strain 536 is not a consequence of dissimilar expressional rate, rather it is dependent on the origin of toxin molecules, i.e., HlyA encoded on *hlyII* appears to be more active than that originated from *hlyI.* Modification of transcriptional rate has only minor effect on the activity deriving from the two distinct HlyA molecules.

Secondly, within the leader sequence of *hlyI*, a regulatory element and/or additional stronger promoter region is presumed that directs stronger expression of the operon. To test the latter hypothesis, *hlyI* determinants containing different lengths of 5' flanking regions were constructed. Evidence has been shown that clones possessing 1500 bps or more of untranslated leader sequences produce larger amount of HlyA than those harbouring 500 or 1000 bps. On the other hand, lower expression in the latter case is coupled by a stronger *in vitro* and *in vivo* activity of α-haemolysin molecules suggesting the presence of a regulatory element in the far upstream region (more than 1000 bps from transcriptional start of gene *hlyC*) of the *hlyI* determinant. From the available sequence data, a potential open reading frame could be disclosed, which has been cloned into a compatible vector. Introduction of the

resulting plasmid into strains harbouring haemolytic determinants of either *hlyII* or *hlyI* with short leader sequences, however, does not alter their haemolytic properties. This way, role of a *trans*-acting protein regulator might be excluded. Hence, the existence of a *cis*-acting regulatory element is assumed.

The two known regulatory proteins influencing expression of *hly* operon (RfaH and Hha) require *cis*-acting DNA sequences, which they bind to. RfaH mediated regulation requires a short upstream sequence termed the *ops* element (12). Similarly Hha-mediated effect is shown to be dependent on the presence of a DNA region termed *hlyM* (80), which lies within the first gene of the operon. In case of plasmid encoded determinants a third *cis*-acting region has been identified, which is termed *hlyR*. *hlyR* does not encode a protein and it contains the *ops* element, so these two terms (*hlyR* and *ops*) might hide the same regulatory DNA region. The possibility that a similar *cis*-acting DNA motif is located somewhere between bps 1500-1000 upstream of the start codon exists, however, the actual protein mediator associated with this region is yet to be identified.

As already mentioned above, expression of α-haemolysin has been reported to be under the control of RfaH in *E. coli* strains (13;99). Further evidence is provided in this study that no considerable haemolysis derives from strain 536*rfaH*::*cat* at any growth phase *in vitro*. *In vivo* haemolysin production was assessed in the mouse lung toxicity test (62). In this assay, mice die from the immediate „toxic effect" of bacteria before extensive bacterial multiplication. Since loss of the pathogenicity islands carrying the *hly* determinants of *E. coli* 536 results in an avirulent phenotype in this model (122), we consider haemolysin as the major factor to determine the outcome of intranasal instillation of bacteria. Mutation within gene *rfaH* significantly decreased death rates in this assay, nevertheless, still caused a 10% mortality. As RfaH affects not primarily transcription of the toxin gene itself, but rather genes coding for its secretory machinery (162), HlyA may be released by other, less effective mechanisms or through disintegration of bacteria.

The ability to use haemin as an iron source has been frequently observed in pathogenic *E. coli* strains (95;181). In most cases, these strains carry a 9.2 kb-long region - termed the haem transport locus – involved in haem utilisation. The locus was first characterised in *Shigella dysenteriae* type 1 (116). However, it was soon identified in pathogenic *E. coli* strains as well, suggesting horizontal transfer of this region (170). In every case, the locus is inserted in a discrete position between two ORFs that corresponds to the 78.7 min region of the *E. coli* K-12 chromosome. The presence of the locus as well as its chromosomal insertion

site in the uropathogenic *E. coli* strain 536 was verified by PCR as described by Wickoff *et al.* (181). Though the entire locus contains 8 open reading frames, the presence of the receptor protein-encoding gene *chuA* alone is sufficient to allow haem uptake when transformed into *E. coli* K-12. Nevertheless, the presence of the whole locus results in an increased efficiency of haem utilisation (116).

The receptor expression was proven to be negatively regulated by iron via the Fur protein (116;117). A Fur-box overlaps the proposed promoter region, the presence of which explains the observed effect of iron availability on *chuA* expression. The transcriptional control of haemin receptors, however, appear to be multifactorial, involving global regulators, as proposed by Lee (98). In this study, evidence is shown that the amount of the ChuA protein is considerably decreased in an *rfaH*-derivative of strain 536. The reduction in ChuA-level is proven to be a consequence of altered transcription of the corresponding gene.

RfaH is a regulator, which influences the transcription of long operons that encode cell surface and extracellular components that are important for bacterial fertility and virulence. Haemin receptor ChuA is located on the surface of virulent *E. coli* strains, anchored in the outer membrane. Direct evidence for its contribution to *in vivo* urovirulence was recently shown (171). Though the haem transport locus contains 8 ORFs, the receptor gene (*chuA*) is transcribed as a monocistronic mRNA. This is in contrast to other operons influenced by RfaH. Despite, the quantity of *chuA* transcript is strongly reduced in the *rfaH* null-mutant strain 536*rfaH*::*cat*. Furthermore, *chuA* transcript level is considerably increased upon *trans*-complementation of gene *rfaH*. This seems to be inconsistent with the present point of view, that RfaH stimulates transcription without transcription initiation (10;99;161). A model has been proposed by Koronakis and co-workers in which RfaH acts post-initiation as a transcriptional antiterminator and reduces the polarity within an operon by increasing the transcription of promoter-distal genes (11). However, Leeds and Welch showed that in case of the *hly* determinant of *E. coli*, transcript levels of even promoter-proximal genes were 2-7.5 fold lower in *rfaH* mutants, in addition to a more striking decrease in the transcriptional rate of the promoter-distal genes (99). The decrease in the mRNA level of promoter-proximal genes was also described in other RfaH-affected operons (19;161). A hypothesis that the RNA polymerase-complex could pause (with a possibility of transcription termination) at certain DNA motifs spread all along these templates was proposed (11). Supposing random termination which could be prevented by RfaH, this model could explain the observed transcriptional pattern.

The operons influenced by RfaH contain a conserved DNA motif (Fig. 18.). This was first identified in the upstream regions of operons involved in polysaccharide synthesis and was therefore termed JUMPStart (**J**ust **U**pstream of **M**any **P**olysaccharide-associated gene **Start**s) sequence (71). The almost identical region within these operons consists of 39 base pairs containing two copies of the motif GGTAGC. The characterisation of additional RfaH-affected operons revealed that these repeats were not always present. Nieto *et al.* showed that the most conserved part of all affected operons was the GGCGGTAG motif covering the 3' repeat of the JUMPStart sequence (125). The deletion of this element resulted in a similar transcriptional pattern of affected operons as that observed in *rfaH* mutant strains (125;161). The presence of this motif seems to be a crucial factor in the RfaH-mediated antitermination, hence the octamer was termed the *ops* element (*o*peron *p*olarity *s*uppressor). When all affected operons are compared, it is evident that the *ops* element with the well-conserved additional base C downstream of *ops* is always proceeded by a direct repeat that shows less similarity to the standard element. (Fig. 18.). In one possible model, the JUMPStart sequence facilitates the formation of a stem-loop structure involving the *ops* element and its imperfect direct repeat (110). It was proven that the *ops* element recruits the RfaH protein to the transcription complex. (12). However, not only deletion of the *ops* element, but also that of the less-conserved repeat located in the 5' region of the JUMPStart sequence or the separation of the repeats by a linker insertion significantly reduces transcription. This suggests that additional elements located in the larger 39 bp-region are also essential for the optimal effect of RfaH (100;110;161). Within the *chuA* gene of *E. coli* 536, we found a region that corresponds to the typical arrangement of a JUMPStart sequence. It contains an *ops*-like motif with a relatively well-conserved direct repeat. The repeats are separated by a spacer, the length of which resembles that of other JUMPStart sequences (Fig. 18.). Almost identical regions to the proposed *chuA* JUMPStart sequence of strain 536 can be found within the formerly described *shuA*/*chuA* determinants as well.

The localisation of the JUMPStart motif is non-typical. Usually, this region lies within the non-translated leader sequence of the first gene of the RfaH-affected operon. In case of the *tra* operon, however, the corresponding sequence is located between two genes, approximately 5 kb downstream of the promoter (49). The position of JUMPStart sequence deriving from various haemolysin determinants is also variable, although the motif is always located in the region upstream of *hlyC*. In case of *chuA,* the potential JUMPStart sequence is localised more than 1 kb downstream of the start codon. Bailey *et al.* demonstrated that the presence of the *ops* element on the template DNA is essential, however, alone not sufficient for the RfaH-

derived effect (12). The way, how *ops* recruits RfaH, as well as the altered function of the RfaH-modified, termination resistant RNAP is not yet clear. It is probable that RfaH is part of a regulatory complex, the additional components of which need to be identified.

The presence of the haemin utilisation locus in both *S. dysenteriae* type 1 and pathogenic *E. coli* suggests that it may be distributed by horizontal gene transfer. The existence of two different 5' flanking regions and their patterned distribution among different pathogroups provides further evidence for the clonality of *E. coli* pathogens. Whether the differences in the *chuA* upstream regions have any influence on the regulation of *chuA* expression still needs to be clarified.

The co-regulation of different determinants involved in pathogenicity is energetically advantageous for pathogenic bacteria. It is especially true for components of a complex system, which are functionally related. α-haemolysin and haemin-uptake are both regulated by iron suggesting that utilisation of haem compounds liberated from eukaryotic cells is an important iron acquisition strategy during infection. Coupled regulation by RfaH gives further evidence that the function of the *E. coli* haemin uptake (*chu*) system is dependent on secreted haemolysin, since free haem-iron can be found neither in blood nor in secretory fluids.

Since the first report describing RfaH as a regulator of the *rfa* locus (104) several other operons have been identified which depend for full expression on the presence of this regulatory protein (for a review see reference (11). Nevertheless, a global regulatory function has not been attributed to RfaH as distinct observations were reported from different pathogenic isolates of *S. enterica* serovar Typhimurium and *E. coli*. In the present study it is shown for the first time that expression of several components (LPS, K15 capsule, α-haemolysin, and haemin receptor ChuA) are coregulated by RfaH in one single *E. coli* strain. What could be the basis of common regulation of these structures? Interestingly, although these factors are different in their composition and function, they all are transported through both membranes of the bacterium; they are anchored in the outer membrane or transported out of the cell. Common regulatory systems for LPS and K15 (group II) capsule synthesis could be explained with similarities in synthesis, assembly, and transport mechanisms, in spite of the distinct genes involved. Furthermore, activity of secreted α-haemolysin was reported to be dependent on intact lipopolysaccharide (16;160;176). Similarly, function of the haemin receptor ChuA is speculated to depend on the activity of α-haemolysin, i.e., the toxin liberating haem from eukaryotic cells (see above). All these observations suggest that these

components have evolved to co-utilise regulatory protein RfaH as proposed by Bailey *et al.* (11).

As all structures whose expressions have been shown to be influenced by RfaH are potential virulence factors, we proposed that mutation of gene *rfaH* should result in a decrease of virulence. Indeed, in an ascending mouse model of urinary tract infection, virulence of wild type strain 536 is almost completely abolished through a mutation within *rfaH*. The decreased *in vivo* virulence of the *rfaH* mutant, however, can not be explained with inappropriate growth potential, since *in vitro* growth curve of 536*rfaH*::*cat* does not differ from that of the wild type strain 536. Moreover, both strains have been shown to be able to grow equally *ex vivo* in the urine of healthy individuals (data not shown) indicating that there are no differences in growth-abilities between the two strains at the beginning of the infectious course.

Colonisation, however, is a more complex process requiring effective adhesion mechanisms and rapid multiplication through utilisation of limited nutrients. By destroying epithelial cells, the possibility of tissue invasion and dissemination arises. On the other hand, bacteria have to face different host defence mechanisms. The inability of bacteria to overcome different „attacks" of the immune system results in localised infections only, or - in most cases – total elimination of the infectious agent. The first line of defence against dissemination is the action of the complement system. In contrast to commensal gram-negative bacteria, extraintestinal clinical isolates of enterobacteria often show resistance to the killing effect of serum, suggesting that the ability of bacteria to cause disease correlate with their resistance to the bactericidal effect of serum. Thus, serum resistance has been a virulence parameter for many pathogens including extraintestinal isolates of *E. coli*. Resistance to serum killing is multifactorial and has been associated with several surface components of *E. coli* among which capsule and lipopolysaccharide are thought to be the most important ones (79). We have shown that loss of regulatory protein RfaH results in high susceptibility to human serum in strain 536*rfaH*::*cat*, which might be explained with altered expressions of both LPS and K15 capsule due to inactivation of gene *rfaH*.

The coinfection assay provides an objective model to compare colonisation-capacity of the *rfaH* mutant with that of the wild type strain without individual differences of the host. Usefulness of similar models was shown recently in investigations aimed to clarify the pathogenic role of cytotoxic necrotizing factor type 1 (134), and different iron transport systems (171) in the virulence of UPEC strains. Even though we had to face a negative selection of our experimental animals (i.e., those mice having the most fulminant infections died within a few days), the difference in colonisation-potential between the two clones has

been clear. Our results provide evidence that - in contrast to the wild type strain - the *rfaH* mutant is not able to cause ascending uroinfection, since it was never detectable in the upper urinary tract.

Bacterial pathogenesis is a complex phenomenon, which is attained through a concerted action of virulence factors. Exclusive role of a single pathogenicity factor is rare; they usually function synergistically, amplifying or complementing the effect of each other. Therefore, total abolishment of virulence is rarely caused by the loss of individual virulence factors (118). Nevertheless, mutation in one single gene can result in significant loss of virulence if the related gene product has an impact on several virulence determinants simultaneously. We have presented evidence that the absence of functional RfaH protein results in parallel underexpression of several virulence factors, which probably all contribute to the complete virulence of *E. coli* strain 536. Further experiments are needed, however, to clarify the potential global regulatory role of RfaH in other pathogroups of *E. coli* and additional gram-negative bacteria.

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