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Implication of extraintestinal pathogenic *Escherichia coli* siderophore receptors in host pathogen interaction

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meiner Familie

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ABBREVIATIONS

AA	amino acid
Afa	AfaDE family of adhesins
Afa/Dra	Afa/Dr family of adhesins
Auf	Auf fimbriae
cDNA	complementary DNA
CDT	cytolethal distending toxin
CNF	cytotoxic necrotizing factor
ChuA	outer membrane hemin receptor
Cva	colicin
°C	degree celsius
DABCO	1,4-diazabicyclo-(2.2.2)octane
DAPI	4'6'-diamidino-2-phenylindole hydrochloride
DNA	desoxyribonucleicacid
dH_2O	distilled water
Dra	Dr fimbriae
DTT	dithio…
ELISA	enzyme linked immunosorbent assay
Ent	enterobactin siderophore system
EPEC	enteropathogenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
FACS	fluorescence activated cell sorting
FepA	siderophore enterobactin receptor
Fim	type I fimbriae
FITC	fluorescein isothiocyanate
Foc	F1C fimbriae
FyuA	siderophore yersiniabactin receptor
Hbp	hemoglobin-protease
Hek	hemagglutinin from *E. coli* K1
HlyA	alpha-hemolysin
Hra	heat-resistant agglutinin
Iha	IrgA-homologue adhesin
IC	intermediate cell

Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranosid
IrgA	putative protein encoded by an iron -regulated gene
IreA	iron-responsive element
Irp	yersiniabactin siderophore system
IroN	siderophore salmochelin receptor
Iss	protein involved in increased serum resistance
Iuc	aerobactin s siderophore system
IutA	siderophore aerobactin receptor
Kb	kilo base pair
kDa	kilodalton
LAMP	lysosomyl-associated membrane protein
LPS	lipopolysaccharide
M	Molar
Mb	mega base pair
Mch	microcin
mM	millimolar
µM	micromolar
µF	microfarad
µg	microgram
µm	micrometer
NCBI	the National Center for Biotechnological Information
NMEC	newborn meningitis associated *E. coli*
nt	nucleotide
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
ORF	open reading frame
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PAI	Pathogenicity Island
Pap	P fimbriae

PCR	polymerase chain reaction
Pic	putative serine protease autotransporter protein
PG	phylogenetic group
PMN	polymorphonuclear leukocyte
PrrA	putative siderophore receptor
PVDF	polyvinylidene fluoride
rpm	rounds per minute
Sat	serine auto-transporter toxin
SDS	sodium dodecyl sulfate
Sfa	S fimbriae
spp.	species
TBS	tris buffered saline
TGN	trans golgi networks
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TonB	ferri-siderophore complex transport protein
tp^r	trimethoprim resistance cassette
TR	Texas Red
TRITC	tetramethylrhodamine isothiocyanate
tRNA	transfer-RNA
SFC	superficial facet cell
Usp	uropathogen specific protein
UTI	urinary tract infection
UT	urinary tract
v/v	volume/volume
w/v	weight/volume

1. INTRODUCTION

1.1. The species Escherichia coli

Escherichia coli was discovered as „*bacterium coli commune*" by the German pediatrician and bacteriologist Theodor Escherich (1857 - 1911) at the end of the 19th century. Meanwhile, *E. coli* represents the probably best-studied prokaryotic organism and serves as an important model organism in diverse research fields such as genetics or biotechnology. *E. coli* is a rod shaped, Gram-negative, facultative anaerobic bacteria belonging to the Enterobacteriaceae family of gamma-proteobacteria. The family of Enterobacteriaceae contains more than 40 different genera (amongst others *Shigella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Erwinia*, *Proteus*, *Yersinia*, *Enterobacter* and *Escherichia*) including apathogenic or commensal as well as pathogenic strains. The species *E. coli* is exceedingly heterogeneous and consists of commensal and pathogenic strains. Most *E. coli* strains colonize as harmless commensals the normal flora of the gut of warm-blooded humans and animals, benefiting their hosts e.g. by producing vitamin K (Bentley and Meganathan, 1982) or by preventing the establishment of pathogenic bacteria within the intestine (Reid et al., 2001). However, depending on their serotype and virulence factor pattern, some *E. coli* strains can cause serious diseases. Thus, *E. coli* is also known as one of the most widespread and clinically significant pathogen. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrhoeal disease. Pathogenic *E. coli* strains can be classified into distinct pathotypes which are characterized by the virtue of their virulence gene patterns and the site of infection. Intestinal pathogenic *E. coli* strains can be divided into five pathotypes dependent on their serological characteristics and virulence properties:

Enterotoxigenic *E. coli* [ETEC] are the causative agent of diarrhoea (without fever) in humans and several animals. ETEC uses fimbrial adhesins to bind enterocyte cells in the small intestine and produce at least one member of two defined groups of enterotoxins. The larger of the two proteins, is the heat labile enterotoxin (LT) that is similar to cholera toxin in its structure and function, while the smaller heat stable enterotoxin (ST) causes cGMP accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen. ETEC strains are non-invasive and do not leave the intestinal lumen (Farthing, 2004).

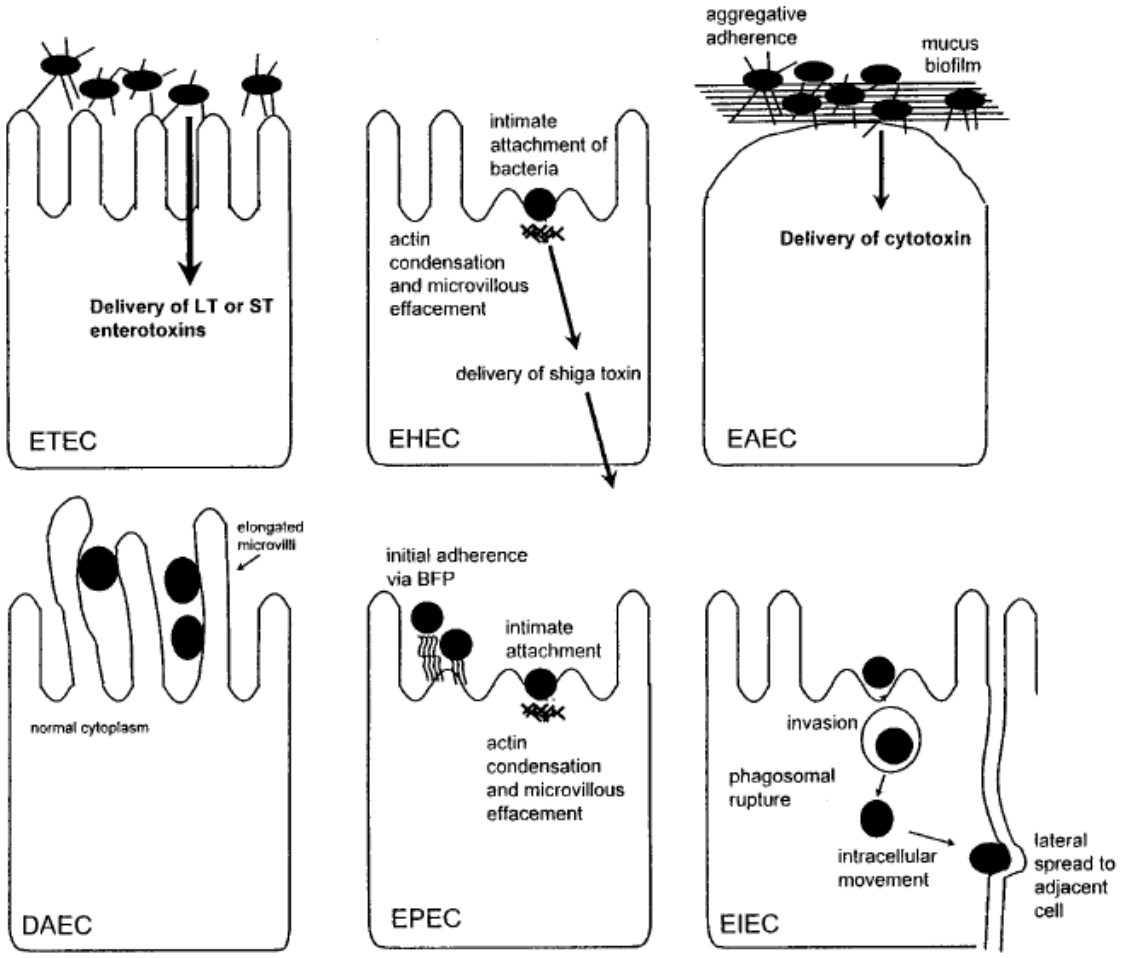

Figure 1. Pathogenic schemes of diarrheagenic *E. coli* (Nataro and Kaper, 1998). The six recognized categories of diarrheagenic *E. coli* each have unique features in their interaction with eukaryotic cells. Here, the interaction of each category with a typical target cell is schematically represented. It should be noted that these descriptions are largely the result of *in vitro* studies and may not completely reflect the phenomena occurring in infected humans. See the text for details.

More than 50 years ago, *E. coli* strains of the serogroup O26 were recognized as causes of infantile diarrhoea and classified as enteropathogenic *E. coli* [EPEC]. The hallmark of infections due to EPEC is the attaching-and-effacing (A/E) histopathology. Effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane are caused by the characteristic adhesive determinant of EPEC strains known as intimin (Levine et al., 1987). Genes responsible for the A/E phenotype are encoded on a pathogenicity island called the locus of enterocyte effacement (LEE). Adherence of EPECs to the intestinal mucosa causes a rearrangement of actin in the host cell, resulting in significant deformation. EPEC strains are moderately invasive and the mediated changes in the intestinal cell ultrastructure are likely the prime cause of diarrhoea in those afflicted with EPEC (Clarke et al., 2003). Furthermore, EPECs possess several virulence factors such as additional fimbrial structures, proteins responsible for the invasion process invasions and the low-molecular-

weight ST called EAST1. However, their significance in EPEC pathogenesis is still unknown, and it is most likey that these virulence determinants are involved in producing attaching and effacing lesions.

In contrast to EPEC and ETEC, the enteroinvasive *E. coli* [EIEC] are found only in humans. EIEC infection causes a syndrome that is identical to Shigellosis, characterized by profuse diarrhoea and high fever. EIEC strains are highly invasive; the binding and internalization into intestinal cells is promoted by adhesins. Although, EIEC strains produce no toxins, the intestinal wall is severely damaged by mechanical cell destruction.

Typically, virulent enteric strains cause no more than a bout of diarrhoea in healthy adult humans, but there are some particular strains, that can cause serious illness or death in elderly, very young or immunocompromised people (Hudault et al., 2001). These bacteria are the enterohemorrhagic *E. coli* [EHEC], represented by a single strain (serotype O157:H7), which causes a diarrhoeal syndrome distinct from EIEC (and *Shigella*), in that there is copious bloody discharge and no fever. A frequent life-threatening situation is its toxic effects on the kidneys, the hemolytic uremic syndrome (HUS) or sudden kidney failure (Caprioli et al., 2005). EHEC strains attach with its fimbriae to intestinal cells and moderately invade host cells. Some strains possess a phage encoded verocytotoxin (also termed shigatoxin; Stx) that can elicit an intense inflammatory response (Karmali et al., 1985). Another major virulence determinant of EHEC strains is the plasmid located EHEC-*hlyA* gene, which encodes EHEC hemolysin (Schmidt et al., 1995) (also termed EHEC toxin; Ehx (Bauer and Welch, 1996)).

A further pathotype, the enteroaggregative *E. coli* [EAEC] are currently defined as *E. coli* strains that do not secrete enterotoxins LT or ST but contain special fimbriae which aggregate tissue culture cells. EAEC strains bind to host cells by aggregative adherence distinguished by prominent autoagglutination of the bacterial cells to each other. EAEC associated diarrhoea is caused by binding of the non-invasive EAECs to the intestinal mucosa resulting in persistent watery diarrhoea without fever and inflammation, especially in young children.

The term “diffusely adherent *E. coli*” [DAEC] was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like microcolonies. With the discovery of EAEC, most authors now recognize DAEC as an independent category of potentially diarrheagenic *E. coli.* DAEC strains possess fimbrial genes with homology to members of the Dr group of bacterial adhesins that promote diffuse adherence to host cells. However, little is known about the pathogenetic features of DAECinduced diarrhea.

1.2. Extraintestinal pathogenic *Escherichia coli* (ExPEC)

In contrast to the intestinal pathotypes, extraintestinal pathogenic *E. coli* [ExPEC] represents a major but little appreciated health threat. ExPEC strains are responsible for the majority of community-acquired infections outside the gastrointestinal tract. These infections range from asymptomatic urinary tract infections (UTIs) to life threatening diseases such as pyelonephritis or sepsis (Johnson and Stell, 2000; Picard et al., 1999). UTIs caused by uropathogenic *E. coli* [UPEC] strains, the major subtype of ExPEC, represent a heterogeneous group of disorders that collectively cause considerable morbidity, lost productivity, and increased health-care costs. UTIs can differ in their severity from asymptomatic UTI and harmless cystitis to systemic infections like severe pyelonephritis. Further pathotypes of ExPEC are the sepsis-associated pathogenic *E. coli* [SPEC] strains that are the most common cause of community-acquired bacteremia and sepsis and the newborn meningitis-associated *E. coli* [NMEC], the leading causes of neonatal meningitis and neonatal sepsis, which often lead to serious sequelae and to death (Russo and Johnson, 2003). ExPEC strains also feature prominently in intraabdominal infections and nosocomial pneumonia and occasionally participate in other extraintestinal infections such as osteomyelitis, cellulitis, and wound infections (Johnson and Russo, 2002).

Phylogenetic analyses revealed that the natural *E. coli* isolates are basically clonal and fall into four main phylogenetic groups: A, B1, B2 and D. ExPEC strains, that are responsible for most ExPEC infections in noncompromised hosts, are as distinct from commensal *E. coli* as the intestinal pathogenic *E. coli* types (Russo and Johnson, 2000). The phylogenetic distribution of ExPEC virulence genes in relation to clinical sources is depicted in Figure 1. Commensal *E. coli* strains typically derive from phylogenetic groups A or B1 and lack most virulence factors (VFs) (Johnson et al., 2001a; Herzer et al., 1990), whereas the various intestinal pathogenic *E. coli* strains, which only rarely cause extraintestinal disease, derive from phylogenetic groups A, B1, or D, or from ungrouped lineages (Pupo et al., 1997). The intestinal pathogenic strains possess distinctive virulence factors (e.g. shigatoxin, ST, LT or intimin) that are characteristic of their particular diarrhoeal syndromes. Unlike commensal and intestinal pathogenic *E. coli*, ExPEC derive predominantly from the phylogenetic group B2 and, to a lesser extent, group D (Johnson et al., 2001a). Moreover, particular virulence genes (*papA*, P fimbriae; *kpsMT*, group II capsule synthesis; *sfa/foc*, S and F1C fimbriae; *iutA*, aerobactin receptor) accumulate within these phylogenetic groups indicating a high association of these VFs to ExPEC strains.

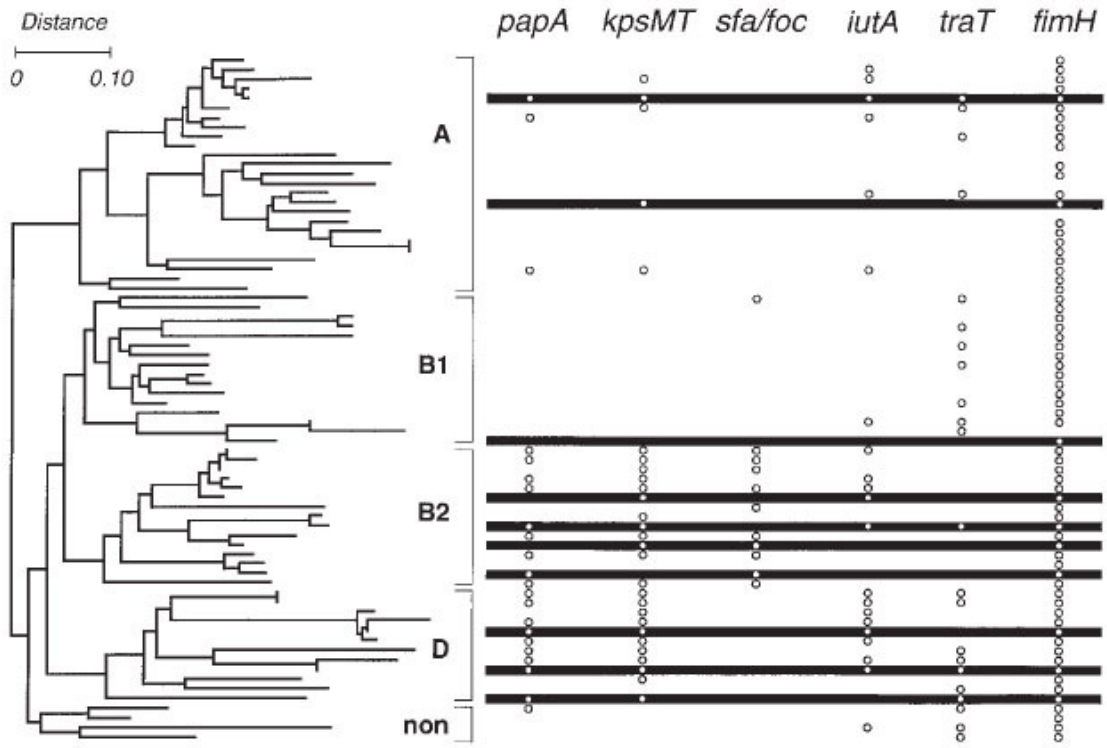

Figure 2. Phylogenetic distribution of *E. coli* extraintestinal virulence genes in relation to clinical sources. The dendogram depicts the phylogenetic relationship for the 72 members of the *E. coli* reference collection (ECOR) collection as inferred from multilocus enzyme electrophoresis (Johnson and Russo, 2002). Horizontal bars at right indicate the 10 ECOR strains from patients with symptomatic UTI. Most remaining strains are faecal isolates from healthy human or animal hosts.

Recent studies have described that most ExPEC strains have evolved from an ancestral strain by a stepwise accumulation of virulence factors through horizontal gene transfer (Dobrindt and Hacker, 2001; Bidet et al., 2005). The specific genetic background may be required for the acquisition and expression of these virulence factors (Dobrindt et al., 2002; Le Gall et al., 2005; Picard et al., 1999). However, the sole association of an *E. coli* strain with a certain phylogenetic group does not necessarily render the strain pathogenic or non-pathogenic as the adaptation of a strain to a host also contributes significantly in determining its virulence (Souza et al., 1999; Duriez et al., 2001).

1.2.1. Virulence factors of ExPECs

Although, ExPEC strains constitute a fraction (20%) of feacal *E. coli*, they do not cause any gastrointestinal disease (Murray et al., 2004; Nowrouzian et al., 2005; Siitonen, 1992; Johnson et al., 1998). However, ExPEC clearly possess a unique ability to effectively colonize the gastrointestinal tract (Johnson and Russo, 2002). ExPEC exhibit a broad range of extraintestinal virulence factors (VFs) that enable the strains to colonize host mucosal surfaces, avoid or subvert local and systemic host defence mechanisms, scavenge essential nutrients such as iron, injure or invade the host, and stimulate a noxious inflammatory response (Bower et al., 2005; Johnson, 1991). The characteristic VFs of ExPEC include diverse polysaccharide coatings (e.g. lipopolysaccharides and capsules), toxins, adhesins, invasins, proteases, serum resistance proteins and siderophores (see Figure 3). Many of these VFs are encoded on so called pathogenicity islands (PAIs) (Groisman and Ochman, 1996). PAIs are defined as chromosomally inserted genetic elements with a distinct G + C content, harbouring virulence associated genes that are absent from commensal *E. coli* (Hacker et al., 1990). PAIs are characteristically acquired via horizontal gene transfer which has likely been a key step in the evolution of the pathogen. However, specific transmissibility of PAIs has not yet been demonstrated and their evolutionary origin remains unknown.

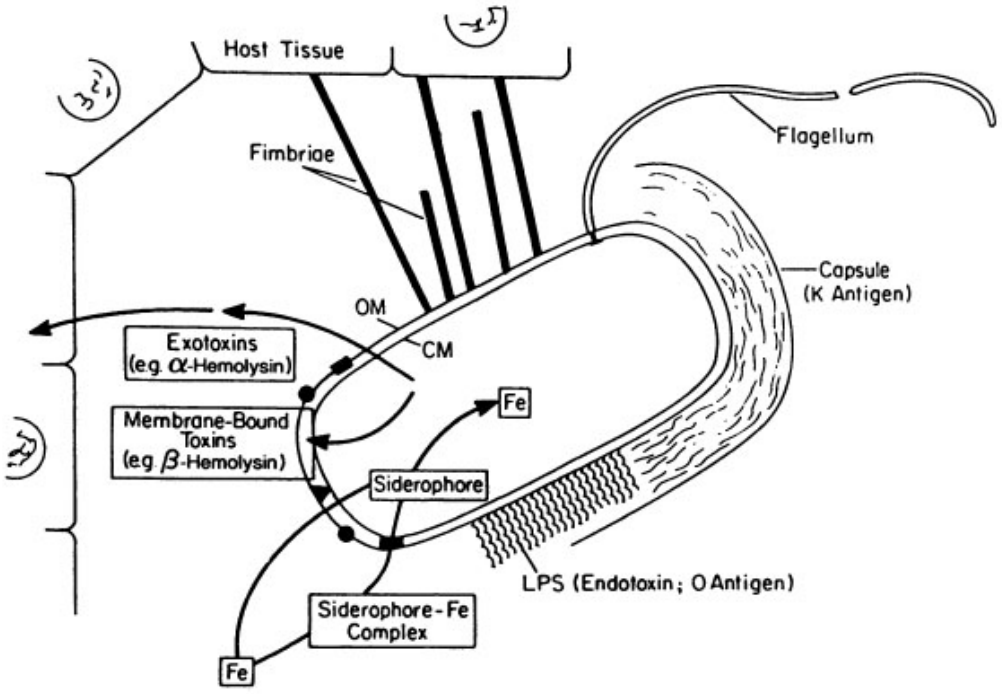

Figure 3. Schematic representation of an *E. coli* cell interacting with host tissue, highlighting features relevant to bacterial pathogenicity. Membrane proteins involved in transport, serum resistance, etc., are indicated by solid black circles, triangles, and rectangles. OM, Outer membrane; CM, cytoplasmic membrane; LPS, lipopolysaccharide (Johnson, 1991).

Polysaccharide coatings

Bacterial surface coatings, i.e. capsular polysaccharides (K-antigens), LPS and O-polysaccharide moieties of LPS (O-antigens) have been implicated as possible virulence determinants in extraintestinal infections (Kusecek et al., 1984; Russo et al., 1996). There are 176 different O-serogroups and more than 80 K-antigens in *E. coli*. In addition, many *E. coli* strains are able to synthesize colonic acid, the main component of the polysaccharide coatings of biofilms (Reisner et al., 2003).

Capsular polysaccharides are linear polymers of repeating carbohydrate subunits coating the cell, interfering with O-antigen detection and protecting the cell from host defence mechanisms. *E. coli* produces four types of capsular polysaccharides (groups I to IV), each of which comprises of a large number of distinct types (Keenleyside et al., 1993). The capsules of most ExPEC strains are thin, patchy, acidic, thermostable, and highly anionic, characteristics that identify group II polysaccharides. Certain ExPEC capsule types (e.g. K1, K5, K10, K54) have been shown to contribute to the pathogenesis of these strains (Horwitz and Silverstein, 1980; Pluschke et al., 1983a; Pluschke et al., 1983b; Schneider et al., 2004; Suerbaum et al., 1994). Similarly, UPEC strains are more likely to belong to the O-antigen serotypes O1, O2, O4, O6, O16, O18, O22, O25, and O75 (Korhonen et al., 1985; Kusecek et al., 1984; Russo et al., 1996; Johnson et al., 1994). The genes responsible for the capsular polysaccharide and LPS synthesis in the UPEC strain CFT073 are highly expressed during experimental UTI, which indicates the importance of surface coatings *in vivo* (Snyder et al., 2004). However, whether certain O-polysaccharide or capsular antigens contribute to the ascending UTI is not clear. Some studies implicate that bacterial coatings may account for the increased serum resistance and thus impede the clearance of ExPEC by phagocytosis (Horwitz and Silverstein, 1980; Pluschke et al., 1983a; Suerbaum et al., 1994) (see also section serum resistance proteins).

Adherence

Adherence is a property common to many pathogenic microorganisms. By attaching to host structures, microbial pathogens avoid being eliminated by the normal flow of body fluids (blood, urine, intestinal contents). Attachment is considered a necessary first step in the colonization of host mucosal surfaces and a precedent to invasive infection. In the late 1970s it was recognized for the first time that strains of *E. coli* causing UTI typically agglutinate human erythrocytes despite the presence of mannose (mannose-resistant hemagglutination) and adhere to human urothelial cells. However, adherence to urothelial cells is also possible

by those strains exhibiting only mannose-sensitive hemagglutination. Among most urinary isolates, both properties are mediated by fimbriae.

Fimbrial adhesins

The familiy of mannose-resistant adhesins includes P fimbriae and X adhesins with the collective property to agglutinate erythrocytes of different species and blood groups.

P fimbriae (*pap*, pyelonephritis associated pili) are UPEC specific adhesins which recognize specifically the P blood group antigens via Galα (1-4)Galβ specific attachment to glycolipid receptors on renal epithelial cells (Korhonen et al., 1982). Thus, P fimbriae mediate adherence to epithelial cells within the urinary tract (UT), thereby permitting bacterial colonization and stimulating inflammation, such as chemokine mediated recruitment of neutrophils into the UT (Wullt et al., 2000; Wullt et al., 2002). P fimbriae are more frequent associated with pyelonephritis (70%) than with cystitis isolates (36%) or faecal strains (19%). These observations suggest that P fimbriae contribute to the ability of ExPEC to cause UTI and that strains lacking P fimbriae are disadvantaged in the UT (Ofek et al., 1982).

The group of X adhesins includes the familiy of Dr adhesins, S, F and M fimbriae, all of which bind to distinct receptors. Dr adhesins are non-fimbrial adhesins and are referred in the section afimbrial adhesions. S fimbriae and FlC fimbriae are closely related adhesins expressed by some urinary strains. S fimbriae bind specifically to terminal sialyl-galactoside residues of human erythrocytes or epithelial cells of the proximal and distal tubules, collecting ducts, and glomerulus, in the renal interstitium, and on renal vascular endothelium (Korhonen et al., 1984; Parkkinen et al., 1989). S fimbriae (*sfa*) contribute to virulence in a number of animal models of infection, including UTI (Marre et al., 1986). In humans, S-fimbriated strains are more closely associated with meningitis and bacteremia than with UTI (Ott et al., 1986). The role of FlC fimbriae (*foc*) in the pathogenesis of UTIs is questionable, especially because they were only rarely found in patients with UTI (Pere et al., 1987). Furthermore, F1C fimbriae bind only to buccal epithelial cells and to some renal tissues but do not mediate hemagglutination or urothelial cell adherence (Orskov and Orskov, 1985).

In contrast to P and X fimbriae, the type 1 pili are mannose-sensitive. Type 1 fimbriae (*fim*) are common among *E. coli* strains from all clinical categories of UTI and among feacal strains (Arthur et al., 1989). Type I pili are adhesive surface fibers which mediate intimate contact between UPEC and host urothelium via FimH, the adhesive tip protein (Abraham et al., 1988). FimH binds to mannosylated uroplakin lining the bladder lumen leading to invasion of the underlying superficial umbrella cells (Mulvey et al., 1998). Type I fimbriae adhere

specifically to host cells in the urinary tract, but are considered to play a more important role in colonization or infection of the bladder than in invasive UTI. It was further reported, that type 1 fimbriae may also contribute to bacterial persistence in patients with *E. coli* UTI during long-term use of an indwelling bladder catheter (Mobley et al., 1987).

Recently, a further fimbrial gene cluster, *aufABCDEFG*, was described to be present in the archetypal UPEC strain CFT073 (Buckles et al., 2004). Auf fimbriae are significantly associated with UPEC when compared to commensal *E. coli* strains. However, no hemagglutination or cellular adherence properties have been described for the Auf fimbriae. Hence, the role of these pili in the ExPEC pathogenesis is still unclear.

Afimbrial adhesins

Dr adhesins (*dra*) are non-fimbrial adhesins that bind to various Dr blood group antigens within the urinary tract and which are structurally distinct from other *E. coli* fimbrial adhesins (Labigne-Roussel and Falkow, 1988; Nowicki et al., 1989; Nowicki et al., 1990). In contrast to P fimbriae, the Dr adhesins (e.g. O75x, AFA-I, AFA-III) are predominantly associated with cystitis strains (50%) (Arthur et al., 1989).

In addition, ExPEC strains possess an IrgA homologue adhesin (*iha*) which is a non-hemagglutinating adhesin originally identified as part of a tellurite resistance associated PAI from an *E. coli* O157:H7 isolate. The ExPEC *iha* gene is similar to the *V. cholerae* iron-regulated gene A (*irgA*) (Goldberg et al., 1992; Tarr et al., 2000). In *V. cholerae* IrgA has a dual function; it serves both as an adhesin and as a receptor for the siderophore enterobactin (Mey et al., 2002). Recently, Johnson and colleagues showed that *iha* is significantly associated with the ExPEC phenotype (Kanamaru et al., 2003; Johnson et al., 2005a). Iha conferred upon a non-adherent *E. coli* the ability to adhere to the uroepithelial cells *in vitro* and *in vivo*. However, whether Iha contributes to adherence directly or indirectly in concert with other bacterial components is not known.

Motility

At present, more than 50 flagellar-antigens (H-antigens, *fliC*) are described for *E. coli* (Prager et al., 2003; Johnson et al., 2005a). The virulence of enteric *E. coli* has been shown to be dependent on the expression of functional flagellae (Nougayrede et al., 2003; Best et al., 2005). In addition, flagellae are supposed to be essential adhesive organs in establishing *E. coli* biofilms (Tenorio et al., 2003; Reisner et al., 2003). However, the impact of motility in colonization of the urinary tract is questionable.

Serum resistance proteins

As reviewed by Taylor (1983) bacteria are killed by normal human serum through the lytic activity of the complement system. Bacterial resistance to killing by serum results from the individual or combined effects of capsular polysaccharide, O-polysaccharide side chains (smooth strains are more resistant than rough strains), and surface proteins such as Iss (increased serum resistance) or TraT (outer membrane lipoprotein) or by the plasmid pColV (encoding for production of and resistance to colicin V) (Perez-Casal and Crosa, 1984).

Toxins

Several bacterial toxins (endo- and exotoxins) or effector proteins have established or putative roles in the virulence of ExPEC. Their various biological roles and activities include for example iron accumulation through cell lyses, adhesion through disruption of the epithelium, modulation of the cell cycle and induction of apoptosis.

α-hemolysin (*hlyA*) is an exotoxin frequently associated with ExPEC strains and is one of the most important virulence factors involved in the pathogenesis of ExPEC. The α-hemolysin belongs to the family of RTX toxins and is secreted by the type I secretion system. The synthesis, activation and secretion of α-hemolysin are determined by the *hlyCABD* operon (Welch, 1991). α-hemolysin is synthesized in an inactive form with a molecular weight of 110 kDa, which is activated in the cytoplasm to the hemolytically active form by HlyC, a fatty acid acyltransferase (Stanley et al., 1998). α-hemolysin is directly secreted from the cytoplasm to the extracellular milieu through a transmembrane channel consisting of HlyB, HlyD and TolC (Koronakis, 2003; Thanabalu et al., 1998; Wandersman and Delepelaire, 1990). The secreted α-hemolysin has a Ca^{2+}-dependent cytolytic and/or cytotoxic activity against a wide range of mammalian cell types (Lally et al., 1999). Furthermore, α-hemolysin was reported to interact with the host cell membrane and to modulate the inflammatory response in target cells e.g. by elevated IL-6 and IL-8 levels (Laestadius et al., 2003; Soderblom et al., 2002; Uhlen et al., 2000).

Some (but not all) hemolytic strains produce the cytotoxic necrotizing factors 1 and 2 (*cnf*) which are considered as important ExPEC virulence factors as they are highly cytotoxic against various cell types (Rippere-Lampe et al., 2001; Hoffmann and Schmidt, 2004). CNF-toxins are composed of two domains, the cell binding and catalytical domain (deaminase) (Lemichez et al., 1997). The toxins were internalized through a common lamin receptor precursor via clathrin-independent endocytosis and translocated into the cytosol (Chung et al., 2003). Once in the cytosol, CNF specifically activates host Rho GTPases influencing

regulatory pathways for actin cytoskeleton, transcription, cell transformation, apoptosis and cell proliferation (Oswald et al., 1994; Peres et al., 1997; Falzano et al., 2003; Fiorentini et al., 1997). Thus, CNF has been shown to modulate and impair immune response by disturbing the cell cytoskeleton, modulating cell cycle control, apoptosis and secretion of inflammatory mediators in cells participating immunity (Fiorentini et al., 1997; Hofman et al., 1998; Hofman et al., 1999; Mills et al., 2000).

The uropathogenic specific protein (*usp*) was recently described as a putative uropathogenic virulence gene (Bauer et al., 2002). Usp is homologue to a *V. cholerae* zonula occludens toxin (*zot*) gene and has a high prevalence in ExPEC strains (93% of pyelonephritis and 80% of cystitis isolates), but is rarely present in stool samples from healthy individuals (24%) (Kurazono et al., 2000). Some studies however, reported only modest association of *usp* with UTI isolates (Bauer et al., 2002). Although the prescise mechanism remains unclear, *usp* was shown to significantly induce virulence in mouse ascending UTI model (Yamamoto et al., 2001) and is is condidered to be a bacteriocin acting as an endonuclease, since it showed high homology to the S-type pyocins produced by *P. aeruginosa* strains (Parret and De Mot, 2002).

Cytolethal distending toxins (*cdt*) are bacterial effector molecules which are able to manipulate the eukaryotic cell cycle (Nougayrede et al., 2005). These toxins consist of the catalytic domain (CdtB) and carrier subunits required for the receptor recognition and delivery of the catalytical domain into the nucleus of the target cells (Lara-Tejero and Galan, 2001). CdtB causes chromatin damage, which in turn activates the cellular DNA damage signaling pathways (Frisan et al., 2002). The most commonly reported response to the CDT intoxication is an irreversible blockade of the cell cycle, but the effect of CDTs vary depending on the cell type (e.g. necrosis *versus* apoptosis) (Bielaszewska et al., 2005; Yamamoto et al., 2004). Although a number of ExPEC strains are positive for CDTs, their function in the pathogenesis of these strains is not known.

Microcins (*mch*) and colicins (*cva*) are antimicrobial peptides secreted by various members of the Enterobacteriaceae family (Gillor et al., 2004; Patzer et al., 2003). They are produced under conditions of nutrient depletion and are active against phylogenetically related microbial strains. Microcins and colicins are considered to play important roles in the microbial competitions within the infecting populations and the intestinal flora (Azpiroz et al., 2001; Snyder et al., 2004).

Among all these exotoxin associated with ExPEC strains, the Gram-negative endotoxin lipopolysaccharide (LPS), a part of the bacterial outer membrane, is one of the main *E. coli*

virulence factors. In the ascending UTI, the O-polysaccharide moiety of the UPEC LPS has an important regulatory function (Svanborg et al., 2001; Fischer et al., 2006). LPS signals through Toll-like receptor 4 (TLR4) of urothelial and inflammatory host cells which induces the production of pro-inflammatory mediators (e.g. cytokines and chemokines) and cellular nitric oxide synthases (Cross et al., 1995; Austin et al., 2003; Backhed et al., 2003). The LPS-induced systemic inflammation breaks mucosal inertia and allows UPEC to enter underlying tissues. Several studies reported that LPS-dependent host cell signaling is dependent of type 1 and/or P fimbriae (Blomgran et al., 2004; Hedlund et al., 2001; Fischer et al., 2006).

Proteases

UPEC strains have usually four outer membrane serine proteases (Sat, PicU, Hbp, and OmpT) with different substrate specificities and functions (Otto et al., 1998; Guyer et al., 2002; Kukkonen and Korhonen, 2004; Parham et al., 2004). Sat (*sat*) is a serine auto-transporter toxin, which displays cytopathic activities on the kidney and bladder epithelium *in vitro* and *in vivo* (Guyer et al., 2000; Guyer et al., 2002; Anderson et al., 2004). PicU (*picU*) is homologous with the Pic (protein involved in intestinal colonization) protein of *Shigella* spp. and enteroaggregative *E. coli*. Pic proteases disrupt mucosal barriers by degrading mucosal pepsin, spectrin and mucin (Bellini et al., 2005; Henderson et al., 1999; Parham et al., 2004). Hemoglobin-protease Hbp (*hbp*) is a homologue of the Tsh hemagglutinin of avian pathogenic *E. coli* (APEC). Hbp and Tsh are heme-binding proteins with serine protease activity targeted at hemoglobin (Tivendale et al., 2004). OmpT (*ompT*), the fourth outer membrane endopeptidase of ExPEC, is homologous with the plasminogen activator Pla of *Yersinia pestis* and degrades antimicrobial peptides secreted by epithelial cells and macrophages (Grodberg et al., 1988; Kukkonen and Korhonen, 2004).

1.2.2. Siderophore systems of ExPECs

Iron is an essential nutrient for most microorganisms. Although iron is one of the most abundant nature elements, it remains unavailable to microorganisms because it forms insoluble polymers of iron oxyhydroxide in the presence of oxygen and physiological pH, whereas the concentration of soluble iron ranges of 10^{-18} M (Raymond et al., 2003). Thus soluble iron cannot get into the cell via diffusion because the concentration of iron in the cytoplasm of metabolically active bacteria is about 1 µM. A similar shortage of iron is created within the host: iron is predominantly intracellular, occurring as heme and heme proteins (e.g. hemoglobin), iron-sulfur proteins or the iron-storage protein ferritin. The limited amounts of extracellular iron are tightly bound to proteins such as transferrin in the serum and lactoferrin on mucosal surfaces. In order to acquire iron from the host organism, ExPEC strains have developed a variety of iron uptake mechanisms such as the synthesis and transport of low-molecular-weight iron chelators, named siderophores (Crosa, 1989; Neilands, 1976). Siderophores are organic molecules that have the capacity to specifically chelate ferric ions. The ferric siderophore complex is then transported via an energy dependent, multicomponent transport system across the membranes into the cytoplasm. The components of such systems include a receptor protein located in the outer membrane, a periplasmic binding protein and an ABC type transporter located in the inner membrane. The energy required for the transport via an OM receptor is provided by the interaction of the receptor with the TonB protein located in the inner membrane, which couples the proton motive force created by the inner membrane for energy transduction (Postle, 1993; Braun, 1995).

ExPEC strains possess several different iron uptake systems including siderophore systems and siderophore independent system (e.g. hemin receptors) which chelate iron bound to the intracellular heme or hemoglobin (Braun, 2003). Recent studies revealed that some siderophore systems are more prevalent in ExPEC than in commensal strains (Johnson et al., 2005b; Schubert et al., 1998) and play an important role in the pathogenesis of UTIs (Johnson et al., 2005c; Russo et al., 2003). Amongst others, this is true for (i) the yersiniabactin siderophore system (*irp-fyuA*), (ii) the salmochelin system (*iroA*), (iii) the aerobactin siderophore system (*iuc-iutA*) and (iv) the hemin uptake (*chu*) system. It has been assumed that the simultaneous presence of these distinct siderophore systems improve the efficiency of growth in the host by allowing the pathogen to take advantage of multiple iron sources in different environments within the host or at different times during the course of an infection

(Valdebenito et al., 2006; Torres et al., 2001). Some of these siderophore receptors and their respective siderophore systems are reviewed in the following section:

The recently identified siderophore receptor IreA (iron-responsive element) first identified from CP9, was previously shown to be virulence associated (Russo et al., 2001). IreA has been further demonstrated to function both as a siderophore receptor and as an adhesin in UTIs. Similarly, a recent study characterized the outer membrane protein Iha as both a catecholate siderophore receptor and an adherence factor in ExPEC strains (Leveille et al., 2006).

The yersiniabactin siderophore system characterized by the *irp* gene cluster and the outer membrane receptor FyuA, encoded on a highly conserved chromosomal DNA region designated High Pathogenicity Island (HPI), was first described in *Y. pestis* (Rakin et al., 1994). However, a unique characteristic of the HPI is its wide distribution in different members of the family of *Enterobacteriaceae*, especially in ExPECs causing urinary tract infection, septicemia, and meningitis in newborns (Clermont et al., 2001; Johnson and Stell, 2000; Schubert et al., 1998). Several studies reported that the HPI encoded yersiniabactin siderophore system is required for full virulence expression in Yersiniae (Carniel, 2001) and in ExPEC (Schubert et al., 2002).

The salmochelin siderophore system including the catecholate receptor IroN is encoded on the iroA locus, the *iroBCDEN* gene cluster, that was originally found in *Salmonella enterica* (Baumler et al., 1998) and later in the UPEC strain CP9 (Russo et al., 1999). The catecholate siderophore salmochelin is synthesized from enterobactin (Hantke et al., 2003), a 2,3 dihydroxybenzoylserine macrotrilactone, which is one of the best studied siderophores produced by enteric bacteria (Figure 4).

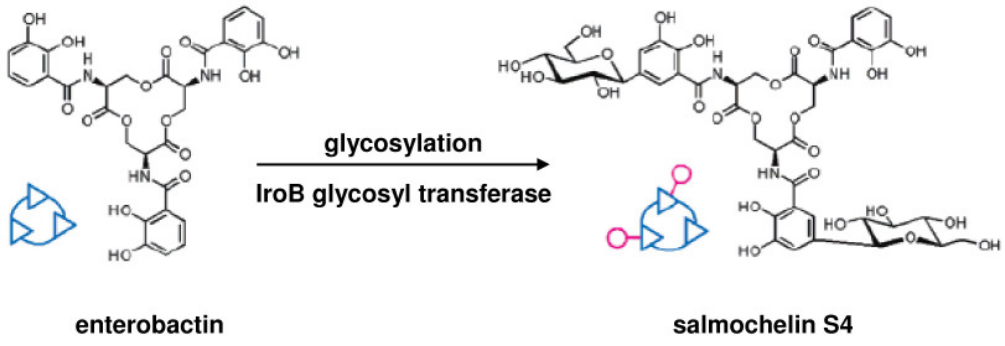

Figure 4. Structures of enterobactin and the glucosylated enterobactin, known as salmochelin. Next to each structure is a schematic representation of the compound, where a blue triangle represents DHB-Ser, and a pink circle represents glucose. Figure was modified from (Lin et al., 2005).

The outer membrane receptor IroN is not restricted to the transport of salmochelin, but also involved in the transport of several other catecholate siderophores, such enterobactin and dihydrobenzoic acid. Moreover, IroN is highly prevalent in virulent ExPEC strains and has an increased expression when grown in urine (Bauer et al., 2002). Interestingly, IroN is supposed to contribute to other virulence traits, such as adherence suggesting that IroN is important for urovirulence (Negre et al., 2004; Russo et al., 2002; Russo et al., 2003).

The aerobactin biosynthetic gene cluster (*iuc*) and the ferri-aerobactin receptor (*iutA*) were first identified in EIEC strains as part of the virulence plasmid pColV (Williams, 1979). The *iuc-iutA* system is distributed among intestinal and extraintestinal pathogenic *E. coli* and other *Enterobacteriaceae* and is associated with increased virulence of enteric bacteria (Vokes et al., 1999). Aerobactin, a hydroxamate siderophore, is produced by many *E. coli* strains isolated from patients suffering from UTIs, bacteremia, or other extraintestinal infections (Johnson, 1991). However, the precise contribution of aerobactin to virulence is not well established (Torres et al., 2001).

The hemin uptake system including the receptor ChuA (*E. coli* heme-utilization protein) was originally identified in the *E. coli* O157:H7 strain EDL933 (Torres and Payne, 1997). This strain synthesized and transported enterobactin in response to iron limitation and had a ferric citrate transport system which used heme and hemoglobin, but not transferrin or lactoferrin, as iron sources in a TonB-dependent, Fur-regulated manner. Although the genes encoding the outer membrane protein ChuA were found in several ExPEC strains of the phlyogenetic group B2, its function and importance for the pathogenesis remains to be elucidated (Clermont et al., 2001; Duriez et al., 2001).

The expression of siderophore systems is up-regulated during bacterial growth in the urinary tract indicating the importance of effective iron assimilation for urovirulence (Snyder et al., 2004). Furthermore, iron or the lack of thereof within the host serves as an environmental signal to regulate the expression of a number of virulence factors. In *E. coli* but also in many other bacteria, the transcriptional regulation by iron depends on the iron-binding repressor Fur (ferric uptake regulation) that acts as a global regulator, governing the expression of several virulence genes (Wee et al., 1988; Schaffer et al., 1985). These virulence genes are often under the control of the same regulators as iron acquisition systems. Thus, genes for iron transport and other virulence factors are simultaneously derepressed when the pathogen encounters the iron-limiting environment of the host. Virulence factors regulated in this manner include a variety of toxins, cell surface proteins, hemolysins, and other secreted enzymes.

Recently, the crystal structures of three outer membrane ferric siderophore receptor proteins from *E. coli*, FepA, FhuA and FecA, which transport ferric enterobactin, ferrichrome and diferric dicitrate, respectively, have been solved (Buchanan et al., 1999; Ferguson et al., 1998; Ferguson et al., 2002). The structures are similar and show the presence of similar domains: e.g., all of them consist of a 22 strand-β-barrel formed by approximately 600 C-terminal residues while approximately 150 N-terminal residues fold inside the barrel to form a plug domain (Figure 5). The beta strands are connected on the extracellular side by loops of variable length, while they are linked with shorter loops on the periplasmic side. The plug domain consists of multiple short beta strands and a few alpha helices (Chakraborty et al., 2007). Multiple alignment studies involving protein sequences of a large number of outer membrane receptor proteins indicated that most of the siderophore receptors are similar in their structure as they contain several conserved residues. For example, IroN and FepA have 52 % sequence identity as determined by pairwise alignment of amino acid sequences from IroN and FepA wit the program CLUSTAL (Baumler et al., 1998).

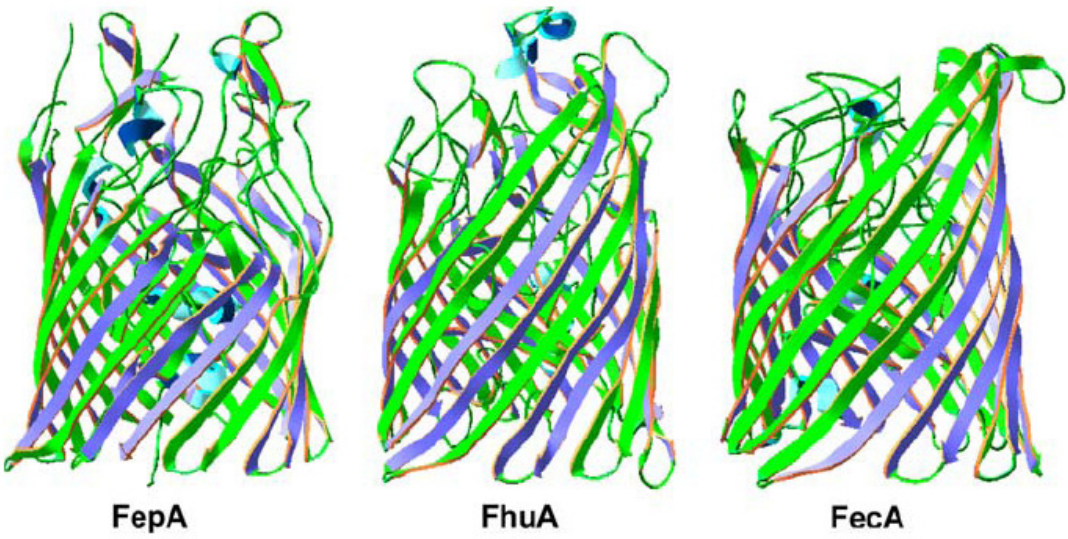

Figure 5. Crystal structures of ferricsiderophore outer membrane receptors FhuA, FepA and FecA from *E. coli* (Chakraborty et al., 2007).

1.3. Outer membrane vesicles

Outer membrane vesicles (OMVs) have been defined as closed proteoliposome which are constitutively shed from the outer membrane of most Gram-negative bacteria during normal growth (Kadurugamuwa and Beveridge, 1995; Kolling and Matthews, 1999; Shoberg and Thomas, 1993; Wai et al., 1995). They are composed of a subset of the outer membrane including outer membrane proteins and lipids, lipopolysaccharides (LPS) and periplasmic components but not inner membrane or cytoplasmic proteins (McBroom et al., 2006; Kesty et al., 2004; Horstman and Kuehn, 2000). Vesicles have been suggested to be involved in several virulence mechanisms like periplasmic enzyme delivery, DNA transport, bacterial adherence, and evasion of the immune system (Pettit and Judd, 1992; Saunders et al., 1999; Dorward et al., 1989; Horstman and Kuehn, 2000; Kadurugamuwa and Beveridge, 1997; Kolling and Matthews, 1999; Shoberg and Thomas, 1993). Vesicles produced by pathogenic bacteria are often associated with active toxins and other virulence factors (Kuehn and Kesty, 2005). These vesicles have been further described to interact with bacteria and mammalian cells by adherence or fusion mechanism (Kadurugamuwa and Beveridge, 1998; Kato et al., 2002; Fiocca et al., 1999) thus playing an important role in the delivery of active virulence factors into host cells during a bacterial infection. For example, *Helicobacter pylori* produce vacuolating cytotoxin A-containing OMVs (Fiocca et al., 1999) and ETEC and *Salmonella typhi* produce cytolysin A-containing vesicles (Wai et al., 2003a; Wai et al., 2003b), which are cytotoxic to mammalian cells. *Pseudomonas aerginosa* vesicles are found to be associated with biofilm matrices (Schooling and Beveridge, 2006), containing the quorum-sensing molecule 2-heptyl-3-hydroxy-4-quinolone (Mashburn and Whiteley, 2005), and fuse with unrelated Gram-negative and Gram-positive bacteria (Kadurugamuwa and Beveridge, 1999; Li et al., 1998), suggesting OMVs function as signals in biofilm formation and intraspecies or interspecies communication. Recently, it was reported that epithelial cells produce IL-8, a cytokine important for neutrophil and monocyte recruitment *in vivo*, in response to *H. pylori* and *P. aeruginosa* vesicles, suggesting that OMVs contribute to inflammation. These biochemical and functional properties of pathogen-derived vesicles reveal their potential to critically impact disease. Despite a history of published reports scanning several decades, the mechanisms regulating OMV production and formation *in vivo* or by which bacterial proteins are selectively expressed in OMVs are only beginning to be clarified (Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006).

1.4. Urinary tract infections

1.4.1. The urinary tract

The urinary tract represents a usually sterile compartment, which is protected from bacterial infections by various mechanisms such as urine flow and immune responses. Moreover, the urinary tract is a hostile environment in terms of supporting bacterial growth. The chemical composition, osmolarity, and pH of urine determine the rate of bacterial growth, but can be very variable, depending on the diet. Normal urine constituents include amino acids and glucose, which are usually present at sufficient concentrations to support rapid bacterial growth. However, other components of urine, such as urea and organic acids, may inhibit growth, mainly by affecting pH and osmolarity (Asscher et al., 1966).

The specialized epithelium, lining much of the urinary tract, including the renal pelvis, the urethras, the bladder, and parts of the urethra, called urothelium and plays an important but conflicting role in the host pathogen interaction: On the one hand, it acts as a permeability barrier, protecting underlying tissues against noxious urine components. On the other hand, the urothelium must be stretchable to accommodate urine pressures. This is warranted by the special structure of the urothelium that is composed of three different cell types: undifferentiated basal cells, intermediate cells, and superficial umbrella cells (Figure 6). Additionally, it is covered with microvilli and mucus. Superficial cell layers contains integral membrane proteins called uroplakin (Ia, Ib, II and III), which is recognized by UPEC (Olsburgh et al., 2003). The urothelium underlies a continual renewal process that is extremely slow and its regeneration in humans is estimated to take 6 to 12 months (Southgate et al., 1999).

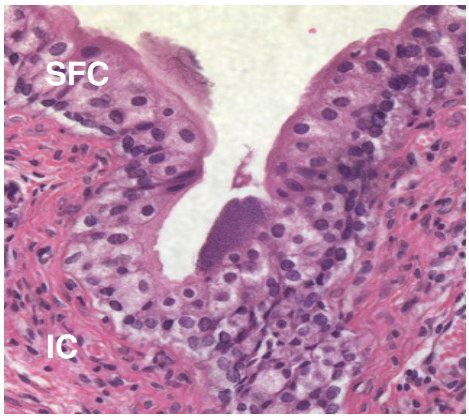

Figure 6. Haematoxylin and eosin stained mouse urothelium. SFC = superficial facet cells, IC = intermediate cells. The figure was kindly provided by K. Hildinger (Max von Pettenkofer-Insitut).

1.4.2. Urinary tract infections caused by ExPECs

Urinary tract infections (UTIs) are bacterial infections that affect any part of the urinary tract. UTIs are one of the most frequently acquired bacterial infections in human world wide (Warren, 1996) and can be caused by several microbial pathogens. However, more than 80% of community-acquired UTIs are caused by UPECs, the major subgroup of ExPEC (Foxman and Brown, 2003). The symptoms of UTI range from asymptomatic bacteriuria to painful and frequent urination, bloody urine, abdominal pain, nausea, and fever (Stamm, 1982). The most common type of UTI is a harmless bladder infection, called cystitis. However, UPEC can also cause life threatening kidney infections, known as pyelonephritis. Acute and recurrent UTIs are prominent health problems in women. UTIs afflict approximately half of all women during their lifetime, and about 25% of these women will experience one or more recurrent infections after their first UTI episode. 5% of these women suffer of persistent infections. UTIs are responsible for serious morbidity and mortality, especially in the elderly, young children, and in immune-compromised patients (Foxman and Brown, 2003; Scholes et al., 2005). Taken together, UTI episodes greatly burden the public health care systems and the national economies (Russo and Johnson, 2003). Further on, UTI have been shown to be an independent risk factor for both bladder cancer and renal cell carcinoma (Parker et al., 2004).

1.5. Host pathogen interaction and modulation of immune response

During establishment of an UTI, UPEC can emerge from the colon and become incorporated into the vaginal and periurethral microbial flora before entering the originally sterile urethra and bladder (Hooton, 2001). Intact superficial facet cells pose a formidable barrier to bacterial colonization, and for decades UPEC was considered strictly an extracellular pathogen. However, superficial facet cells express integral membrane proteins called uroplakins, which can serve as the receptors for UPEC, on their luminal surface. In the first phase of UTI, pathogens bind to the mucosal surfaces coating the urothelium thereby avoiding elimination with the normal flow of urine. Adherence and subsequently invasion into epithelial cells is probably enhanced by fimbriae, such as tye I pili and P fimbriae binding to uroplakin or glycoconjugate receptors respectively (Schilling et al., 2001; Wullt et al., 2002). Mouse models show that UPEC invade terminally differentiated superficial facet cells upon binding of UPEC to those receptors. Once within the intracellular milieu, UPECs rapidly replicate and undergo a complex developmental pathway leading to the formation of intracellular bacterial communities (IBCs), morphologically distinct structures with biofilm-like properties that ultimately protect UPEC from a multitude of host defences (Anderson et al., 2003; Mulvey et al., 1998). An intracellular location also protects bacteria against many common antibiotics and helps to persist in the bladder epithelium for weeks following the acute infection. The persistence of *E. coli* in bladder tissue may be relevant to more chronic diseases of the urinary tract such as recurrent UTIs and interstitial cystitis (Schilling et al., 2001).

However, attachment and invasion also activates a cascade of innate host defences, leading to exfoliation of bacteria-laden superficial facet cells, followed by rapid reconstitution of the urothelium through differentiation of underlying basal and intermediate cells (Mysorekar et al., 2002; Mysorekar and Hultgren, 2006; Mysorekar et al., 2002), and to the production of inflammatory mediators. As a first line of host defence, different urothelial cells secrete unspecific antimicrobial protein, such as defensins and complement proteins, as well as cytokines and inflammatory cells (Schilling et al., 2003b). The main cytokines secreted by the host epithelium include IL-1 α, IL-6, IL-8, transforming growth factor (TGF) β, tumour necrosis factor (TNF) α and β and interferon (IFN) γ (Svanborg et al., 1993). These mediators are also present in highly elevated levels in the urine of patients with acute UTIs (Funfstuck et al., 2000; Schilling et al., 2001). In response to cytokines and leukocyte adhesion molecules expressed by infected urothelial cells, phagocytic cells such as polymorphonuclear neutrophils (PMN) and macrophages, migrate through capillary cell walls to the site of

infection (Godaly et al., 2001). Once there, phagocytes opsonize infecting bacteria through specific receptors and secrete cytokines and reactive oxygen intermediates, which further enhance the inflammation. Taken together, epithelial cells play an active role in the mucosal immune response: attachment and invasion of bacteria trigger the innate immune response and lead to active exfoliation of infected cells, thereby eliminating the bacteria-laden epithelium (Mulvey et al., 1998).

Another part of the innate immune response comprises the expression of Toll-like receptors (TLRs) on the surface of epithelial cells. These receptors are involved in the recognition of pathogen associated molecular pattern (PAMP) of microorganisms such as bacterial LPS, flagellin, viral and bacterial RNA and DNA (Takeda and Akira, 2005). During UTI, the LPS-specific signalling through TLR4 plays an important role in preventing UTI. *In vivo*, UPEC pathogenesis initiates with bacterial binding of superficial bladder epithelial cells via the adhesin FimH at the tips of bacterially expressed type 1 pili (Mulvey et al., 2000). Initial colonization events activate inflammatory and apoptotic cascades in the epithelium, which is normally inert and only turns over every 6 to 12 months (Hicks et al., 1974). Bladder epithelial cells respond to invading bacteria in part by recognizing bacterial LPS via TLR4 - CD14 pathway, which led to the activation of NF-kappaB and p38 mitogen-activated protein kinase resulting in secretion of pro-inflammatory and anti-apoptotic substances (Schilling et al., 2003a). In addition, FimH-mediated interactions with the bladder epithelium stimulate exfoliation of superficial epithelial cells, causing many of the pathogens to be shed into the urine. Genetic programs are activated that lead to differentiation and proliferation of the underlying transitional cells in an effort to renew the exfoliated superficial epithelium (Mysorekar et al., 2002). Despite the robust inflammatory response and epithelial exfoliation, UPEC are able to maintain high titers in the bladder for several days (Mulvey et al., 1998; Mulvey et al., 2001). However, there are also some studies providing evidence that UPECs efficiently block NF-kappaB activation via TLR4 and enhances type 1 pili-induced apoptosis as a component of the uropathogenic programme (Schaeffer et al., 2004; Cirl et al., 2008). Furthermore, a recent publication described that the prototypic ExPEC strain NU14 modulates the host innate immune response by suppressing TNF-α mediated IL-8 and IL-6 secretion from urothelial cells. The reduced stimulation of inflammatory chemokine production resulted in increased bacterial colonization demonstrating how UPECs evade the activation of innate immune response in the urinary tract, thereby providing a pathogenic advantage (Billips et al., 2007).

Recently, a TLR that specifically recognizes uropathogenic bacteria, named TLR11, was recovered in mice (Zhang et al., 2004). Meanwhile the formerly TLR11 was renamed TLR12. The role of this TLR12 in the development of UTIs remains unclear: TLR 12 was found in mice, where it is necessary for cytokine production and the activation of immune cells in response to bacterial infections. In humans however, TLR12 is presumably not expressed. In this regard, the absence of TLR12 has removed a defence pathway with the unique ability to specifically recognize UPECs which may explain why humans are particularly susceptible to UTI (Zhang et al., 2004).

1.6. Aims of the study

Recently, our working group developed an ExPEC pathoarray, a DNA microarray specific for the ExPEC genotype, in order to estimate the extraintestinal virulence potential and accordingly the risk of clinically relevant ExPECs and fecal *E. coli* isolates (Sorsa et al., prepared for submission). This array contained 100 DNA probes homologous to known and putative ExPEC virulence determinants. Interestingly, several virulence factors could be identified that are significantly more prevalent among ExPEC when compared to commensal *E. coli* isolates. These highly virulence associated factors included toxins (*cnf*2, *hbp*, *hlyA*, *usp*), iron acquisition genes (*chuA*, *fyuA*, *ireA1*, *ireA2*, *iroD*, *iroE*, *iroN*, *iucC*, *iutA*, *modD*, *prrA*) and adhesins or fimbrial subunits (*aufA*, *aufC*, *aufG*, *focA*, *hek*, *matB*, *papA*, *papF*, *papGII*, *papGIII*, *prfG*, *sfa/foc*, *sfaS*) (Sorsa et al., manuscript in preparation). The simultaneous presence of so many different iron uptake systems in ExPEC strains could be a hint that siderophore systems fulfil additional tasks in the ExPEC pathogenesis. In this regard, recent studies provided evidence that ExPEC siderophore receptors may contribute to other virulence traits such as adherence (Russo et al., 2001; Russo et al., 2002). This is of particular interest, as it has recently been shown that ExPEC are able to form intracellular, biofilm-like structures in bladder epithelial cells of mice (Anderson et al., 2003).

The aim of this study was to investigate a possible involvement of the ExPEC siderophore receptors IroN and FyuA in host pathogen interaction. Therefore, the following questions were addressed during this work:

- Can the presence of the highly virulence associated determinants found in the microarray be confirmed by PCR?
- Focusing on the siderophore receptors IroN and FyuA, under which conditions are they transcribed and expressed?
- Play the siderophore receptors FyuA and IroN a role for invasion of urothelial cells *in vitro*?
- Which host mechanisms are involved in ExPEC invasion *in vitro*?
- Are FyuA and IroN present in outer membrane vesicles produced by ExPECs?
- Can ExPEC vesicles possibly interact with host cells *in vitro* and which determinants are involved in this interaction?
- What is the function of such outer membrane vesicles?

2. MATERIALS

2.1. Strains

All bacterial strains used in this study and their relevant genotypes are listed in Table 1.

Table 1. Bacterial strains used in this study.

***E. coli* strain**	**Relevant properties**	**Reference**
MG1655	*E. coli* K12, $^-$, λ^-, ilvG, rfb-50 rph-1	(Blattner et al., 1997)
DH5α	F^-, *endA*1, *hsdR*17 (r_k^-, m_k^-), supE44, thi-1, recA1, GyrA96, relA1, λ^-, Δ(argF-lac)U169, Φ80 lacZΔM15	Invitrogen
BL21	*E. coli* K-12, F^-, *ompT hsdSB* - ($r_B^-m_B^-$) *gal dcm* (DE3) *endA* Hte (pLysS Cm^R)	Paisley, UK
HB101	Laboratory strain, fimH-	(Petzoldt et al., 1995) (Boyer and Roulland-Dussoix, 1969)
MC1061	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsr hsm*$^+$ *strA*	(Casadaban, 1976)
TH2	Laboratory strain, A *supE4 hsdS20* ($r_B^-m_B^-$) *recA13 ara-1 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 thi trpR624*	Takara
TH2spec	spontaneous spectinomycin mutant	(Sorsa et al., 2004)
Top10	Laboratory strain, A	Invitrogen
DH10B	Laboratory strain	Invitrogen
H5058	*aroB malt tsx thi cir fiu fepA*	(Baumler et al., 1998)
SCS110	*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F' *traD36 proAB lac1* Z*ΔM15*]	Stratagene
536	pyelonephritis isolate, B2, O6:K15:H31	(Berger et al., 1982)
CFT073	pyelonephritis isolate, B2, O6:K2:H1	(Mobley et al., 1990)
NU14	cystitis isolate, B2, O18:K1:H7	(Johnson et al., 2001b)
RS218	Newborn meningitis isolate, B2, O18:K1:H7	(Achtman et al., 1983)
AD110	Cystitis isolate, B2, O6:K2:H1:F7	(Labigne-Roussel and Falkow, 1988)
C5	neonatal meningitis isolate, B2, O18:K1:H7	(Kim and Anthony, 1983)
J96	pyelonephritis isolate, B2, O4:H5	(Hull et al., 1981)
CP9	bacteremia/sepsis isolate, B2, K54:O4:H5	(Russo et al., 1993)
U3366		
HE300	pyelonephritis isolate, R :H-, A	(Sorsa et al., 2003)
JS293	chronic cystitis, B2, host suffers of *diabetes mellitus*	S. Schubert
JS297	cystitis, B2, host suffers of *diabetes mellitus*	S. Schubert

JS299	Cystitis isolate, B2, O6:H-	(Sorsa et al., 2004)
JS322	pyelonephritis isolate, D, O77:H18	(Sorsa et al., 2004)
JS323	pyelonephritis isolate, B2	S. Schubert
JS326	pyelonephritis isolate, B2	S. Schubert
Do768	Cystitis isolate, B	U. Dobrindt
S27710	Pyelonephritis, B2	S. Schubert
Nissle 1917, Mutaflor® (DSM6601)	Probiotic *E. coli* strain, stool isolate, B2, O6:K5:H1	(NISSLE, 1959)
NU14Δ*iroN*	chromosomal insertion of Kn^R cassette in *iroN* gene	this study
NU14Δ*fyuA*	chromosomal insertion of Cm^R cassette in *fyuA* gene	this study
536Δ*iroN*	chromosomal insertion of Kn^R cassette in *iroN* gene	this study
536Δ*fyuA*	chromosomal insertion of Cm^R cassette in *fyuA* gene	this study
536Δ*fyuA*	chromosomal insertion of Kn^R cassette in *fyuA* gene SphI site	this study
NU14Δ*iroN*/p*iroN*	NU14Δ*iroN* mutant complemented with p*iroN*	this study
NU14Δ*fyuA*/pfyuA	NU14Δ*fyuA* mutant complemented with p*fyuA*	this study
536Δ*iroN*/p*iroN*	536Δ*iroN* mutant complemented with p*iroN*	this study
536Δ*fyuA*/p*fyuA*	536Δ*iroN* mutant complemented with p*fyuA*	this study
536Δ*fyuA*/p*fyuA*	536Δ*iroN* mutant complemented with p*fyuA*	this study
CFT073Δ*iroN*	chromosomal insertion of Kn^R cassette in *iroN* gene	this study
CFT073Δ*fyuA*	chromosomal insertion of Kn^R cassette in *fyuA* gene	this study

2.2. Cell culture collection

All cell lines used in this study and their description are listed in Table 2.

Table 2. Cell lines used in this study.

Cell line	description	Reference
HCV29	human ureter cells	(Masters et al., 1986)
J82	bladder cancer cells	(O'Toole et al., 1983)
RT112	urothelial cancer cell line	(O'Toole et al., 1978)
UROtsa	human ureter cells	(Petzoldt et al., 1995)
HEK 293	human embryonic kidney cells (ATCC: CRL-1573)	(Graham et al., 1977)
GD25	beta 1 integrin-deficient mouse fibroblasts	(Fassler et al., 1995)
GD25β	mouse fibroblasts expressing the A isoform of the beta 1 integrin subunit	(Armulik et al., 2000)
J774.A1	murine Macrophages (ATCC: TIB-67)	(Ralph et al., 1976)

2.3. Cell culture media and solutions

For growth and cultivation of the different cell lines following media and solutions were used:

Dulbecco's modified Eagle' medium (DMEM) (Invitrogen)

DMEM (incl. 4 mM L-glutamine, 25 mM glucose, 500 ml without sodium pyruvate)

RPMI medium (PAN Biotech)

RPMI (incl. 4 mM L-glutamine, 25 mM glucose, 500 ml without sodium pyruvate)

Trypsin solution (PAN Biotech)

trypsin 1.5 g in phosphate buffered saline (PBS) 500 ml

Freezing solution

90% FCS

10% dimethylsulfoxid (DMSO)

The solution was stored in aliquots of 10 ml at -20°C.

2.4. Plasmids

All plasmids used and constructed during this study are listed in Table 3. Maps of the newly constructed plasmids are found in section 4.2.

Table 3. Plasmids used in this study.

Plasmid	Relevant properties	Reference
pKD46	*repA101* (ts), *araBp-gam-bet-exo* (l red recombinase under the contol of *araB* promotor), Ap^R (*bla*)	(Datsenko and Wanner, 2000)
pKD3	oriRg, Ap^R , *cat*-gene flanked by FRT sites	(Datsenko and Wanner, 2000)
pKD4	oriRg, Ap^R , *npt*-gene flanked by FRT sites	(Datsenko and Wanner, 2000)
pCP20	Yeast Flp recombinase gene (FLP, aka exo) *ts-rep*, Ap^R, Cm^R	(Datsenko and Wanner, 2000)
pASK33p	high-level expression vector with a Tc-inducible tet $^{p/o}$ promoter containing C-terminal $(His)_6$ tags	(Skerra, 1994)
pRF12	pASK33p.*fyuA*; His_6-tagged *fyuA* subcloned in pASK33p	K. Hildinger (Max von Pettenkofer-Insitute)
pQE-30	high-level expression low copy vector with a IPTG inducible T5 promoter containing N-terminal $(His)_6$ tags	Qiagen
pRF11	pQE-30.*iroN* His_6-tagged *iroN* subcloned in pQE-30	K. Hildinger (Max von Pettenkofer-Insitute)
pRF13	pQE-30.$iroN_{1-372}$ His_6-tagged N-terminal part of *iroN* subcloned in pQE-30	this study
pRF14	pQE-30.$iroN_{372-726}$ His_6-tagged C-terminal part of *iroN* subcloned in pQE-30	this study
pREP4	low-copy plasmid with chromosomal *lac* repressor	Qiagen
pRS1	IPTG inducible expression plasmid carrying *Yersinia enterocolitica* invasin	(Schulte et al., 1998)
pBAD-B	pBAD/His B Arabinose dose-dependent inducible expression vector containing N-terminal $(His)_6$ tags	Invitrogen
pSL13	pBAD.*chuA*; *chuA* subcloned in pBAD-B	S. Loos (Max von Pettenkofer-Insitute)
pCR4-TOPO	TA cloning vector	Invitrogen
p300	wild-type plasmid of *E. coli* HE300 carrying *iroBCDEN* gene cluster	(Sorsa et al., 2003)
pSuperCos 1	Cosmid vector	Stratagene
pJS332	pSuperCos 1 with 31,7 kb of p300, carrying *iroBCDEN*	(Sorsa et al., 2003)
pJS12	pCR4-TOPO with *iroEN*	(Sorsa et al., 2003)
pJS13	pCR4-TOPO with *iroBCDE*	(Sorsa et al., 2003)
pJS14	pJS12 with trimethoprim cassette in *iroN*	(Sorsa et al., 2003)
pJS15	pJS12 with trimethoprim cassette in *iroE*	(Sorsa et al., 2003)
pACYC184	low-copy plasmid, Tc^R, Cm^R	(Chang and Cohen, 1978)
p*fyuA*	pACYC184 with *fyuA* native expression, Tc^R, Cm^R	this study
p*iroN*	pACYC184 with *iroN* native expression, Tc^R, Cm^R	this study

2.5. Oligonucleotides

All oligonucleotides used for gene mutation using the λ Red-based method (Datsenko and Wanner, 2000), cloning of PCR fragments, as well as for PCR verification of the mutants and for TaqMan analyses were purchased from Metabion (Martinsried, Germany). The sequences and the application of all oligonucleotides are listed in Table 4.

Table 4. Oligonucleotides used in this study.

Name	Sequence (5'→3')	T_m (°C)	Application
iroN.28.for	CTAACTGTGCTCCTGGTTGGGTTGA	59	screening
iroN2053.rev	TGACGCCGACATTAAGACGCAGATT	59	screening
fyuA507.for	CAGTAGCCGCGACAGTTATC	60	screening
fyuA1080.for	CTACGACATGCCGACAATGCC	62	screening
fyuA.1330.rev	CGGGTATACACTCTCCAGTC	60	screening
fyuA.1709.rev	TGCTTCCCGCGCCATAACGTG	60	screening
chuA.255.for	CGGCGTGCTGGTTCTTGTCGATGGT	64	screening
chuA.1464.rev	CGTGGAGATATAATCTTTCGCTTTG	56	screening
chuA.16.for	GTTTCTGCCACCTTGCCAACG	56	screening
YbtA.for	ATGATGGAGTCACCGCAAAC	52	screening
YbtA.rev	GGAGAGGCAACTGGCGTCTT	56	screening
ORF36-PAI III-536.362.for	ATCTGGATCCGGCACTCGTCA	58	screening
ORF36-PAI III-536.1021.rev	ACGGTAATATTCAGCGCCATA	53	screening
Ag43-fluA.2418.for	TCACGATAACAACGGCGGTAT	54	screening
Ag43-fluA.2879.rev	CACTGCCATGCCCGAACTTCA	56	screening
ag43.28.for	GGAACAACAGAGCCACACGTC	56	screening
aufA.124.for	AATTGTATAAGCGGCGGTGGA	60	screening
aufA.636.rev	TGCTGATGTCTACTGCGGTAA	60	screening
sat.498.for	CCCGGCAGAGACCAACCCTAC	60	screening
sat.1083.rev	AAGATAATACCACCGCTACCA	60	screening
aufG.187.for	CGCATCTGTATCTTTATTCGT	60	screening
aufG.882.rev	CATATTGTTATCCGGGTTCAC	60	screening
mchF.222.for	AATCAATGGAATAGCGGAGCA	60	screening
mchF.656.rev	GGCCAGCAGAAATAAGCGTCA	60	screening
pKD4.143.for	CTAAAGGAAGCGGAACACGTAGAAA	60	screening
pKD4.1233.rev	CCGCTCAGAAGAACTCGTCAAGAAG	60	screening

pKD3.153.for	TTCATTAAGCATCTGCCGACA	60	screening
pKD3.977.rev	GCGCCTACCTGTGACGGAAGA	60	screening
pACYC184.1361.for	CAAGAGATTACGCGCAGACC	56	screening
pACYC184.1669.rev	GACGATATCCCGCAAGAGGC	58	screening
F1.iroN-SstI.for	ATCAgagctcAGAATTAACAAAATAATATGGTC	55	protein expression
R1.iroN-PstI.rev	AAActgcagGAATGATGCGGTAACTCCGGCATA	55	protein expression
F2.iroN-SstI.for	ATCAgagctcGTGTCGGTTATTACCAGCGAGGA	60	protein expression
F1.fyuA-SstI.for	ATCAgagctcAAAATGACACGGCTTTATCCTCT	60	protein expression
R1.fyuA-PstI.rev	AAActgcagGAAGAAATCAATTCGCGTATTGAT	60	protein expression
F2.fyuA-SstI.for	ATCAgagctcTCTACTGTTGTCAGCGCGCCGGA	60	protein expression
iroN.1117.sstI.for	ATCAgagctcATGGTTGATCAAACGCTGAC	55	protein expression
iroN.1161.pstI.rev	AAActgcagATCGCGGTTCCACTCTGCAC	55	protein expression
P1_iroN-3.for	ATACCCGCGGTGACACCAACTGGGTGCCACC GGAACAGGTTGAGCGTATTGAAGTGATgtgtagg ctggagctgcttc	60	mutagenesis
P2_iroN.rev	TCCCCACCAGTGAATAAGCGCCCAGCTCTTTA CCTGACAGACCGCCAGTATCTTCcatatgaatatcctc ctta	60	mutagenesis
P1_fyuA.for	GGCGACATGATTAACCCCGCGACGGGAAGCG ATGACTTAGgtgtaggctggagctgcttc	60	mutagenesis
P2_fyuA.rev	CGATATCAAAACGATCGGTTAAATGCCAGGT CAGGTCACTcatatgaatatcctcctta	60	mutagenesis
YbtP.8825.NotI.for	CAGTCgcggccgcCAGCCAGAACAAGATAACGA	56	screening
Irp2.10802.NotI.rev	ATTTTgcggccgcTACGAAACCAGTGTAACCAT	54	screening
iroE.XbaI.rev	GCtctagaGAAAGATAAAGGCGTCAATGC	56	complementation
ORF14.HindIII.for	CCCaagcttTGGTCAGGTTGCGGAGGCTAT	55	complementation
FyuA.HPI.29136. HindIII.for	CCCaagcttCGCCACCTTTGACTGTACCCT	60	complementation
HPI32054.XbaI.rev	GCtctagaGTCCTAATCGCAATTTCATT	58	complementation
FyuA.HPI29136.rev	AGGGTACAGTCAAAGGTGGCG	60	complementation
HPI-fyuA.2947.for	GTCACTATAACGGAAGCAGTTG		complementation
TaqMan			
mdh_F	GCAGGCGTAGCGCGTAA	58	TaqMan
mdh_R	CACGATGCCGGCGTTAAC	60	TaqMan
mdh_S	CCGGGTATGGATCGTTCCGA	69	TaqMan
fyuA_1055.F	ACACCCGCGAGAAGTTAAATTC	60	TaqMan
fyuA_1131.R	AGCGGTGGTATAGCCGGTACT	63	TaqMan
fyuA_1079.S	CCTACGACATGCCGACAATGCCTTATTTAA	69	TaqMan
iroN_536.F	GCTGACCGTTGGTGCAGAGT	63	TaqMan

iroN_536.R	CATTCACCGTCAGGCTGGTA	64	TaqMan
iroN_536.S	CCGCGATAAGCTCGATGATCCTTCC	69	TaqMan
iutA.190.F	AGCCGCAGCAATCGAACT	56	TaqMan
iutA.247.R	CATTTTCGATAACCCAGGTGGTT	61	TaqMan
iutA.209.S	TAGCGGAGATGGCG	47	TaqMan
chuA.733.F	AGTCATCCCCGCACGAGAT	59	TaqMan
chuA.792.R	AGCAGCGACGGCTACAAAGA	60	TaqMan
chuA.756.S	ATTTGTCGGCATCAAC	46	TaqMan
Taq-usp-80.for	TTGGTAATCGCTGGCAGGTT	58	TaqMan
Taq-usp-143.rev	ATGGCGTTGGTGAGTGCAA	57	TaqMan
Taq-usp-103.S	ATTTGTCGGCATCAAC	54	TaqMan
hlyA_536.F	GCAGATGAACTGGGAATTGAAGT	61	TaqMan
hlyA_536.R	TTCTCTGCTGTGCCGAATACC	61	TaqMan
hlyA_536.S	CAGTATGATGAAAAGAATGGCACGGCGATTA	70	TaqMan
tolC.855.F	ACACCTCTTATAGCGGTTCGAAA	61	TaqMan
tolC.922.R	GCCCATATTGCTGTCGTCATAC	62	TaqMan
tolC.879.S	CCCGTGGTGCCGCTGGTACC	69	TaqMan
ompA.561.F	CCAGACGGGTAGCGATTTCA	58	TaqMan
ompA.628.R	CGGCGTTTCTCCGGTCTT	60	TaqMan
ompA.585.S	TGATCGCATACTCAACACCGCCAGC	69	TaqMan
ompT.for	TAGATATAGGAACCACCTCTGGCTGTA	67	TaqMan
ompT.rev	CAATTACCGCCTGGGACTCA	60	TaqMan
ompT.S	AGCTATAACGGCTTTCCTGATATCCGGCC	72	TaqMan
waaV_245.F	GTATGAATTTCTTCTTTCCGGTTACC	63	TaqMan
waaV_322.R	TGAGTCATTTGCGCACGAA	55	TaqMan
waaV_272.S	TTGTATAACTGCATCACAGAAGAGTAGATTC	67	TaqMan
waaL_773.F	GTTGTAGCGTGGGAAGCTGATA	62	TaqMan
waaL_846.R	TTTGCGCATGCCATGTG	52	TaqMan
waaL_796.S	TGCAACACGGTCGACGTTTAAATAGAAATGT	68	TaqMan
waaA_529.F	CGCTTGCTGCGTCGTATTAC	60	TaqMan
waaA_592.R	CCACAAAACGTGCACCATCT	58	TaqMan
waaA_550.S	CTGATTGCCGCCCAAAATGAAG	62	TaqMan
fimH.F	GGCGATTAAAGCTGGCTCATT	59	TaqMan
fimH.R	CTGGAAATCATCGCTGTTATAGTTGT	63	TaqMan
fimH.S	TTGCCGTGCTTATTTTGCGACAGACC	68	TaqMan

fliC_536.F	CAGGCGATTGCTAACCGTTT	58	TaqMan
fliC_536.R	ATACCATCGTTGGCGTTACGT	59	TaqMan
fliC_536.S	TTCTAACATTAAAGGCCTGACTCAGGC	67	TaqMan
fliD_536.F	GCGTAAGCGCAAGCATCATT	58	TaqMan
fliD_536.R	GCCGGTGTCATTTGATGTGA	58	TaqMan
fliD_536.S	ACGTGGGTAACGGTGAATATCGTCT	66	TaqMan
flgK_536.F	GGGAATAAAACCGCGACGTT	58	TaqMan
flgK_536.R	GGAAAGCTGCGTCACCACAT	60	TaqMan
flgK_536.S	AAAACCAGTAGCGCCACGCAAGGT	67	TaqMan

2.6. Chemicals and Enzymes

All chemicals and enzymes used in this study were purchased from the following companies: New England Biolabs (Frankfurt am Main), Invitrogen (Karlsruhe), MBI Fermentas (St. Leon-Roth), Roche Diagnostics (Mannheim), Gibco BRL (Eggenstein), Dianova (Hamburg), Difco (Augsburg), Merck Biosciences(Darmstadt), Oxoid (Wesel), GE Healthcare/Amersham Biosciences(Freiburg), Biomol (Hamburg), BioRad (Munich), (Darmstadt), Roth (Karlsruhe), Serva and Sigma-Aldrich (Taufkirchen).

The following commercial kits were used:
- QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany)
- QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)
- QIAquick Gel extraction kit (Qiagen, Hilden, Germany)
- DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)
- UltraClean Gel Spin DNA Purification Kit (MoBio)
- Wizard® SV Gel and PCR Clean-up System (Promega, Mannheim, Germany)
- Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany)
- The Expressionist Vector System (Qiagen, Hilden, Germany)
- SuperSignal® West Pico Chemiluminescent Substrate (Perbio Science, Bonn, Germany)
- mouse IL-1beta ELISA (Promokine, Heidelberg, Germany)
- mouse IL-18 ELISA Kit (MBL, Naka-ku Nagoya, Japan)
- mouse KC (IL-8) ELISA Kit (R&D Systems, Minneapolis, MN)
- mouse inflammation Kit (BD, Heidelberg, Germany)
- Apo-One Homogeeous Caspase-3/7 Assay (Promega, Mannheim, Germany)

2.7. Antibodies

The following antibodies were used for immunoblotting and immunofluorescence staining (Table 5).

Table 5. Antibodies used in this study.

Specificity	Dilution	Description	Source
Primary antibodies			
anti-*E. coli*	1:1000	polyclonal antiserum against *E. coli* Nissle 1917 (O6:K5:H1) from rabbit	S. Schubert
anti-IroN	1:100	polyclonal anti-IroN antiserum from rabbit	this study
anti-FyuA	1:100	polyclonal anti-FyuA antiserum from rabbit	this study
anti-HlyA	1:1000	polyclonal anti-α-hemolysin from rabbit	Balsalobre et al.,1996
anti-TolC	1:5000	polyclonal anti-TolC antiserum from rabbit	Thanabalu et al.,1998
anti-OmpA	1:10000	polyclonal anti-OmpA antiserum from rabbit	Henning et al., 1979
anti-Crp	1:6000	polyclonal anti-Crp antiserum from rabbit	Johansson et al., 2000
anti-lamp-1	1:100	polyclonal IgG developed in goat	Santa Cruz
anti-golgin 97	1:100	monoclonal IgG_1 developed in mouse	Santa Cruz
Secondary antibodies			
anti-rabbit	1:20000	polyclonal anti-rabbit IgG horseradish peroxidase-conjugate antibody	Amersham
anti-mouse	1:10000	polyclonal anti-mouse IgG horseradish peroxidase-conjugate antibody	Dako
anti-rabbit	1:10000	polyclonal anti-rabbit IgG developed in goat; peroxidase conjugate	Sigma
anti-rabbit	1:40	polyclonal anti-rabbit antiserum developed in swine; FITC conjugate	Dako
anti-rabbit	1:100	polyclonal anti-rabbit IgG developed in donkey; TR conjugate	Santa Cruz
anti-mouse	1:100	polyclonal anti-rabbit IgG developed in donkey; TR conjugate	Santa Cruz
anti-goat	1:100	polyclonal anti-rabbit IgG developed in donkey; TR conjugate	Santa Cruz
Fluorescence labelled molecules			
DAPI	1:1000 1µg/ml	4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain binding strongly to DNA	Sigma
phalloidin-rhodamine	1:300	phallotoxin is produced by *Amanita phalloides* labeling F-actin	Molecular Probes, Invitrogen
phalloidin-FITC	1:200	phallotoxin is produced by *Amanita phalloides* labeling F-actin	Molecular Probes, Invitrogen

2.8. DNA and Protein Markers

To determine the size of DNA fragments in agarose gels the Generuler™ 1 kb (A) and 100 bp (B) DNA ladder were used. To determine the molecular weight of protein fractions separated by polyacrylamide gel electrophoresis, the PageRuler™ Prestained Protein Ladder was used. All markers were purchased from Fermentas (Figure 7).

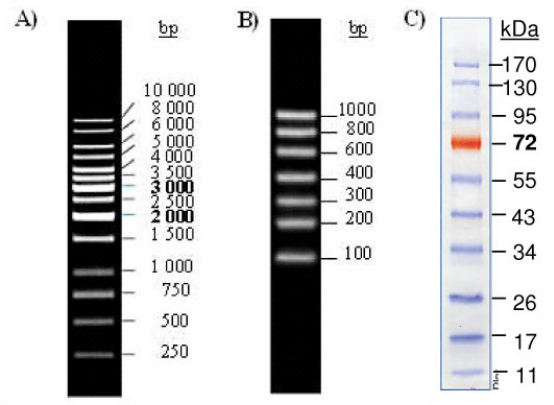

Figure 7. DNA and protein ladders. (A) Generuler™ 1 kb and (B) 100 bp, (C) PageRuler™ Prestained Protein Ladder were purchased from Fermentas.

2.9. Inhibitors

Inhibitors were added to cells 30 min prior to infection in the following concentration (Table 6) if not stated otherwise.

Table 6. Inhibitors used in this study.

Inhibitor	Stock	Solvent	Working concentration	Inhibited pathway
wortmannin	0.1 mM	DMSO	200 nM	phosphoinositid-3-kinase (PI3K)
cytochalasin D	1 mg/ml (2 mM)	DMSO	2 µM	actin polymerisation
nocodazole	2mg/ml (6.6mM)	DMSO	10 µM	tubulin polymerisation
filipin	1 mg/ml	DMSO	1 µg/ml	cholesterol-rich lipid raft (caveolae; endocytosis)
chlorpromazine	1 mg/ml	DMSO	1 µg/ml	clathrin-coated pits (endocytosis)
amiloride	10 mM	H_2O	10 µM	macropinocytosis

2.10. Media, agar plates and antibiotics

All media were autoclaved for 20 min at 120 °C, if not stated otherwise. Supplements for media and plates were sterile filtered through a 0.22 µm pore-filter and added after cooling down the media to <50 °C.

2.10.1. Media

LB medium (Luria-Bertani) (Sambrook *et al.*, 1989)
10 g tryptone from casein
5 g yeast extract
5 g NaCl
ad 1 l dH_2O

SOC medium (Sambrook *et al.,* 1989)
10 g tryptone (Oxoid, L42)
0.5 g yeast extract
0.2 g $MgCl_2$ x 6 H_2O
0.25 g $MgSO_4$ x 7 H_2O
0.36 g glucose
ad 1 l dH_2O, pH 7.0

NB medium
8 g nutrient broth (Difco Laboratories)
5 g NaCl
ad 1 l dH_2O

NBD medium
For iron depletion, NB medium was supplemented with 2,2-dipyridyl (Sigma) at a final concentration of 200µM.

2.10.2. Agar plates

LB, NB and NBD agar plates

Medium + 1.5 % (w/v) agar (Difco Laboratories)

Blood agar plates (BD Bioscience)

LB plates containing 5 % (v/v) washed sheep erythrocytes

Mueller-Hinton agar plates (BD Bioscience)

2.10.3. Antibiotics

When appropriate, media and plates were supplemented with the antibiotics listed in Table 7 in the indicated concentrations. Stock solutions were sterile filtered and stored at –20 °C until usage.

Table 7. Antibiotic substances used in this study.

Antibiotic	Stock	Solvent	Working concentration
Chloramphenicol (Cm)	50 mg ml^{-1}	MeOH	30 µg ml^{-1}
Ampicillin (Ap)	100 mg ml^{-1}	dH_2O	100 µg ml^{-1}
Kanamycin (Kn)	50 mg ml^{-1}	dH_2O	50 µg ml^{-1}
Spectinomycin (Spec)	30 mg ml^{-1}	dH_2O	30 µg ml^{-1}
Trimethoprim (Tp)	5 mg ml^{-1}	70% EtOH	75 µg ml^{-1}
Tetracyclin (Tet)	5 mg ml^{-1}	70% EtOH	12 µg ml^{-1}
Streptomycin (Str)	30 mg ml^{-1}	dH_2O	30 µg ml^{-1}
Rifampicin (Rif)	20 mg ml^{-1}	MeOH	150 µg ml^{-1}
Nalidixin (Nal)	6 mg ml^{-1}	0,5 M NaOH	60 µg ml^{-1}
Tobramycin	40 mg ml^{-1}	dH_2O	40 µg ml^{-1}
Anhydrotetracycline (AHT)	2 mg ml^{-1}	EtOH	0.2 µg ml^{-1}
isopropyl-β-D-thiogalactopyranosid (IPTG)	200 mM	dH_2O	1 mM
Arabinose (Ara)	20%	dH_2O	0.002-0.2 %

2.11. Technical Equipment

Autoclaves	Integra Bioscience, H+P Varoclav
Balances	Sartorius Type 1801
	Kern 440-33N
Clean bench	HERA safe K12, Thermo Electron Corporation
Bunsen burner	FireBoy plus, Integra
DNA-/protein software	Lasergene 5.08, DNA-Star Inc., Madison, Wi, USA
Gel documentation	Molecular Imager ChemiDoc XRS, BioRad
	Quantity One software, BioRad
Electrophoresis systems	Mini-PROTEAN 3 Electrophoresis System, Bio-Rad
Electroporator	Gene Pulser II, BioRad
	Pulse Controller II, BioRad
Incubators	Heraeus T12, Heraeus
	Heraeus T20, Heraeus
	Cytoperm2, Heraeus
	BBD 6220, Heraeus
Hybridization oven	PersonalHyb, Stratagene
Heater	IKA-RCT basic, IKA-Werke
Heat block	Unitek HB-130, Miyachi Unitek Corporation
Centrifuges	Eppendorf 5810 R
	Eppendorf 5417 C
	Eppendorf 5417 R
	Sorvall Super T21 (rotor: ST-H750), Thermo Electron Corporation
Magnetic stirrer	IKAMAG REO, IKA-Werke
Micropipettes	Eppendorf Reference
Microscope	Axiovert 25, Zeis
	Leica Leitz DM RBE with camera system (SPOT)
Microscope photo software	Meta-Morph 6.0; Spot Basic
Microwave	M1712N Samsung
Power supplies	Power Pac 200 and 300, BioRad
PCR-Thermocycler	GeneAmp PCR System 9700, Applied Biosciences
	Mastercycler gradient Eppendorf
TaqMan	ABI prism 7500 fast technology (Applied Biosystems)
Photometer	Ultrospec 3100, Amersham Biosciences
pH-meter	pH3000, Wissenschaftlich-Technische-Werkstätten
Documentation	BioRad GelDoc2000 + MultiAnalyst Software V1.1
Photometer	Pharmacia Biotech Ultrospec 3000
Shakers	Thermomixer comfort, Eppendorf
	Certomat H, – R and BS-1
Sonicator	Branson Sonifier 250, Sonicator 102C; Tip UW70
Speedvac	Savant SC110
Videoprinter	UP-895 CE, Sony Corp.
Vortexer	Vortex-Genie 2^{TM} Scientific Industries
UV-Transiluminator	TFX-20.M, Vilber Lourmat

3. METHODS

If not stated otherwise, all methods followed the instructions described in the CSH Laboratory Manual (Sambrook 1989). Centrifugations with no other indications were carried out in a table top centrifuge at 13,000 rpm and 4°C.

3.1. Bacterial strains

Bacteria were grown shaking at 37°C with aeration in Luria- Bertani (LB) medium if not stated otherwise. For plasmid maintenance, antibiotics were added to the culture medium as required.

3.2. Cell culture

3.2.1. Cultivation of cells

HEK293 and GD25 cells were grown in DMEM, all other cell lines were cultured in RPMI medium. If not stated otherwise, the medium was supplemented with 10 % heat inactivated fetal calf serum (FCS), 10,000 IE penicillin and 10 mg/ml streptomycin. The supplemented medium was stored up to four weeks at 4°C. For cultivation, cells were grown in 80 cm^2 tissue culture flasks containing 20 ml medium in a humidified atmosphere with 5 % CO_2 at 37°C. All used cell lines grow adherent on the ground of the flasks. If required, the medium was replaced with fresh medium to remove dead cells and catabolites. For passaging, the medium was removed and the cells were incubated with 7 ml trypsin at 37°C for 5 minutes. After detachment from the flask's ground, cells were harvested in 10 ml medium to stop the proteolytic activity of trypsin. After centrifugation (1,200 rpm, 5 min, 20°C), supernatant was discarded and cells were suspended and seeded with a density of 10,000 cells/cm^2 in a new flasks. J774 cells were scraped from the bottom of the flask instead of trypsinization and seeded into fresh medium every three days.

3.2.2. Freezing and thawing of cells

For long-term storage cell lines should be stored in liquid N_2 using this method. Cultures should be only frozen if they are healthy and in the mid-log phase of growth (e.g. 2-5 x 10^5 cells/ml). Confluent cells were trypsinized or scraped as described before. After centrifugation, 1-5 x 10^6 cells were suspended in 1 ml freezing solution and filled as 500 µl aliquots into cryovials. The presence of DMSO in the freezing solution prevents the crystal growth within cells. Freezing of cells has to be a slow process, so that the freezing tubes were packed in a cryocontainer (Nalgene) which was filled with isopropanol (The isopropanol must be changed after five uses). The container with the vials was placed for 24 hrs into a -80°C freezer before it was transferred to the liquid nitrogen tank for long-term storage at -172°C.
For thawing, cells were removed from liquid nitrogen and immediately transferred to a 37°C water bath with slightly agitation. When the cells are completely thawed, they were transferred to 10 ml pre-warmed complete medium and spun down (1,200 rpm, 2 min, 20°C). After decanting the medium, the cells were suspended in 10 ml complete media and transferred to a 25 cm^2 flask for propagation at 37°C, 5% CO_2 and 95% humidity.

3.2.3. *In vitro* Gentamicin protection assay

Invasion of host cells by bacterial strains was studied by means of the gentamicin protection assay (Mulvey et al., 2001). The premise of this assay is, that the bacteria which are able to invade host cells can be identified by adding gentamicin, which kills all extracellular, but not the intracellular bacteria since gentamicin is unable to enter host cells. In brief, HCV29 bladder epithelial cells were seeded into 24 well plates and grown to confluency. In three sets of duplicate wells, cells were infected with a multiplicity of infection (MOI) of 50-100 bacteria per host cell. To advance bacterial contact with host cells, plates were centrifuged at 200 rpm for 2 min. After 1h incubation time at 37°C, 5% CO_2 and 95% humidity, three fractions of bacteria were separated: First, the total number of added bacteria was determined after direct lysis of the host cells with 0.1 % Triton X-100. In the second set of duplicate wells, the cells were washed three times with PBS prior to lysis representing the adherent and the invaded bacteria. In the third set, medium was replaced with fresh medium containing 100 µg/ml gentamicin to kill extracellular bacteria. After additional 1 hour incubation, cells were washed and lysed, resulting in the number of invaded bacteria. All these lysates were diluted in ten-fold series and plated on Müller Hinton II agar plates to determine the total number of bacteria, the number of adherent and invaded and the number of surviving intracellular

bacteria. For each panel, experiments were repeated at least three times obtaining comparable results. The amounts of invading bacteria were expressed as the mean and standard deviation of six independent samples. The Student's *t* test for unpaired data was used to test significance between the means, with P values of < 0.05 considered statistically significant.

3.2.4. Assay for cell detachment

Cell detachment was assayed on semiconfluent monolayer of HCV29 cells cultured inRPMI medium supplemented with 10% fetal calf serum in 24-well tissue culture plates. HCV29 cells monolayers were washed two times with PBS-CM (phosphate buffered saline with 0.01% $CaCl_2$ and 0.01% $MgCl_2$), and 0.5 ml of PBS-CM was added to each well. In total, 10 µl samples (bacterial culture, culture supernatant, vesicles suspension or buffer) was added and the plate was incubated at 37°C for 90 min under 5% CO_2. Cell monolayers were washed two times, fixed with 70% methanol for 10 min, stained with 0.13% crystal violet for 10 min, and then briefly destained in water. Cell detachment was quantified by eluting crystal violet with a solution of 50% ethanol and 1% SDS, and measuring the absorbance of the eluate at 590 nm.

3.2.5. Cytotoxicity assay with HCV29 cells

The cytolytic activity of bacterial strains, supernatant and vesicle samples was measured as the release of cytoplasmic lactate dehydrogenase (LDH) from infected HCV29 cells after 6 h incubation using the CytoTox96 assay (Promega) according to the manufacturers' instructions.

3.2.6. Immunofluorescence staining

For Immunofluorescence staining cells were grown on glass slides in 24 well plates until semi-confluency. Cells were infected with bacteria, latex beads or outer membrane vesicles as indicated. At designated time points the cells were washed with 1x PBS and fixed with 2 % paraformaldehyde for 10 min. Fixed cells were washed five times with 1x PBS and treated with 0.1 M glycine. Then, cells were washed, permeabilized with 0.1 % Triton for 3 min, washed and subsequently blocked with 5% BSA-PBS for 15 min. Incubation with the first antibody was carried out in a steam chamber for 2 h at 37°C or overnight at 4°C. After

washing, cells were blocked with 5% donkey or pig serum for 30 min at room temperature and further incubated with a secondary antibody for 30 min at 37°C. If indicated, cells were then stained with DAPI (3 min) or F-actin was labelled with phalloidin (30 min). After ten final washing steps, cells were embedded in Mowiol Mounting Medium and stored at 4°C in the dark until analysed.

Mowiol Mounting Medium (Heimer and Taylor, 1974; Osborn and Weber, 1982)
2.4 g Mowiol 4-88 (Calbiochem)
6 g glycerol
6 ml dH_2O
12 ml 0.2 M TrisHCl pH 8.5

Mowiol was dissolved in glycerol and dH_2O while stirring for 2 h at room temperature. TrisHCl was added and the solution was incubated at 53°C until Mowiol has dissolved. After centrifugation (4,300 rpm, 20min, 20°C), the cleared supernatant was frozen in 1ml aliquots at -20°C for up to 12 month. Once defrosted the Mowiol medium is stable for at least one month. Just before use, 2.5% (w/v) 1,4-diazabicyclo-(2.2.2)octane (DABCO) as antifade reagent was added to prevent photo bleaching.

3.2.7. Inside-outside staining

The inside-outside staining was performed in two steps: First, the outside staining was carried out. For this, cells were fixed with paraformaldehyde and glycine as described above, blocked and directly incubated with the first antibody for 1h (without permeabilization). Next, cells were washed and further incubated with a secondary antibody (e.g. labelled with Texas Red) for 30 min. Prior to the inside staining, cells were permeabilized and incubated again with the first antibody for 1h. After washing, cells were stained with a different labelled (e.g. FITC) secondary antibody for 30 min and the staining was finished as described before.

3.3. Manipulation of DNA

3.3.1. Isolation of plasmids

Plasmids were isolated from bacterial cells grown overnight with the appropriate antibiotics. For small scale plasmid isolation the QIAGEN Plasmid Mini Kit and for large scale plasmid purification the Nucleobond AX-20 columns were used. Protocols were carried out as indicated by the manufacturer and the eluted DNA was analysed on agarose gels.

3.3.2. Isolation of chromosomal DNA

Bacteria from 50 ml of an over night culture were harvested by centrifugation (4,300rpm, 20 min, 4°C), suspended in 10 ml 1x TE buffer pH 8.0 supplemented with 0.1 mg/ml RNaseA and frozen for 1h at -20°C. Then, 30 µl lysozyme (5 mg/ml) were added and samples were incubated for 1 h at 37°C. Afterwards, 2 ml STEP solution containing 1 mg/ml proteinase K were added and the solution was further incubated for up to 2 h at 50 °C until it became clear. Next, the genomic DNA was precipitated by addition of 1 vol dH_2O and 1.5 vol phenol (10 min, room temperature) and then collected by centrifugation (4,300 rpm, 30 min, 4°C). Next, 100 ml ethanol were mixed with 10 ml 3M Na Ac and this mixture was added to the upper phase resulting from centrifugation. The genomic DNA was collected by stirring with a sterile gals pipette, washed with 70% ethanol, briefly dried and finally dissolved in 2 ml TE buffer pH 8.0 and stored at 4°C.

STEP solution
50 mM Tris, pH 8.0
0.5 % SDS
0.4 M EDTA, pH 8.0
1 mg/ml proteinase K

3.3.3. Precipitation of DNA

DNA was either precipitated with ethanol or with isopropanol. For ethanol precipitation, 0.1 vol 3 M Na-acetate (pH 4.8) and 2.5 vol ice-cold 100 % (v/v) ethanol were added to the samples. For isopropanol precipitation, 0.7 vol isopropanol was added and samples were

incubated for at least 1 h at –80°C. After centrifugation (13,000 rpm, 4°C, 30 min), the DNA pellet was washed with 70 % (v/v) ethanol, vacuum-dried for 10 min and suspended in an appropriate vol of dH_2O.

3.3.4. Determination of nucleic acid concentrations

Nucleic acid concentrations were measured at 260 nm in quartz cuvettes with a diameter of 1 cm. Absorption of 1.0 at 260 nm corresponds to 50 µg/ml double-stranded DNA or 40 µg/ml RNA. The purity of the preparations was determined by measuring the absorption of the sample at 280 nm. DNA and RNA were considered sufficiently pure when the ratio A260/A280 was higher than 1.8 or 2.0, respectively.

3.3.5. Polymerase chain reaction (PCR)

This method, first described by Saiki et al. (1988) allows the exponential amplification of DNA regions *in vitro* by using a heatstable DNA polymerase from *Thermophilus aquaticus* (*Taq*). This way, even small amounts of template DNA can be amplified to high copy numbers and easily visualized during screening assays. Another application of PCR is the site-directed mutagenesis by using oligonucleotides with adapted sequences, e.g. restriction sites.

Standard PCR

For routine PCR-amplification, the *Taq* DNA polymerase kit of Q-BIOgene was used and the reaction was usually performed in a final volume of 25µl.

Mix for one sample:
2.5 µl 10 × reaction buffer
1 µl 20mM dNTP mix
4 µl 25 mM $MgCl_2$
0.5 µl 20 pM primer solution 1
0.5 µl 20 pM primer solution 2
1 µl 100 ng/µl template DNA or boiled cells
0.125 µl *Taq* DNA polymerase
ad 25 µl dH_2O

The thermal profile was designed according to the annealing temperature of the individual primers and the elongation time depended on the length of the expected amplification product (1 min for about 1000 nt):

Initial denaturation	10 min, 95 °C	
1. Denaturation	45 sec, 95 °C	35 cycles
2. Annealing	45 sec (see Table 4)	
3. Elongation	30 sec x min, 72 °C	
Final elongation	10 min, 72 °C	

PCR with proof-reading polymerases

For site-directed mutagenesis using PCR products and for gene amplification prior to cloning into expression plasmids, the LA Taq polymerase (Takara) or the Amplitaq Gold (Applied Biosystems), both with 3'→5' proofreading activity were used in order to prevent misincorporations during extension. The composition of a typical PCR mix for the λRed mutagenesis (see section 3.3.11) is given below:

Mix for 1 sample using the LA Taq polymerase (Takara):
5 µl 10 × buffer
10 µl dNTP mix (2,5mM each)
1 µl 20 pM forward primer
1 µl 20 pM reverse primer
1 µl 100 ng/µl template plasmid DNA
1 µl LA Taq
ad 50 µl dH_2O

After amplification, PCR products were purified using a commercial PCR purification kit (QIAGEN) and analysed by performing horizontal gel electrophoresis (section 3.3.6).

3.3.6. Horizontal Gel Electrophoresis

For routine analytical and preparative separation of DNA fragments, horizontal gel electrophoresis was performed using agarose gels under non-denaturing conditions. Depending on the size of the DNA fragments to be separated, the agarose concentration

varied between 1 and 2 % (w/v). 5 µl ethidium bromide were added to the gel and the electrophoresis was carried out in 1 × TAE as running buffer. In order to have a visible running front and to prevent diffusion of the DNA, 0.2 vol loading dye was added to the samples before loading. Electrophoresis was carried out at a voltage in the range between 100-140 V. The gels were photographed under a UV-transilluminator.

50 x TAE buffer
2 M Tris
6 % (v/v) acetic acid (99.7 %)
50 mM EDTA (pH 8.0)
ad 1 l dH_2O

5 x Loading Dye
50 % glycerol
0.2 M EDTA
0.05 % bromophenol blue

3.3.7. Isolation of DNA fragments from agarose gels

Agarose pieces containing the DNA fragment of interest were cut out of the gel and purified using the UltraClean Gel Spin DNA Purification Kit (MoBio) or the QIAquick Gel Extraction Kit (QIAGEN).

3.3.8. Enzymatic digestion of DNA with restriction endonucleases

DNA was dissolved in dH_2O and mixed with 0.1 vol 10 × reaction buffer and 1 U of restriction enzyme per µg DNA, so that the final volume of the sample was 50 µl, each for plasmid and genomic DNA. The mixture was incubated at 37°C, depending of the specific requirements of the enzyme indicated on the product sheets. Whereas plasmid DNA was digested for three hours, digestion of genomic DNA was carried out over night. When appropriate, inactivation of the restriction enzyme was carried out by heating the samples for 20 min at 65 °C. All digests were analysed and purified by means of horizontal gel electrophoresis.

3.3.9. Ligation of DNA fragments

Linearized vector and insert DNA after restriction digest can be ligated either due to the presence of sticky ends or by blunt-end ligation. The modifying enzyme for ligation process was a T4-DNA ligase (Fermentas). Best efficiencies were obtained using an insert/vector ratio of 3/1. Reactions were performed over night at 16°C in a final volume of 10 µl containing 1 µl 10 x ligation buffer and 1 µl T4 ligase (5U).

3.3.10. Transformation of bacterial cells

Preparation of electrocompetent bacterial cells

200 ml LB medium were inoculated with 1 ml of an over night culture of the strain of interest. Cells were grown to an OD_{600} of 0.6-0.8 and cooled on ice. The culture was centrifuged (4,300 rpm, 30 min, 4°C) and the pellet was suspended in 100 ml ice-cold dH_2O. This washing step was repeated three times. After, the pellet was suspended in 50 ml ice-cold 10 % (v/v) glycerol, centrifuged and finally suspended in an appropriate volume of 10 % glycerol. Competent cells were stored in aliquots at -80°C.

Transformation by electroporation

DNA solution with a high salt content were dialyzed for 20 min on a nitrocellulose membrane against sterile dH_2O (filter type 0.025 µm, Millipore) prior to transformation. Meanwhile, 90 µl of competent cells were thawed on ice, ~ 0.5 µg of plasmid DNA were added and the cells were incubated on ice for 30 min. Then, the mixture was applied into an electroporation cuvette (Molecular BioProducts) with a distance between the electrodes of 1 mm and electroporated using a Gene pulser II transfection apparatus (Bio-Rad) at the following conditions: 2.5 kV, 25 µF, and 200 Ω. Immediately after electroporation, 1 ml of prewarmed SOC medium was added to the cuvettes, the mixture was transferred into a new tube and incubated at 37°C for 1 h, allowing the expression of the selection marker before the cells were plated on selective agar plates.

3.3.11. Gene inactivation by λ Red recombinase-mediated mutagenesis

Site-directed mutagenesis of *E. coli* strains was performed using linear DNA fragments for recombination as described by Wanner and Datsenko (2000). This method relies on the

replacement of a chromosomal sequence with an antibiotic marker that is generated by PCR using primers with homology extensions to the flanking regions of the target sequence. Recombination is mediated by the λ Red recombinase derived from the λ phage. In brief, a linear DNA fragment containing an antibiotic marker cassette flanked by FRT sites and 45-nt homologous extensions to the target genes (e.g. up- and downstream of *iroN* and *fyuA*) was amplified by PCR as described in section 3.3.5 using the plasmids pKD3 (harbouring a chloramphenicol resistance cassette) or pKD4 (harbouring a kanamycin resistance cassette) as template. PCR products were purified and transformed in λRed recombinase expressing ExPEC wild-type strains. Transformants were selected from agar plates supplemented with the appropriate antibiotic (Cm or Kn, respectively).and were re-streaked onto ampicillin containing agar plates at 37°C to confirm loss of the temperature-sensitive helper plasmid pKD46. All mutants were verified by PCR and Western blotting.

3.3.12. Sequence analysis

Sequencing of unknown DNA fragments was carried out by MWG. Alignments of retrieved nucleotide sequences were performed using the Lasergene sequence analysis software (DNASTAR Inc., Madison, Wisconsin, USA). Open reading frames (ORFs) were selected by means of the Lasergene software or using the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). For standard sequence or protein comparisons and similarity searches, the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information Homepage (http://www.ncbi.nih.gov/BLAST/) was used. Putative promoter predictions of sequences, including their up- and downstream regions were performed using the program NEURONAL NETWORK PROMOTER PREDICTION (http://www.fruitfly.org/seq_tools/promoter.html).

3.4. Manipulation of RNA

For working with RNA, special care had to be taken in order to prevent contamination of the samples with exogenous RNases. Gloves were worn throughout the experiment and double autoclaved pipette tips and reaction tubes were used. For all buffers and solutions, dH_2O pre-treated with 0.1 % (v/v) diethylpyrocarbonate (DEPC) was used.

3.4.1. Isolation of total RNA

Total RNA was isolated from late log-phase cultures using TRIZOL reagent (Invitrogen) as recommended by the manufacturer, starting with 1 ml TRIZOL per 1×10^7 bacterial cells. Final RNA pellets were dissolved in 50 µl RNase-free water and RNA preparations were stored at –80°C. As a control for successful RNA isolation 2 µl of total RNA preparations were analysed on a 1% agarose gel.

3.4.2. DNase I treatment

Contaminating DNA was routinely removed from total RNA preparations by DNase I treatment (DNase I, Amplification Grade, Invitrogen). In brief, 10 µl RNA in a final volume of 50 µl were incubated with 5 µl 10 x DNase I reaction buffer and 5 µl DNase I for 15 min at 21°C. Inactivation of DNase I activity was performed by addition of 5 µl 25mM EDTA followed by incubation for 10 min at 65°C. As a control for successful removal of all DNA from the samples, 2 µl of the DNase treated RNA or 1 µl DNA as positive control were used as template in a PCR reaction with primers binding within the housekeeping gene malate dehydrogenase (*mdh*). The DNase digest was considered as complete if no product could be amplified from the RNA samples.

3.4.3. Reverse transcription (RT) for cDNA synthesis

After successful elimination of contaminating DNA, 1 µg RNA preparation was used for cDNA synthesis by means of the SuperScript II reverse transcriptase (Invitrogen) using random hexamers.

3.4.4. Quantitative RT-PCR (TaqMan)

cDNA samples derived from reverse transcription were diluted 1:5 with nuclease-free dH_2O and quantified on an ABI prism 7500 fast technology TaqMan (Applied Biosystems) according to manufacturers' instructions by using FAM/TAMRA labelled probes and 5 µl cDNA. Control reactions included one reaction without template and one control sample processed without reverse transcriptase for each primer combination. TaqMan PCR reactions were carried out in MicroAmp™ Fast 96-Well Reaction plates (Applied Biosystems) using the TaqMan® Universal PCR Master Mix (Applied Biosystems) to minimize pipetting errors in a final volume of 25 µl per well.

Mix for 10 wells:
125 µl TaqMan® Universal PCR Master Mix
4.5 µl primer F (50µM)
4.5 µl primer R (50 µM)
6.25 µl probe (10 µM)
59.75 µl H_2O

Thermal profile

UNG activation	2 min; 50 °C	
Activation of AmpliTaq Gold	10 min, 95 °C	
Denaturation	15 sec, 95 °C	45 x
Annealing	1 min, 60 °C	

Primers and probes for TaqMan analyses were selected with the Primer Express® Software (Version 2.0, Applied Biosystems) with the following options: product length ranged from 50-150-nt; annealing temperature range from 58-60°C for primers and 68-70°C for probes. Probes were labelled with FAM (5') and TAMRA (3'). The sequences of all oligonucleotides used are listed in Table 4. For comparative, quantitative analysis, transcript levels were normalized to the level of the endogenous reference gene *mdh* (malate dehydrogenase). Data were analysed by the $2^{-\Delta\Delta}CT$ method (Livak and Schmittgen, 2001) and presented as a change difference (*n*-fold) between strains.

3.5. Working with Protein

3.5.1. Expression of recombinant proteins

Bacterial cultures were grown in LB medium at 37°C with aeration to an OD_{600} of 0.6 prior to induction with anhydrotetracycline (AHT, 0.2 µg/ml final concentration), IPTG (1 mM final concentration) or 0.2 % arabinose, respectivly. Protein synthesis was carried out for 3 h.

3.5.2. Preparation of cell lysates

For the preparation of crude cell extracts, 1 ml bacterial culture were centrifuged (5 min, 6,500 rpm, 4°C) and the pellet was suspended in 150 µl 1 x Laemmli buffer and 50 µl 4 x loading dye. The samples were loaded onto the SDS-gel after heating for 10 min at 95°C in 1 × loading dye.

1 x Laemmli buffer
3.09 g Tris base
14.41 g glycine
1 g solid SDS
ad 1 l dH_2O

4 x SDS-loading dye
0.25 M TrisHCl (pH 6.8)
0.4 M ß-mercaptoethanol
8 % (w/v) SDS
40 % (v/v) glycerol
0.4 % bromophenol blue

3.5.3. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were separated and analysed in denaturing polyacrylamide gels as described by (Laemmli, 1970). This was done using the detergent sodium dodecylsulfate (SDS), which binds to proteins and leaves them unfolded and negatively charged. Therefore, the protein mixture can be separated in the meshwork of the polyacrylamide gel due to variable migration

speed depending on the size of the protein. Since most of the proteins of interest in this study were more than 70 kDa in size, in most cases a 10 % resolution polyacrylamide gel was used. The size of the gels was 10 × 7 × 0.75 cm, and electrophoresis was performed at room temperature at 200 V per gel in 1 x Laemmli buffer using the Mini-PROTEAN 3 Electrophoresis System (Bio-Rad). The acrylamide gel consists of two parts: a lower part mediating the separation of the proteins, and an upper part, which is used for concentration of the sample in a single running front after entering the gel. For the mixture which was sufficient for 4 mini gels see Table 8.

Table 8. Mixture for 4 mini gels.

	lower gel			**upper gel**
	8%	**10%**	**15%**	**5%**
30% acrylamide (37.5:1)	4 ml	5 ml	7.5 ml	1260 µl
1.5 M TrisHCl (pH 8.8)	4.4 ml	5.6 ml	5.6 ml	1880 µl 0.5 M TrisHCl (pH 6.8)
dH_2O	4.4 ml	4.4 ml	1.875 ml	4.4 ml
10% SDS	150 µl	150 µl	150 µl	75 µl
10% APS	112.5 µl	112.5 µl	112.5 µl	22.5 µl
TEMED	9.45 µl	9.45 µl	9.45 µl	18.5 µl

3.5.4. Coomassie Blue staining

To visualize proteins in polyacrylamide gels, gels were incubated for 15 min in Coomassie Blue staining solution. Protein bands were visualized after removing unbound Coomassie dye by incubating the gel in destaining solution I for 30 min, followed by incubation in destaining solution II or with dH_2O.

Coomassie Blue staining solution

0.125% Coomassie Brilliant Blue R-250

50% methanol

10% acetic acid

***Destaining solution I*:** 7 % (v/v) acetic acid; 40 % (v/v) methanol

***Destaining solution II*:** 7 % (v/v) acetic acid; 5 % (v/v) methanol

3.5.5. **Silver nitrate staining** (Blum et al., 1987)

Based on the methodology of Heukeshoven and Dernick (1985), this sensitive silver staining protocol which is 100-fold more sensitive than Coomassie Blue staining was performed for detection of proteins in the nanogram range. Typical sensitivity is 0.2-0.6 ng proteins/band. All solutions were prepared freshly (not older than 24 hrs) to give best results. 125 ml of solutions are needed per gel. All steps were performed with gentle shaking of the staining tray.

The samples were run in 10, 13.5 or 15 % SDS-gels as indicated. After electrophoresis, gels were fixed for 30 min in fixing solution. This solution was replaced by sensitizing solution, in which the gels were incubated for at least 30 min. Subsequently, the gels were washed three times for 15 min with dH_2O, and then incubated for 20 min in silver nitrate solution. The gels were rinsed two times for 1 min with dH_2O and then developed in developing solution for up to 30 min. As soon as the intensity of the appearing bands was satisfying, the reaction was stopped by incubation in stopping solution for 10 min. After three final washing steps, the gels can be stored in dH_2O up to one week.

Fixing solution

40% (v/v) ethanol

10% (v/v) glacial acetic acid

Sensitizing solution

30% (v/v) ethanol

5% (w/v) sodium thiosulfate

17 g sodium acetate

ad 250 ml dH_2O

Silver nitrate solution

0.625 g silver nitrate

100 µl 37 % Formaldehyde

ad 250 ml dH_2O

Developing solution

6.25 g sodium carbonate

50 µl 37 % Formaldehyde

17 g sodium acetate
ad 250 ml dH_2O

Stop solution
5% (v/v) acetic acid

3.5.6. Western blotting

Separated proteins from SDS-PAGE were transferred to a nitrocellulose membrane (PROTRAN® BA-85; pore size 0.45 µm; Schleicher&Schuell) as described by Towbin et al., (1979). The transfer was carried out in the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) as recommended by the manufacturer by applying an electric current of 90V (0.8 mA cm^{-2}) for 1 h. After transfer of the proteins, the membrane was blocked over night at 4°C (or 1 h at room temperature) in TBS-T solution (0.05 M TrisHCl, pH 7.5; 0.15 M NaCl; 0.1 % Tween 20) supplemented with 5 % fat-free dry milk. Subsequently, the membrane was incubated with a primary antibody for 1-2 h at room temperature. The concentration of the primary antibodies diluted in TBS-T buffer is listed in table Table 5. After washing the membrane with TBS-T three times for 5 min, a secondary peroxidase-conjugated antibody (1:10000 diluted in TBS-T) was added and incubated for 45 min at room temperature. After washing, the membrane was reacted with the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instruction and signals were detected using X-ray films.

3.5.7. Stripping and reprobing membranes

Membranes can be stripped and reprobed up to 3 times, if stored at 4°C in wet wrap. In brief, the membrane was submerged in stripping buffer (100 mM ß-mercaptoethanol, 2% SDS, 62.5 mM TrisHCl ph 6.7) for 30 min at 60°C, with occasional agitation. Then, the membrane was washed two times for 10 minutes in TBS-T buffer at room temperature using large volumes of wash buffer. After blocking the membrane for 1 hour in 5% non-fat dried milk in TBS-T at room temperature immunodetection was performed as usual.

3.5.8. Production and purification of polyclonal antibodies

Polyclonal antibodies production was performed together with K. Hildinger (AG Schubert, Max von Pettenkofer Institut). In brief, the proteins FyuA and IroN were recombinantly expressed and purified. Antibodies directed against FyuA and IroN were generated in cooperation with C. Giller (Max von Pettenkofer Institut) using New Zealand White Rabbits immunised with a total amount of 2-3 mg protein that was applied by 6 immunisations within 77 days. After final exsanguination of the rabbits, antisera were tested for specific recognition of FyuA and IroN. As antisera usually crossreact with several other proteins, anti-FyuA and anti-IroN antisera were purified to enhance specific binding of the antibodies and to prevent unspecific crossreaction with other proteins.

For purification of polyclonal antibodies directed against FyuA or IroN from rabbit antisera, 1µg of the respective protein was blotted onto a nitrocellulose membrane as described in the previous section (3.5.6). The membrane was stained in Panceau S to visualize the protein band, the localization of the protein was marked, the membrane destained and blocked in 1% BSA in PBS for 1h at room temperature, Then, the membrane was washed three times with dH_2O and cutted into small pieces. The slices were incubated overnight at 4°C with the respective rabbit antiserum diluted 1:10 in TBS-T buffer under rotation. During this step, antibodies that are specific for the protein were bound to the membrane whereas unspecific moelcules remain unbound and can be removed by washing the membrane three times with TBS-T. The antibodies which were specifically bound to the protein on the membrane slices were carefully eluted during vortexing (600 rpm, 30 sec) in 1 ml elution buffer (500 mM NaCl, 50 mM HCl, pH 2.3). This mixture was immediately neutralized by addition of 100 µl TrisHCl pH 9.0. A second eluate was generated by an additional washing step. Both samples, the eluate and the last wash were tested for specific antigen recognition by Western blotting.

3.5.9. Determination of protein concentrations

Protein concentrations were determined using BioRad protein assay solution (BioRad) following the manufacturer's instructions. 200 µl protein samples were mixed with 800 µl 1 x assay solution, and absorbance was measured at 590 nm and 450 nm. Protein concentrations were then calculated using the following formula (based on a BSA calibration curve):

$$\mu g \text{ protein} = (A_{590nm} / A_{450nm} - 0.4475) / 0.1132$$

3.5.10. Trichloroacetic acid (TCA) precipitation

TCA precipitation was used to concentrate proteins prior to SDS-PAGE (e.g. precipitation of proteins from culture supernatant). For this, culture supernatant was sterile filtered (0.45 µm-pore-size filter, Millipore) and precipitated by adding 1/3 volumes of ice cold 50% TCA (final concentration ~15% TCA). Then, the mixture was vortexed and incubated for 30 min up to over night on ice. After centrifugation (14,000 rpm, 30 min, 4°C), the supernatant was completely removed, the pellet was suspended with 100 % acetone (1/10 vol) and precipitated at least 1 h on ice. After centrifugation (10,000 rpm, 30 min, 4°C), the supernatant was discarded and the pellet suspended in an appropriate volume of 20 mM TrisHCl pH 8.0 or 1x Laemmli and stored at -20°C.

Small volumes of supernatant were precipitated as follows:

300 µl 50% TCA were carefully added to 1ml sterile filtered supernatant and precipitated 30 min on ice. After centrifugation (20 min, 14,000 rpm, 4°C), the supernatant was completely removed, the pellet suspended 30 µl 20 mM TrisHCl pH 8.0 and stored at -20°C.

3.5.11. N-terminal protein sequencing

Determination of the N-terminal amino acid sequence of proteins was achieved by Alphalyse A/S (Odense, Denmark). The peptide masses obtained were determined using the NCBI BLAST database.

3.6. Isolation of outer membrane proteins (OMPs) (modified from Hantke, 1981)

Bacteria from 100 ml culture were harvested by centrifugation (4,300 rpm, 30 min, 4°C) and the pellet was suspended in 500 µl 0.2 M TrisHCl pH 8.0. Next, 1 ml lysis buffer (0.1 M TrisHCl pH 8.0, 1M sucrose, 1 mM EDTA) and 200 µg lysozyme dissolved in 2 ml H_2O were added and the mixture was incubated for 20 min at room temperature. After, cells were disrupted by 4x 4 min sonication (10 s pulses, 30% amplitude) in between the mixture was cooled on ice. Afterwards, 5 ml extraction buffer (2% Triton X-100, 50 mM TrisHCl pH 8.0, 10 mM $MgCl_2$) and 300 µl freshly prepared DNaseI (1 mg/ml) were added and incubated for 15 min at room temperature. After centrifugation (6,000 rpm, 20 min, 4°C), the supernatant containing the fraction of the outer membrane was transferred in a fresh tube and centrifuged (14,000 rpm, 50 min, 4°C). The crystalline protein pellet was washed with 1 ml dH_2O, centrifuged (14,000 rpm, 50 min, 4°C), suspended in 200 µl dH_2O and stored at -20°C.

An alternative method for isolation of OMPs is described here:
Bacteria were grown in a shaking oN culture at 37°C. 9 ml of this oN culture were placed on ice and sonicated for 3 min (20% amplitude; pulse: 3 sec on, 3 sec off). To remove the unbroken cells, the mixture was centrifuged (6,000 rpm, 20 min, 4°C) and the supernatant containing the fraction of the outer membrane was further treated with N-lauryl sarcosyl at a final concentration of 2%. After incubation for 30 min at room temperature the mixture was transferred to an ultracentrifugation tube, filled up to 40 ml with dH_2O and centrifuged (29,000 rpm, 120 min, 4°C). The crystalline protein pellet was washed with 40 ml dH_2O and centrifuged again (29,000 rpm, 120 min, 4°C). The pellet containing the outer membrane proteins was suspended in 200 µl H_2O and stored at -20°C.

3.7. Isolation of bacterial outer membrane vesicles (OMVs)

OMVs were isolated from bacterial cultures grown aerobically at 37°C to late log-phase in LB or NBD broth, as indicated. Bacterial cells were removed by centrifugation (6,000 g, 15 min, 4°C) and the supernatants were filtered through a 0.45 µm-pore-size vacuum filter (Durapore Stericup, Millipore). Vesicles were collected by ultracentrifugation (150,000 g, 120 min, 4°C) in a 45 Ti rotor (Beckman Instrument) using polycarbonate tubes (Beckman). The supernatants were carefully removed and the vesicles were suspended in 20 mM Tris HCl (pH 8.0), unless otherwise stated. Vesicles preparations were kept at −20°C.

3.7.1. Outer membrane vesicles fractionation

Fractionation of OMVs was carried out as explained elsewhere (Horstman and Kuehn, 2000). In brief, OMV samples were adjusted to 45% Optiprep (Sigma) in 0.4 ml, transferred to the bottom of a 14 ml ultracentrifugation tube and layered with Optiprep/TrisHCl as follows: 3 ml 35%, 3 ml 30%, 2 ml 25%, 2 ml 20%, 1 ml 15% and 1 ml 10%. Gradients were centrifuged (180,000 g, 18 h, 4°C) in a SW 41 Ti rotor (Beckman Coulter). Fractions of equal volumes were sequentially removed from the top. A portion of each fraction was precipitated with TCA and analysed by SDS-PAGE and immunoblot, while the remaining samples were used for the hemolytic contact assay, LPS isolation and electron microscopy.

3.7.2. Dissociation assays

A dissociation assay was performed as described by Horstmann and Kuehn (2000). In brief, vesicle preparations in 50 mM HEPES (pH 7.3) were treated in absence or presence of either 1 M NaCl, 0.1 M Na_2CO_3, 8 M Urea or 1% SDS for 1 h on ice. After centrifugation (150,000 g, 180 min, 4°C), both soluble (supernatant) and particulate (pellet) fractions were analysed by SDS-PAGE. Prior to loading, the soluble proteins present in the supernatants were concentrated by trichloroacetic acid precipitation (see section 3.5.10.).

3.8. Isolation of lipopolysaccharides (LPS)

For analysis of the LPS composition, cells from 1 ml shaking oN culture were pelleted by centrifugation (10 min at 6,000 rpm, 4°C), suspended in 200 µl 1x sample buffer without dye (50 mM TrisHCl (pH 6.8), 100 mM β-mercaptoethanol, 2 % (w/v) SDS, 10 % (v/v) glycerol) and incubated at 100 °C for 10 min. After brief cooling, 40 µl 10mg/ml proteinase K (final concentration 1- 2.5 mg/ml) were added to the samples, which then were incubated at 60 °C for 1 h for removal of proteins. The LPS preparations were kept at 4°C and boiled with 4x sample buffer for 10 min prior to loading on SDS-PAGE. 15 µl of the LPS preparations were used for electrophoresis. Separation of the LPS components was performed by SDS-PAGE using 15 % SDS gels. After electrophoresis, the polyacrylamide gels were stained with silver nitrate.

3.9. Microscopy

3.9.1. Fluorescence microcopy

Fluorescence staining was performed as described in section 3.2.6. Imaging was carried out using the Leica DM RBE microscope and the Leica Meta-Morph or the 6.0 Spot Basic photo software. Antibodies used for fluorescence staining are listed in Table 5.

3.9.2. Electron microscopy

Ultrastructural analysis of vesicles was performed by negative staining technique using 0.5% uranyl acetate and examined by a JEM2000ET electron microscope (JEOL, Akishima, Tokyo, Japan) at 100 KV as described by (Wai et al., 1995). Immunogold localization using purified anti-IroN antibody was performed using 10 nm diameter gold particles as previously described (Wai et al., 1998).

3.9.3. Atomic force microscopy

Outer membrane vesicles preparations or bacterial cells were diluted with ultrapure water (Millipore) and immediately placed on a freshly cleaved mica surface as described previously (Balsalobre et al., 2006; Kouokam et al., 2006). The samples were incubated at room temperature for 5 min, gently washed with ultrapure water and dried in a desiccator for at least 2 h. Imaging was performed on a Nanoscope IIIa Atomic Force Microscope (Digital Instruments, Santa Barbara, CA) using Tapping Mode. The pictures are presented in amplitude mode.

3.10. Assay of hemolytic activity

Quantitative hemolytic assay was performed essentially as described previously (Sansonetti et al., 1986). Briefly, a 20% sheep blood suspension was freshly prepared in either 0.9% NaCl or 0.9% NaCl containing 10 mM $CaCl_2$. 50 µl of this mixture was incubated with an equal volume of vesicle samples in serial dilutions in a 96-well microtitre plates for 120 min at 37°C on a rocking platform. After, 100 µl of ice-cold 0.9% NaCl was added and the plate was centrifuged (400 g, 15 min, 4°C). After centrifugation, the supernatant was carefully transferred into a fresh microtiter plate and the hemolytic activity was monitored as the release of hemoglobin into the supernatant, measured spectrophotometrically at 540 nm. The amount of all vesicles samples used in the hemolytic assay was standardized by the amount of the OmpA protein.

3.11. Cytokine ELISAs

For the measurement of the cytokine profile of murine J774 macrophages, 150 µl of J774 cells ($5x10^5$/ml) were seeded into 96 well plates and grown 24h at 37°C, 5% CO_2 and 95% humidity. After stimulation with OMVs, cell supernatants were carefully removed at indicated time points, centrifuged to clear the samples and stored at -20°C until they were analysed. For this, the following commercial cytokine ELISAs were carried as described by the manufacturers:

- mouse IL-1beta ELISA (Promokine, Heidelberg, Germany)
- mouse IL-18 ELISA Kit (MBL, Naka-ku Nagoya, Japan)
- mouse KC (IL-8) ELISA Kit (R&D Systems, Minneapolis, MN)
- mouse inflammation Kit (BD, Heidelberg, Germany)

4. RESULTS

4.1. Prevalence of ExPEC virulence factors

ExPEC strains are diverse, regarding their genetic and their clinical properties, and are characterized by a broad range of virulence factors, such as adhesins, toxins, and iron accumulation systems (Russo and Johnson, 2003; Hochhut et al., 2005; Johnson et al., 2005b). However, ExPEC strains are known to possess neither a specific virulence factor nor a set of factors, which are obligatory for the infection of a certain extraintestinal site (Johnson et al., 2005b; Welch et al., 2002).

The first intention of this work was to characterize the virulence gene profiles of distinct ExPEC strains, including the archetypal strain CFT073 and the well characterized cystitis isolate 536, the genomes of which are sequenced and available in the NCBI database. The apathogenic strain MG1655 was used as negative control. The selection of oligonucleotides included known virulence genes encoding (i) toxins (*cnf*1, *hlyA*, *mchF*, *ompT, sat, usp*), (ii) components of the iron accumulation systems (*chuA, fyuA, ireA, iroN, iutA, irp*, *ybtA*), and (iii) adhesion factors (*aufA, aufG, iha-irgA*) and a gene with so far unknown function (*tir*), which is supposed to have an impact on urovirulence. The presence of these virulence genes was examined by PCR using oligonucleotides that are listed in Table 4 (p.41). The results are summarized in Table 9. Interestingly, the PCR data indicated that nearly all strains carried different genes for iron uptake systems, which are characterized by their respective siderophore or hemin receptors (FyuA, IroN, IutA and ChuA), whereas most of the other virulence determinants examined were predominantly present in single, but not in all strains. These findings are in agreement with the results obtained from a diagnostic DNA microarray specific for ExPEC that was recently developed by our working group (Sorsa et al., prepared for submission). This microarray defined virulence factors as “highly virulence associated” if they have a high prevalence in ExPEC, but not in commensal strains. A subset of the most virulence associated determinants is given in Table 10.

Table 9: Presence of selected virulence factors in ExPEC strains.

gene / strain	*fyuA*	*irp*	*iroN*	*chuA*	*ireA*	*iutA*	*ybtA*	*hlyA*	*cnf1*	*usp*	*sat*	*ompT*	*mchF*	*aufG*	*aufA*	*iha-irgA*	*tir*	serotype
536	+	+	+	+			+	+		+		+	+	+	+	+	+	O6:K15:H31
CFT073	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	O6:K2:H1
NU14	+	+	+				+	+		+		+			+			O18:K1:H7
Do768	+	+		+	+		+	+		+	+		+	+	+	+		
C5	+	+	+	+			+			+		+			+			O18:K1:H7
RS218	+	+	+	+		+	+	+		+		+		+	+			O18:K1:H7
J96	+	+	+	+			+	+	+	+		+	+	+	+	+	+	O4:H5
CP9	+	+	+	+	+		+	+	+	+		+		+	+	+	+	O4:K54:H5
AD110	+	+	+		+	+	+	+		+		+	+		+	+	+	O6:K2:H1:F7
JS299	+	+	+	+			+	+	+	+		+	+	+	+		+	O6:H
HE300	+	+	+				+											H
Mutaflor	+	+	+	+		+	+			+	+		+	+	+	+	+	O6 :K5 :H1
MG1655																		

Table 10: Prevalence of virulence factors.

	prevalence of *E.coli* strains			
Target gene	**ExPEC n=53**	**commensal n=37**	**P[a]**	**Correct classification rate[b]**
fyuA	43 (81%)	8 (22%)	$7.061e^{-08}$*	0.80
aufA	38 (72%)	3 (8%)	$9.187e^{-09}$*	0.80
chuA	38 (72%)	9 (24%)	$2.524e^{-05}$*	0.73
iroE	37 (70%)	6 (16%)	$1.635e^{-06}$*	0.76
usp	36 (68%)	3 (8%)	$6.012e^{-08}$*	0.78
iroD	35 (66%)	8 (22%)	$8.278e^{-05}$*	0.71
papF	31 (58%)	3 (8%)	$3.660e^{-06}$*	0.72
iucC	31 (58%)	4 (11%)	$1.388e^{-05}$*	0.71
aufG	31 (58%)	4 (11%)	$1.388e^{-05}$*	0.71
iroN	31 (58%)	5 (14%)	$4.765e^{-05}$*	0.70
iutA	29 (55%)	4 (11%)	$5.562e^{-05}$*	0.69
aufC	28 (53%)	3 (8%)	$3.077e^{-05}$*	0.69
hlyA	27 (51%)	4 (11%)	$2.017e^{-04}$*	0.67

Table kindly provided by L.J. Sorsa.

a The significance of association with the extraintestinal virulent or commensal *E. coli* phenotype was measured by $\chi 2$-test with Yate's continuity correction. A P value <0.05 was considered as statistically significant and is indicated with an asterisk.

b The correct classification rate of a DNA probe (1.0 = perfect indicator of the ExPEC phenotype, 0.0 = indifferent gene, not representative of the ExPEC phenotype)

The coincidental presence of these different iron uptake systems in ExPEC strains prompted us to quest if siderophore receptors may have further functions in the pathogenicity of ExPECs. Based on these considerations, the present study focused on the presence of siderophore receptors in ExPEC strains. In the following sections, the siderophore receptors IroN and FyuA will be further characterized and their role in the pathogenesis of ExPECs will be determined.

4.2. Characterization of the siderophore receptors FyuA and IroN

The siderophore receptor FyuA is encoded by the *fyuA* gene (2021 nt) on the yersiniabactin gene cluster located on the High Pathogenicity Island (HPI) (Figure 8). FyuA is synthetized as a 71 kDa sized transmembrane protein, which is responsible for ferric bound yersiniabactin uptake.

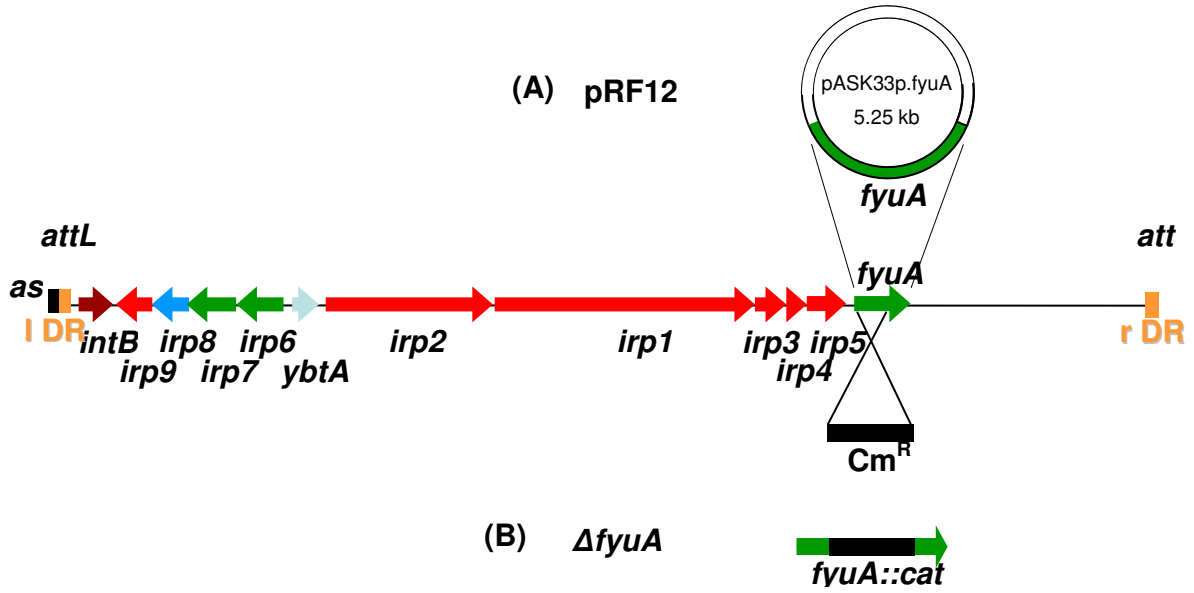

Figure 8: The yersiniabactin siderophore system located on the HPI. (A) Construction of the recombinant plasmid pRF12 (*fyuA*) and (B) mutagenesis of the *fyuA* gene. Genes of the *irp-fyuA* gene cluster encode for yersiniabactin transport (green), yersiniabactin synthesis (red), regulator (grey), integrase (brown).

Similarly, *iroN*, the gene for the salmochelin receptor (2178 nt) is part of the *iroBCDEN* gene cluster, called *iroA* locus, located on the pathogenicity island (PAI) III in the ExPEC strain 536 (Figure 9). The 81 kDa receptor is located in the outer membrane and specifically recognizes and incorporates iron bound salmochelin, a catecholate siderophore, into the bacterial cell.

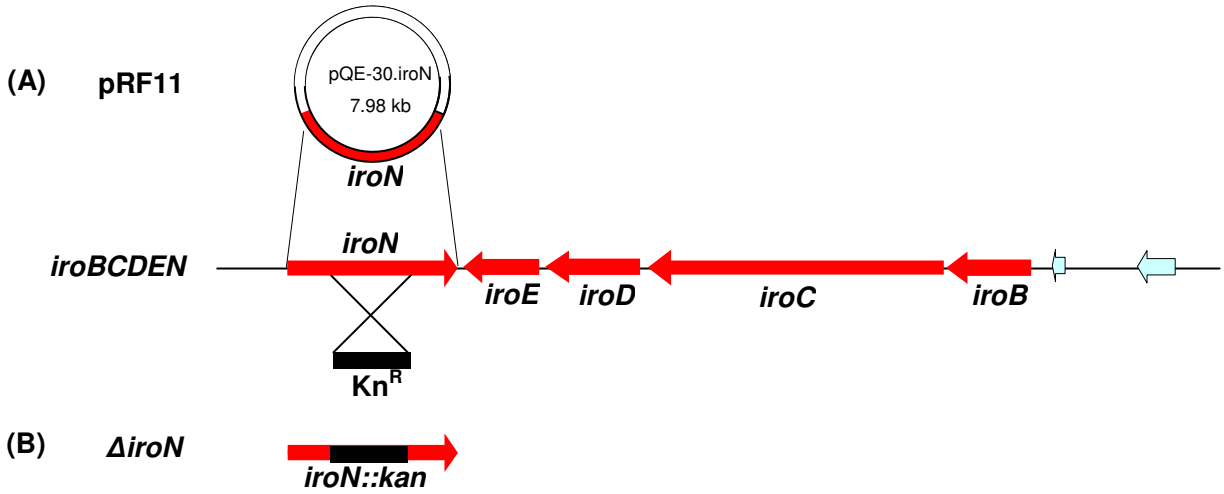

Figure 9: The salmochelin siderophore system is located on the PAI III. (A) Construction of the recombinant plasmid pRF11 and (B) mutagenesis of the *iroN* gene. Genes of the *iroBCDEN* gene cluster encode for the salmochelin receptor (*iroN*), hydrolase (*iroE*), ferric enterrochelin esterase (*iroD*), ABC transporter (*iroC*), glycosyl transferase (*iroB*).

Based on the *fyuA* and the *iroN* gene, different constructs were generated for further characterization of their respective function.

First, single mutants of the genes *fyuA* and *iroN* were constructed in the uropathogenic *E. coli* isolates 536 and NU14. The construction of the mutants was performed using linear DNA for recombination, as described by Datsenko and Wanner (2000). This method relies on the replacement of a chromosomal sequence by an antibiotic marker that is generated by PCR using primers with homology extensions to the flanking regions of the target sequence (see method section 3.3.11.). In detail, 506 nt of the *fyuA* gene were replaced by insertion of a chloramphenicol cassette (chloramphenicol acetyltransferase, *cat*) at positions 646 to 1152 nt within the *fyuA* gene by the λ red recombinase encoded by the plasmid pKD46 in the ExPEC strains 536 and NU14. For *iroN* mutants a kanamycin resistance (*kan*) gene was inserted at position 444 and 1920 to replace a 1476 nt sized part of the *iroN* gene. The allelic replacement of the target gene with theses antibiotic resistance markers resulted in a shift in the fragment size, which could be visualized by PCR amplification. The results of PCR

verification confirmed that both, the *fyuA* and the *iroN* loci, were successfully mutagenized in the strains 536 and NU14 (data not shown).

For expression of recombinant proteins, the plasmids pRF11 for *iroN* and pRF12 for *fyuA* were constructed. For this, the *iroN* gene of the strain CFT073 and the *fyuA* gene of the strain 536 were amplified by PCR using the primers F1.iroN-SstI.for and R1.iroN-PstI.rev or F1.fyuA-SstI.for and R1.fyuA-PstI.rev (see Table 4). The PCR products were cloned into the expression plasmids pQE-30 and pASK-IBA33p resulting in the recombinant plasmids pRF11 (*iroN*) and pRF12 (*fyuA*), respectively. These plasmids were subsequently transformed into the *E. coli* strain HB101 and the strains were induced for protein expression with 0.2 µg/ml anhydrotetracycline (AHT) for pRF12 and 1mM IPTG for pRF11 for 3h. Successful protein expression was determined by SDS-PAGE (Figure 10).

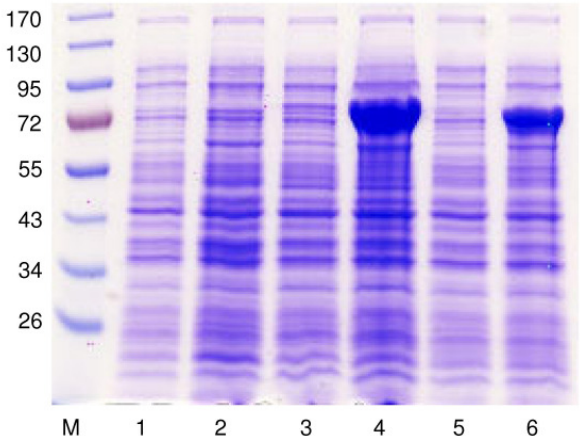

Figure 10. Expression of recombinant FyuA and IroN are shown in a Coomassie Blue stained 10% SDS-PAGE. Whole cell lysates of HB101 with pQE (lane 1-2), pRF12 (lane 3-4) and pRF11 (lane 5-6), untreated (1,3,5) and induced (2,4,6) samples are loaded in alternated order. M: Marker, numbers indicate molecular weights in kDa.

4.2.1. Transcriptional analyses of the siderophore receptor genes *fyuA* and *iroN*.

Recently, it was described that the siderophore receptors IroN and FyuA have a high prevalence in ExPEC isolates (Johnson et al., 2005b; Schubert et al., 1998). However, the expression level of different siderophore receptors may vary depending on the strain and on the environmental condition at the site of infection. Thus, siderophore receptors are known to be up-regulated when grown under iron starvation conditions such as in urine or inside the host (Alteri and Mobley, 2007). To investigate the transcription levels of *iroN* and *fyuA* in 536 and NU14, total RNA was isolated and used for cDNA synthesis. cDNA samples derived from reverse transcription were quantified by real time PCR analyses (TaqMan) and the transcriptional levels between growth in LB and in the iron depletion medium NBD were compared. The results indicated that the transcriptional level of *fyuA* in NU14 and 536 was 10 to 20-fold less than that of *iroN* when grown in LB medium (Figure 11). Moreover, growth of bacterial cultures under iron depletion conditions (NBD medium) led to a 20 to 50-fold increase in the transcription level of *fyuA* whereas that of *iroN* was only 6 to 8-fold induced in NU14 and 536 transcripts versus the transcription level in LB medium. However, although transcription of *fyuA* was stronger up-regulated than that of *iroN* during defined iron limitation (20 to 50-fold compared to 6 to 8-fold), the level of *fyuA* was significantly less than that of *iroN* in both strains and conditions.

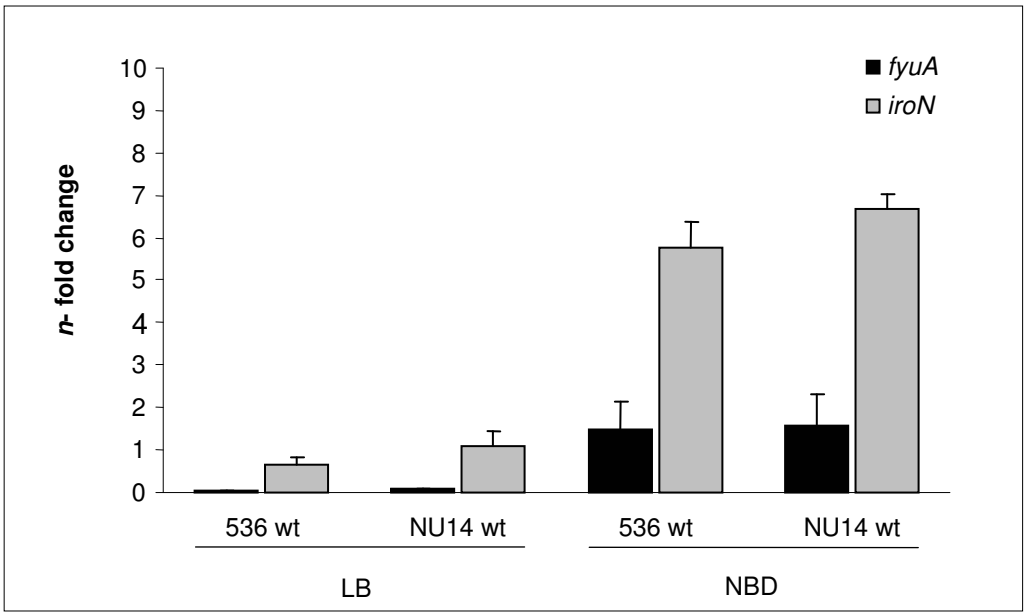

Figure 11. Transcriptional levels of *fyuA* and *iroN* were examined by TaqMan analyses. For comparative, quantitative analyses, transcript levels were normalized to the level of the endogenous reference gene *mdh* (malate dehydrogenase). Data were analysed by the $2^{-\Delta\Delta}$ CT method (Livak and Schmittgen, 2001) and presented as a change difference (*n*-fold) between strains.

4.2.2. Expression and subcellular localization of IroN and FyuA.

To confirm the transcriptional findings we used Western blot analyses and examined the expression of *iroN* and *fyuA* under different growth conditions. First, whole-cell lysates from 536 and NU14 wild-type strains grown in LB medium were analysed by immunoblotting with purified polyclonal anti-IroN and anti-FyuA antibodies. As a control we used whole cell preparations from *iroN* and *fyuA* mutant and complemented strains to verify the specific binding of the respective antibody to the protein (data not shown). Consistent with the transcriptional findings, *iroN* was expressed by both ExPEC strains (Figure 12).

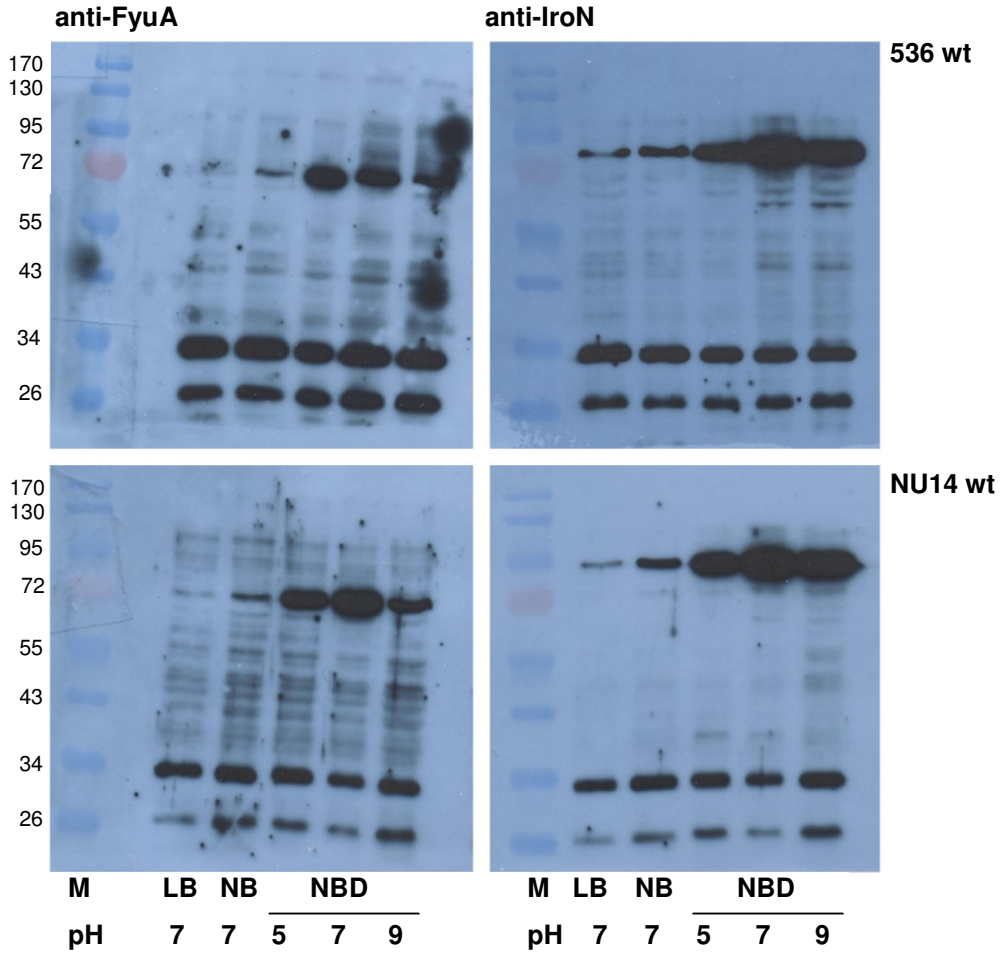

Figure 12. Expression profiles of *fyuA* and *iroN* in 536 and NU14 wild-type strains under different growth conditions. Whole cell lysate were prepared from LB, NB and NBD cultures. *fyuA* was significantly lower expressed than *iroN*, growth under iron depletion condition increased the expression of both genes to comparable expression levels of *iroN* and *fyuA*. M: Marker, numbers indicate molecular weights in kDa.

In contrast, FyuA could be not detected in 536 wt, but was visible as a weak but recognizable immunoreactive band in whole cell extracts from strain NU14. This observation indicated that FyuA was expressed, but the amount of the protein was near the detection limit. Growth under iron depletion conditions increased expression of both genes *fyuA* and *iroN* to a level which was comparable in the strains 536 and NU14. In detail, expression of *fyuA* and *iroN* was slightly increased compared to LB when the strains were grown in the minimal medium NB. Moreover, addition of the iron chelator dipyridyl to the NB medium, resulting in the iron depletion medium NBD, led to a significant increase in the expression rate of *fyuA* and *iroN*. Interestingly, the pH of the medium was also important for the expression of siderophore receptors. Thus, samples prepared from NBD medium of pH 5 and 9 showed an increase in the expression compared to NB and LB medium, but the highest expression level was induced by growth in NBD medium at pH 7. This observation is in agreement with a recent publication (Valdebenito et al., 2006), indicating that yersiniabactin and salmochelin show an expression maximum under neutral to alkaline iron depletion conditions.

Further, the subcellular localization of the siderophore receptors IroN and FyuA was determined. In general, *E. coli* siderophore receptors are outer membrane proteins that are similar in their structure and show the presence of similar domains, consisting of a membrane integrated β-sheet structured barrel (compare Figure 5). To confirm the presence of FyuA and IroN in the outer membrane, outer membrane proteins (OMPs) from the strains 536 and NU14 were isolated and analysed by immunoblotting with anti-IroN and anti-FyuA antibodies. As expected, IroN was present in comparable amounts in OMP preparations as in whole cell samples. In agreement with previous observations, FyuA could be detected in OMP samples, but the amount was significantly less than that of IroN (data not shown). These findings confirmed the presence of IroN and FyuA in the outer membrane of the ExPEC strains 536 and NU14.

Taken together, analysing the amount of IroN and FyuA in the OM of the ExPEC strains 536 and NU14 by Western blotting, we found that *iroN* was stronger expressed than *fyuA*, but that the expression levels of both genes were induced to comparable amounts under iron depletion conditions. These data are in good agreement with the levels of induction seen by TaqMan analyses and immunoblotting.

4.3. Functional relevance of FyuA and IroN: invasion assays

4.3.1. IroN contributes to urothelial cell invasion *in vitro*

The aim of this study was to investigate a possible involvement of FyuA or IroN in mediating invasion of urothelial cells by ExPEC strains. As a first approach, we investigated invasion of HCV29 cells by recombinant HB101 strains by means of the gentamicin protection assay (Mulvey et al., 2001). For this, IroN and FyuA proteins were recombinantly expressed in the laboratory *E. coli* strain HB101 and the invasion of these strains into the urothelial cell line HCV29 was assessed. In order to exclude invasion artefacts caused by other bacterial invasion factors such as type 1 fimbriae, we selected the apathogenic *E. coli* strain HB101 as background strain for recombinant expression and invasion study, for HB101 lacks the adhesive subunit FimH, the tip protein of type 1 pili. Gentamicin protection assay was carried out as described in Methods (section 3.2.3.). The premise of this assay is that the bacteria which are able to invade host cells can be identified by adding gentamicin, which kills all extracellular, but not the intracellular bacteria since gentamicin is unable to enter host cells. In brief, HCV29 cells were infected with bacteria and after incubation three different fractions of bacteria were separated: First, the total number of infecting bacteria, the second fraction represented adherent and invaded bacteria, and the third fraction indicated the number of invaded bacteria. The results are shown in Figure 13. The total amount of infecting bacteria (dotted bars) served as a control for the input of bacteria, indicating that similar MOIs were added. Grey bars depict adherent bacteria and black bars represent the level of invaded bacteria. SDS-PAGE was performed and stained with Coomassie Blue in order to confirm expression of the respective protein (Figure 10, p. 80). *E. coli* HB101/pRS1 expressing the *Yersinia enterocolitica* O:8 WA-C invasin gene *inv* (Schulte et al., 1998) was used as positive control and revealed a 103-fold increase of invaded bacteria compared to the control strain, HB101 carrying the pQE vector alone. Interestingly, expression of IroN was associated with an increased invasion rate by the *E. coli* HB101 host strain of between 5- to 10-fold. However, FyuA failed to increase the invasion rate of HCV29 cells. These data indicate that IroN, but not FyuA was able to mediate invasion into urothelial HCV29 cells. Notably, the adherence levels were comparable between all strains. We therefore suggest that increased invasion is not a result from enhanced attachment of *iroN* expressing bacteria to HCV29 cells rather it is a more direct effect on invasion.

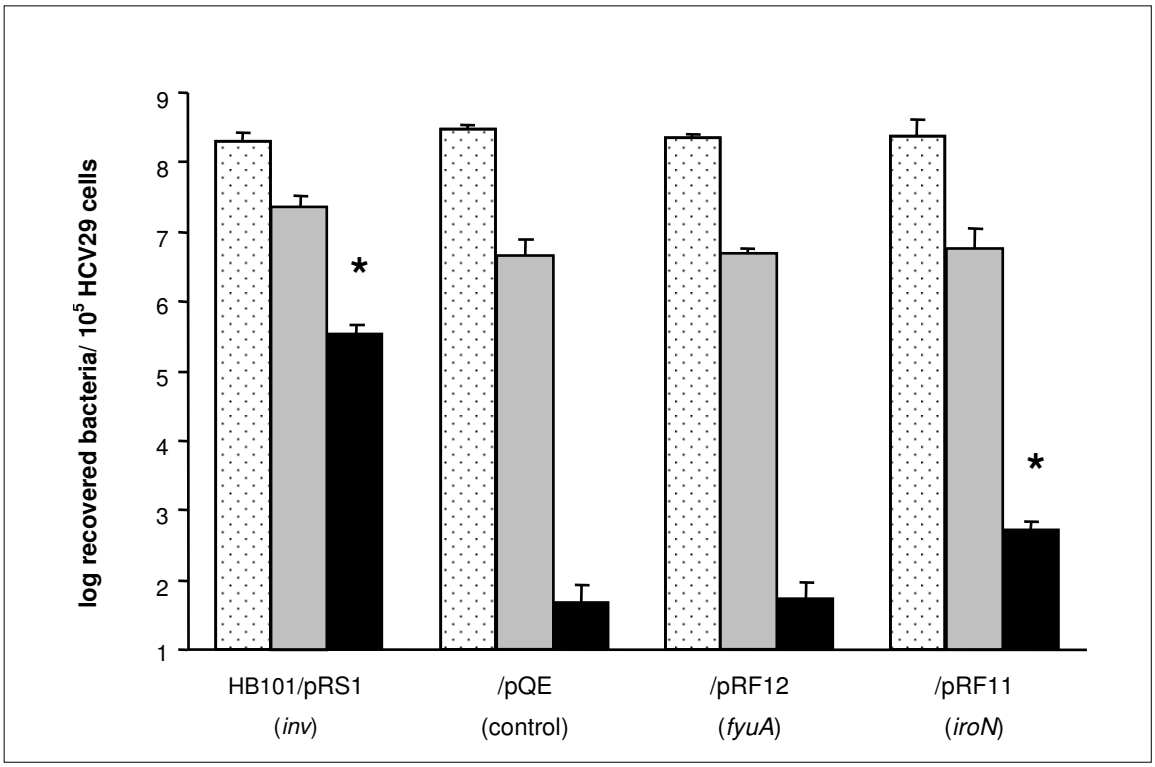

Figure 13. Gentamicin protection assays using HCV29 urothelial cells and recombinant *E. coli* HB101 strains carrying expression plasmids for the proteins Inv (pRS1), IroN (pRF11) and FyuA (pRF12), respectively. The columns represent the total number of added bacteria (dotted bars), the number of the adherent (gray bars) and of the invaded bacteria (black bars). Data are means and standard errors of the means (error bars) obtained from six wells. Repeated experiments gave comparable results. Statistical differences compared to the vector control were marked with an asterisk when considered significant ($P < 0.05$). HB101/pRS1 served as positive control, showing a high invasion level compared to HB101/pQE which was used as negative control. Expression of FyuA did not increase the invasion level compared to the negative control, whereas expression of IroN significantly increased the invasion rate.

These results are not unique for HCV29 cells as we observed similar invasion rates using different cell lines including the urothelial cell lines UROtsa, J82 and RT112 or the kidney cell line HEK293 (data not shown).

In order to investigate if an antibody directed against IroN possibly inhibits IroN mediated invasion, the IroN-expressing *E. coli* strain HB101/pRF11 was incubated prior to infection of urothelial cells with a polyclonal antibody raised against IroN. In agreement with the results of other invasion experiments, the invasion level of the pre-treated *E. coli* strain HB101/pRF11 was considerably decreased in a dose dependent manner (Figure 14).

To determine if the intact IroN protein is necessary for invasion or if invasion is possibly mediated by a single domain of the IroN protein, *iroN* was cloned and expressed in two different parts: the N-terminal part representing the first 387 AA of IroN ($IroN_{1-387}$) and a 354 AA comprising C-terminal part ($IroN_{372-726}$) were cloned into the expression plasmid pQE-30 and expression of the intact and truncated forms of IroN were confirmed by Western blotting using an anti-IroN antibody (data not shown).

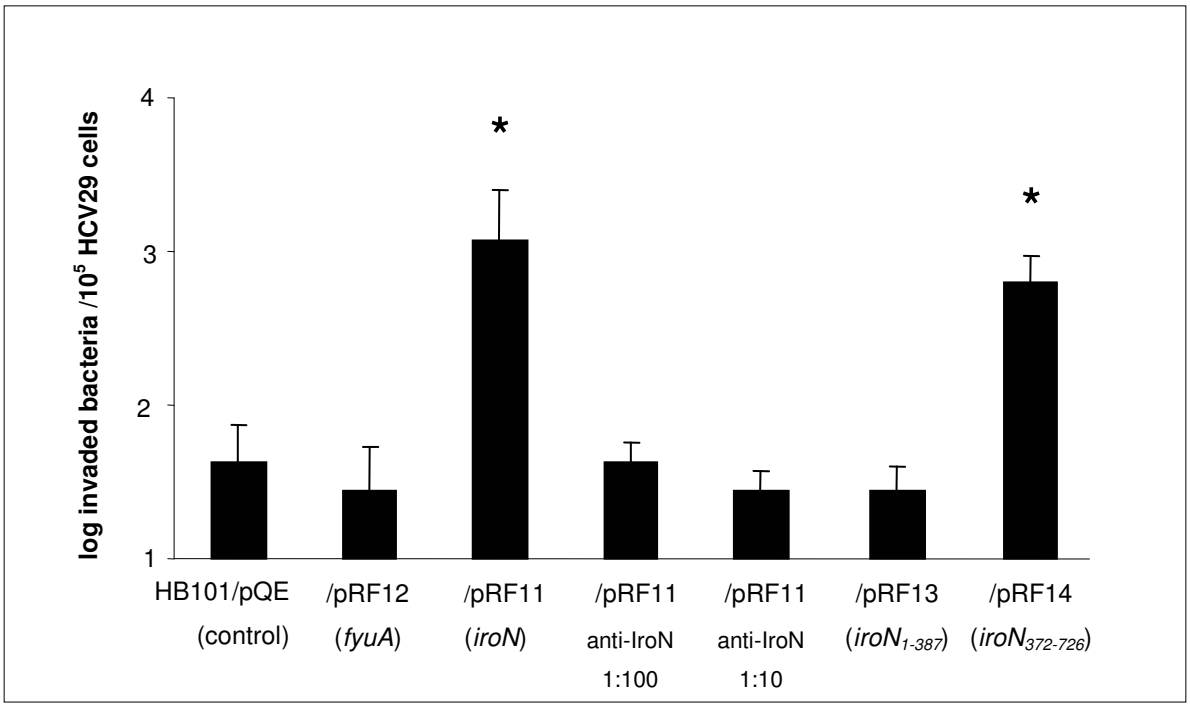

Figure 14. Gentamicin protection assay with HB101 strains expressing *fyuA*, *iroN*, the N-terminal (*iroN*$_{1\text{-}387}$) or the C-terminal part of *iroN* (*iroN*$_{372\text{-}726}$). Statistical differences compared to the vector control were marked with an asterisk when considered significant ($P < 0.05$). Incubation with polyclonal anti-IroN antibody inhibited IroN mediated invasion into urothelial HCV29 cells. Further, the C-terminal (IroN$_{372\text{-}726}$), but not the N-terminal (IroN$_{1\text{-}387}$) part of IroN, contributed to an invasion level which was comparable with that of HB101/pRF11 (*iroN*).

As shown in Figure 14A, the C-terminal (IroN$_{372\text{-}726}$), but not the N-terminal (IroN$_{1\text{-}387}$) part of IroN, contributed to an invasion level which was comparable with that of HB101/pRF11 (*iroN*). These observations indicated that not the intact form of IroN is necessary for invasion of host cells, suggesting that not the functional protein, but rather special structures or domains are important for invasion.

4.3.2. Internalization of Latex Beads

To exclude the impact of other *E. coli* virulence traits such as fimbriae or adhesins on the observed phenotype, invasion studies were performed using latex beads coated with recombinant proteins. For this, latex beads (Sulfate Microspheres, 4-µm, blue fluorescent, Molecular Probes, Invitrogen) were incubated with the purified recombinant proteins FyuA, IroN, Inv (Wiedemann et al., 2001) or with bovine serum albumin (BSA) according to the manufacturer's recommendations. Coated beads were centrifuged (200 g for 2 min) onto HCV29 cells grown on glass slides followed by incubation at 37°C for 1 hour and Immunofluorescence microscopy was performed. Ten areas per slide were then examined and the present beads were counted to determine the amounts of intracellular beads. The number of intracellular beads was expressed as a percent of all blue fluorescent latex beads present in the sample. The varying uptakes of the protein-coated latex beads by urothelial cells are shown in Figure 15A. Inv-coated beads showing an internalization rate of about 95% were used as positive control for successful coating and uptake. In agreement with previous results, latex beads coated with recombinant IroN were internalized by the HCV29 cells at a between 10- to 20-fold higher frequency compared to that of FyuA- or BSA-coated beads, which served as negative control. In addition, the uptake of IroN-coated beads could be inhibited by preincubation with a polyclonal antibody raised against IroN (Figure 15A). These findings further corroborate the direct involvement of IroN in the invasion process of urothelial cells. In Figure 15B, fluorescence pictures of IroN-coated latex beads placed on HCV29 cells is shown.

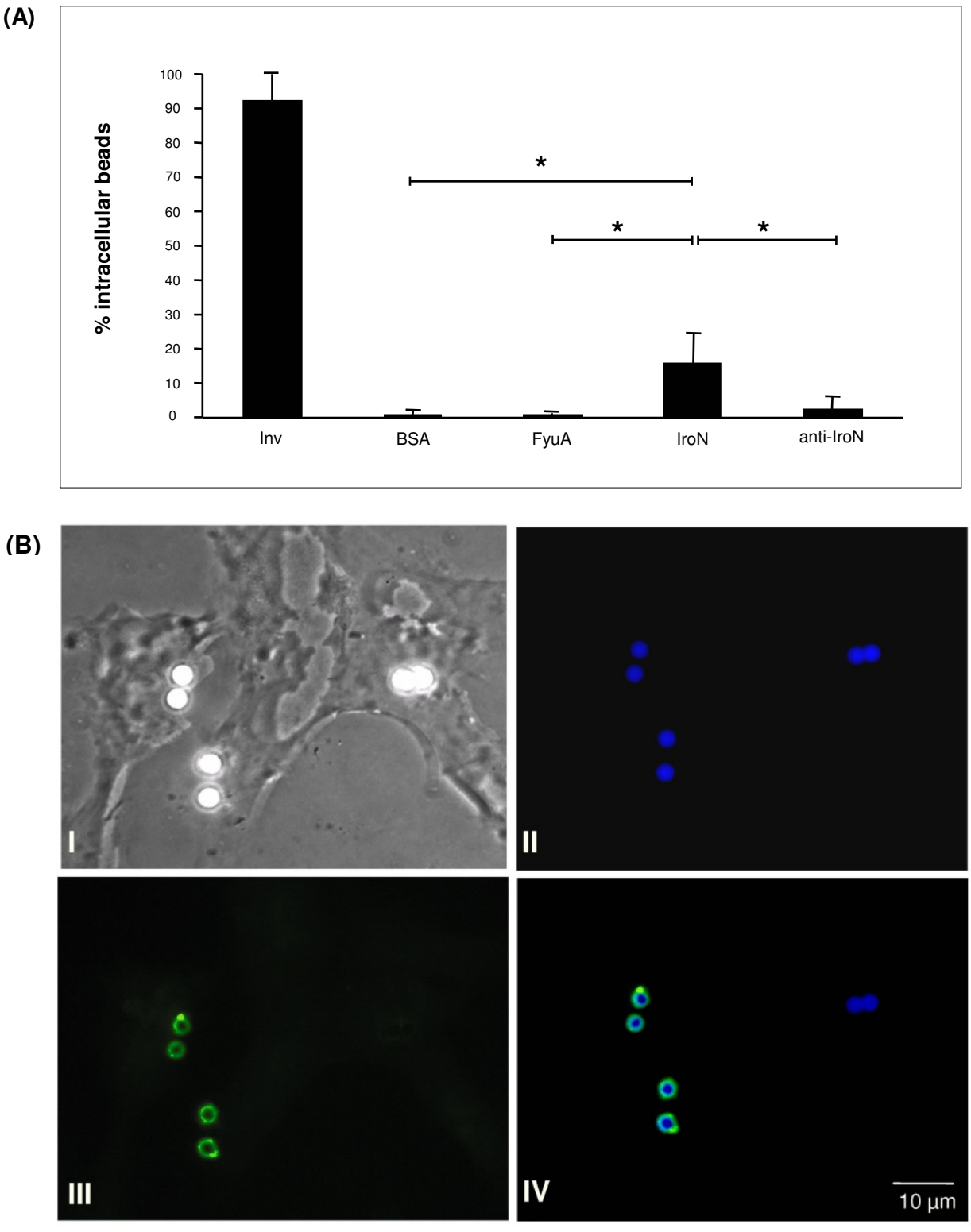

Figure 15. Internalization of protein-coated latex beads by HCV29 urothelial cells. (A) The columns represent the number of intracellular beads as a percentage of all beads present in the sample. The values are means ± standard deviations and the asterisk indicates a P value of < 0.05 which was considered statistically significant. The data indicated that IroN-coated beads were internalized in a significantly higher rate compared to FyuA- and BSA-coated beads. (B) Fluorescence microscopy of IroN-coated latex beads placed on HCV29 cells. Picture I represents the phase contrast micrograph, corresponding fluorescent images reveal the total number of IroN-coated latex beads (blue) (II) and the fraction of extracellular beads (green) (III). Merging of pictures II and III displays intracellular beads in blue, whereas the extracellular beads appear turquoise (IV).

4.3.3. Natively expressed IroN mediated comparable invasion levels

In order to exclude artefacts caused by the high IroN expression of the recombinant plasmid pRF11, we determined the invasion phenotype mediated by IroN expressed under the control of a native promoter. Therefore, invasion assays were performed using the laboratory *E. coli* strain TH2 transformed with a recently identified 80-kb wild-type plasmid (p300) of the ExPEC isolate HE300, which contained a functional *iroBCDEN* gene cluster (Sorsa et al., 2003). A 30-kb subfragment of this plasmid containing among others the *iroBCDEN* gene cluster was subcloned, resulting in the plasmid pJS332. To exclude that any of the unknown genes present on p300 and pJS332 caused an invasive phenotype, we further examined *E. coli* TH2 strains with plasmids carrying parts of the *iro* gene cluster. For this, PCR products representing parts of the *iroBCDEN* gene cluster with their respective native promoters were cloned into a pCR4-TOPO vector, resulting in the plasmids pJS12 for *iroEN* and pJS13 for *iroBCDE*, respectively. In addition, the plasmid pJS12 was mutagenized *in vitro* using a DHFR-1 EZ::TN insertion kit (Epicentre Technologies) giving rise to two plasmids with the EZ::TN insertion in *iroN* and *iroE* genes named pJS14 (*iroN*::Tp^r-*iroE*) and pJS15 (*iroN*-*iroE*::Tp^r), respectively. Expression of IroN by these plasmids was confirmed by Western blot with anti-IroN antibodies (Figure 16B).

In agreement with the results of the recombinantly expressed IroN, we observed a significant difference between the invasion rate of the TH2 strains carrying either plasmids with the entire *iro* gene cluster (p300 and pJS332) or only the pCR4-TOPO vector (control) (Figure 16A). We further found that the plasmid, which carried the *iroEN* genes (pJS12) consistently gave rise to the invasive phenotype, whereas the *iroBCDE* carrying plasmid pJS13 did not. To emphasize the IroN mediated invasion of urothelial cells, we compared the invasion rate of *E. coli* TH2 strain carrying the intact *iroEN* genes (plasmid pJS12) with TH2 strains carrying pJS14 and pJS15, which were derived from the plasmid pJS12 (*iroEN)* carrying transposon insertions in the *iroN* and *iroE* gene, respectively (Sorsa et al., 2003). Concordantly, the transposon insertion in the *iroE* gene did not influence the invasive phenotype of TH2/pJS15, whereas the mutation of *iroN* led to a significant decrease of invasion of TH2/pJS14 compared to pJS12.

Noteworthy, all, the IroN-positive (TH2/pJS12 and TH2/pJS15) and the IroN-negative (TH2/pJS13 and TH2/pJS14) *E. coli* strains, revealed comparable adherence levels (data not shown). We can therefore exclude that the increased invasion phenotype of IroN-expressing

strains is the consequence of enhanced attachment to HCV29 cells. This is in agreement with results presented in a recent study (Leveille et al., 2006).

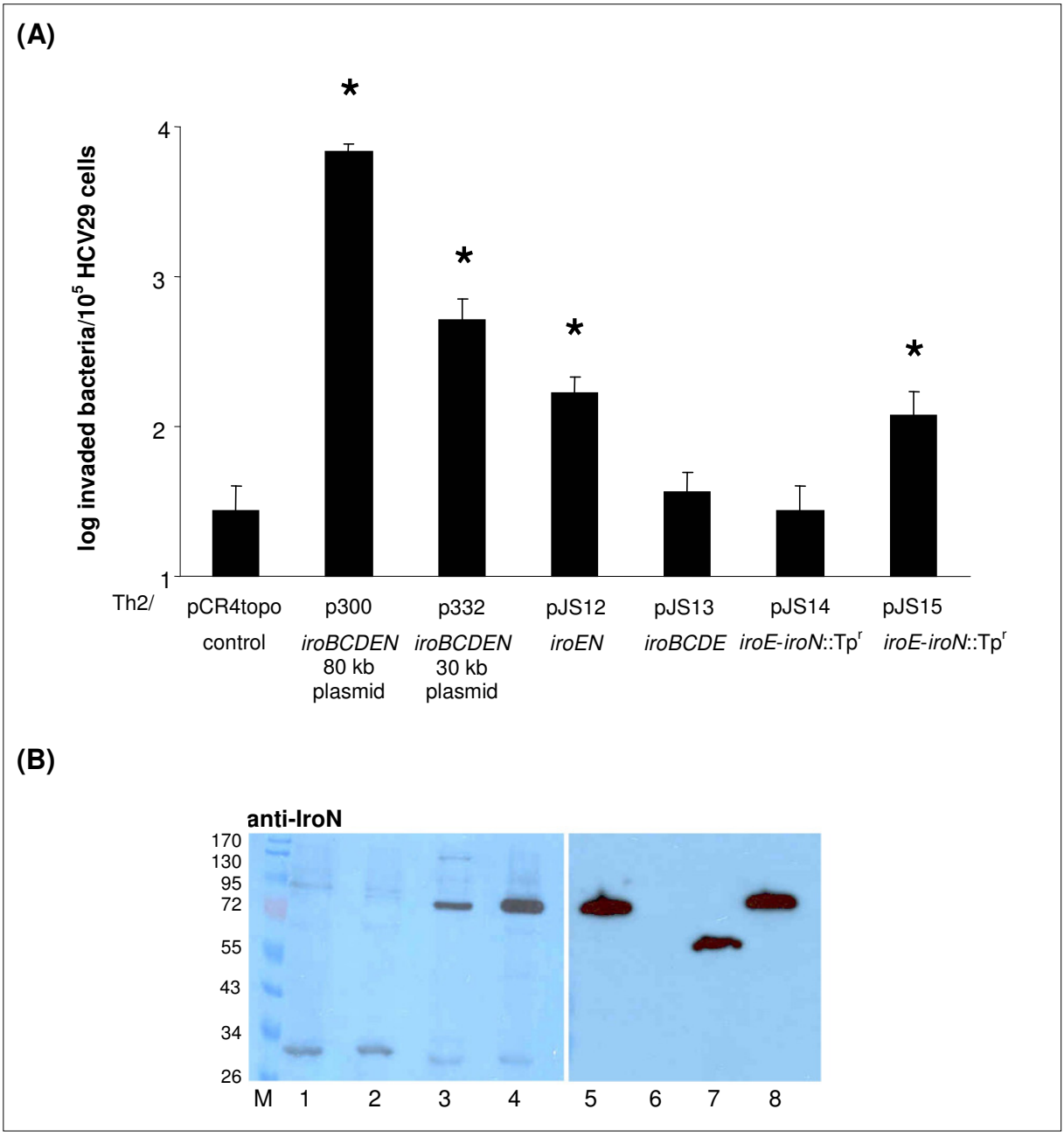

Figure 16. (A) Invasion level of *E. coli* TH2 with the entire *iro* region or with parts of the *iro* gene cluster, all expressed under control of their native promoter were examined by gentamicin protection assay. *E. coli* TH2 carrying the empty vector (pCR4-TOPO) served as negative control. Statistical differences compared to the vector control were marked with an asterisk when considered significant ($P < 0.05$). The data indicated that only IroN-carrying strains mediated invasion, whereas IroN-lacking strains did not. (B) Corresponding expression level of IroN revealed by Western blotting using anti-IroN antibody confirmed the presence or absence of IroN. M: Marker, numbers indicate molecular weights in kDa. Lane 1-8: TH2 wt, TH2 carrying pCR4-TOPO, p300, pJS332, pJS12, pJS13, pJS14, pJS15.

4.3.4. The role of IroN within an ExPEC background

In order to confirm the IroN mediated invasion effect in an ExPEC background, we determined invasion of urothelial HCV29 cells by the ExPEC strains CFT073 and 536 wt in comparison to the apathogenic *E. coli* strain HB101. We found that the invasion rates of CFT073 and 536 were 10^2-fold higher than those of HB101 (HB101: 5 x 10^1, CFT073: 2 x 10^3) (data not shown). However, no significant differences in the invasion rates were detectable comparing CFT073Δ*iroN* mutant and CFT073 wt or 536Δ*iroN* mutant and 536 wt strains Figure 17A). This observation is most likely due to the coincidental presence of several redundant factors involved in the invasive phenotype of the ExPEC strain CFT073.

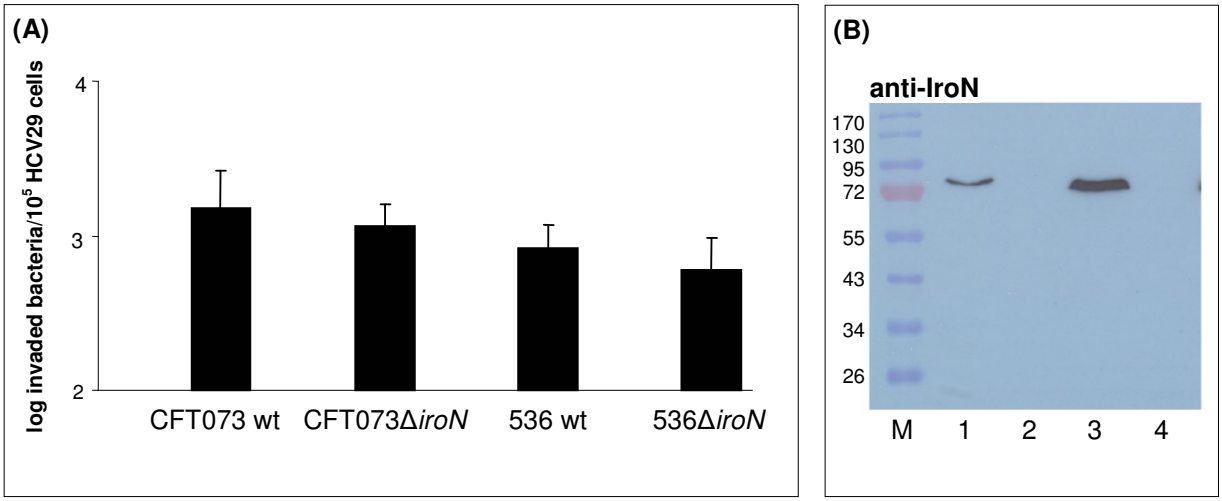

Figure 17. (A) Comparison of invasion levels of CFT073 and 536 wt and *iroN* mutant strains revealed no significant differences in the invasion rates of mutant and wt strains. (B) Corresponding expression level of IroN revealed by Western blotting using anti-IroN antibody. M: Marker, numbers indicate molecular weights in kDa. Lane 1-9: CFT073 wt, CFT073Δ*iroN*, 536 wt, 536Δ*iroN*.

As an alternative to the isogenic *iroN* mutant strains, we used an *iroN*-deficient ExPEC strain, the strain Do768. This strain represents a clinical isolate harboring several typical ExPEC virulence genes (e.g. *fyuA*, *chuA*, *usp*, *iha*, *pap*, *auf*), but not the *iroN* gene. Interestingly, the strain Do768 was about ten times less invasive compared to the *iroN*-positive ExPEC strain CFT073 (Figure 18). Introduction of *iroN*-carrying plasmids (pJS12, pJS332 and pRF11) into the strain Do768 resulted in a significant increase of the invasion rate and gave rise to intracellular bacterial counts comparable to those of the strain CFT073. These data further corroborate our findings that IroN contributes to the invasion of urothelial cells by ExPEC.

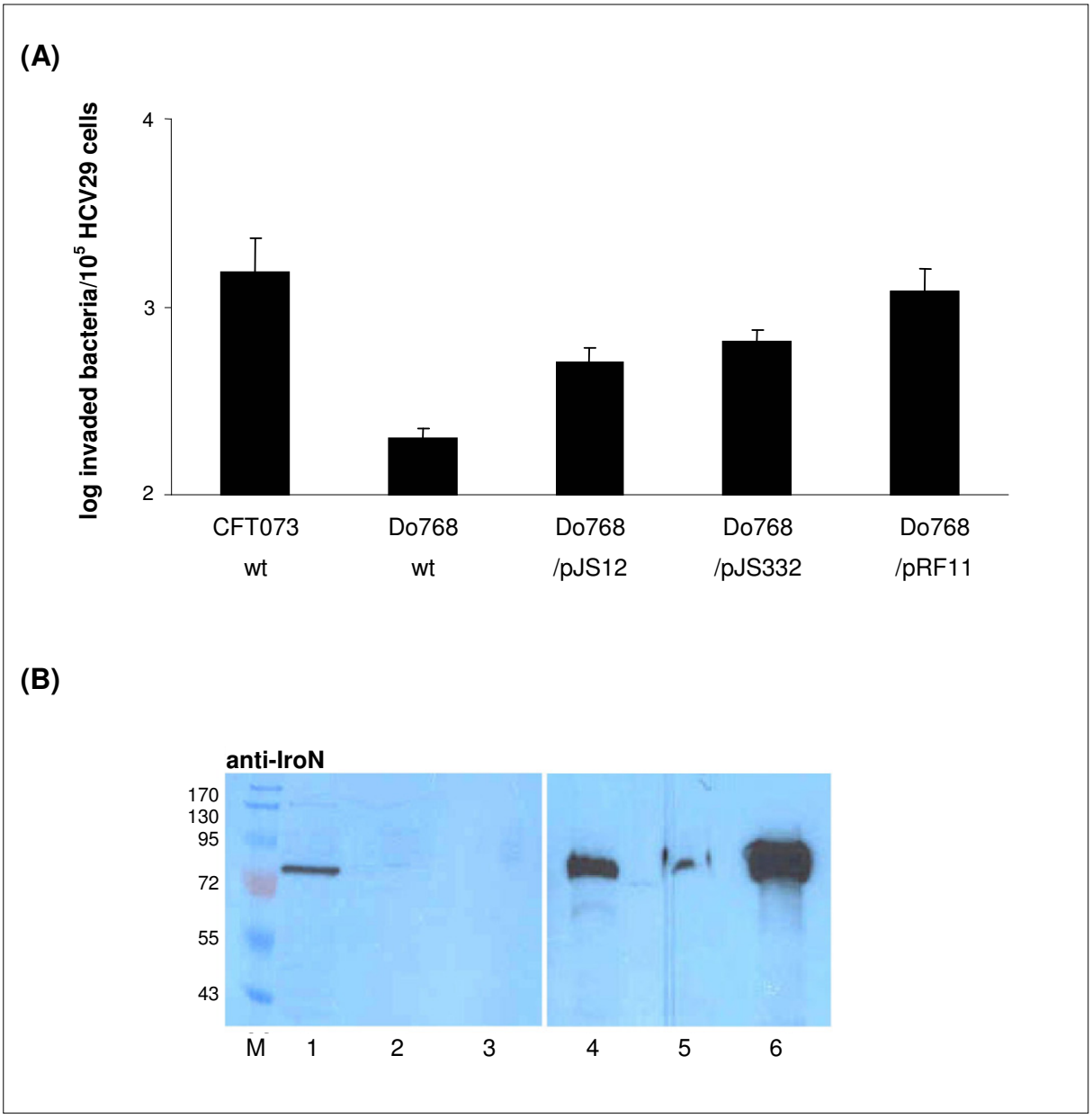

Figure 18. (A) Results of the gentamicin protection assay indicated that the IroN-positive CFT073 was more invasive than the IroN-lacking strain Do768. Complementation of the *iroN*-negative Do768 with *iroN*-containing plasmids (pJS12, pJS332, pRF11) led to invasion levels comparable to that of CFT073. (B) Corresponding expression level of IroN revealed by Western blotting using anti-IroN antibody. M: Marker, numbers indicate molecular weights in kDa. Lane 1-6: CFT073, DO768 wt, Do768 with pCR4-TOPO, pJS12, pJS332, pRF11.

4.3.5. Mechanism of IroN mediated invasion

This section is focused on the cellular mechanism of IroN mediated host cell invasion. The first approach was to investigate if *iroN* expressing strains were possibly internalized via the same mechanism as *inv* expressing strains, namely the invasin/β1 integrin triggered phagocytosis. For this, gentamicin protection assays were performed with the mouse fibroblast cells GD25, which naturally lack the β1 integrin subunit. As a control, GD25 beta cells, which are complemented with recombinant β1 integrin were used. As shown in Figure 19, the absence of β1 integrin led to a decreased invasion level of the *inv* expressing strain HB101/pRS1 into GD25 cells compared to GD25 beta cells, confirming that *inv* expressing strains are internalized by invasin/β1 integrin triggered phagocytoses. Complementation of GD 25 with recombinant β1 integrin (GD25 beta) revealed similar invasion rates of HB101/pRS1 as obtained by using HCV29 cells. In contrast, the *iroN* expressing strain HB101/pRF11 showed similar invasion rates either using GD25 or GD25 beta cells. In addition, control strains and *fyuA* expressing HB101 strains were also not influenced by the lack of the β1 integrin subunit. Taken together, we could exclude the β1 integrin triggered phagocytosis as internalization pathway for *iroN* expressing strains.

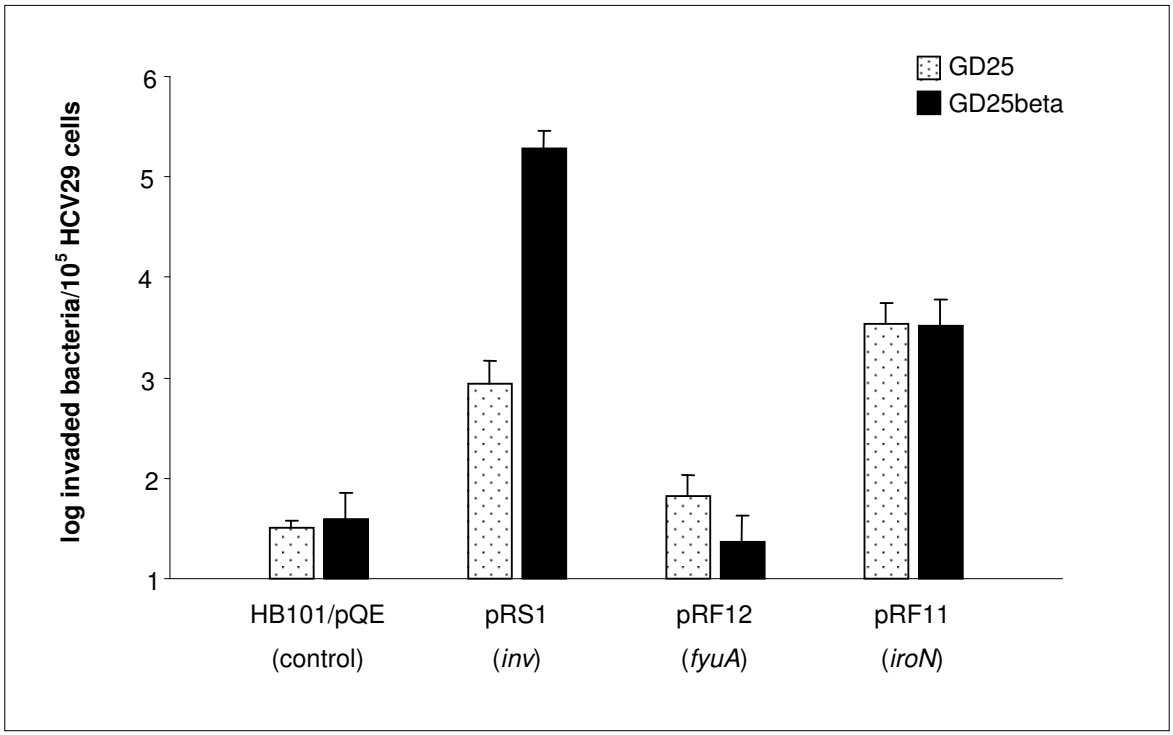

Figure 19. Invasion assay using the β1 integrin negative fibroblast cell line GD25. The absence of β1 integrin led to a decreased invasion level of *inv* expressing strains, whereas IroN mediated invasion was not influenced (dotted bars). Complementation of GD 25 with recombinant β1 integrin (GD25 beta, black bars) revealed similar results as obtained by using HCV29 cells.

In general, bacteria and bacterial products can be internalized by host cells via different pathways. These include endocytic pathways such as lipid rafts and clathrin-coated pits, tubulin or actin polymerization, which lead to a changed conformation of the cytoskeleton and result in the uptake of the pathogen. Other invasion mechanisms comprise macropinocytosis or phosphatidylinositol 3-kinase (PI3K) mediated intracellular trafficking. To further decipher whether *iroN* expressing strains enter host cells via one of these mechanisms, we performed invasion studies with different inhibitors that selectively block specific invasion pathways (Figure 20). The inhibitors used were wortmannin, a metabolite of the fungi *Penicillium funiculosum*, which specifically inhibits PI3K mediated trafficking, filipin which blocks the formation of cholesterol-rich lipid raft and chlorpromazine preventing clathrin-coated pits mediated endocytosis. Further inhibitors interfered with tubulin (nocodazole) or actin (cytochalasin D) polymerization and macropinocytosis (amiloride).

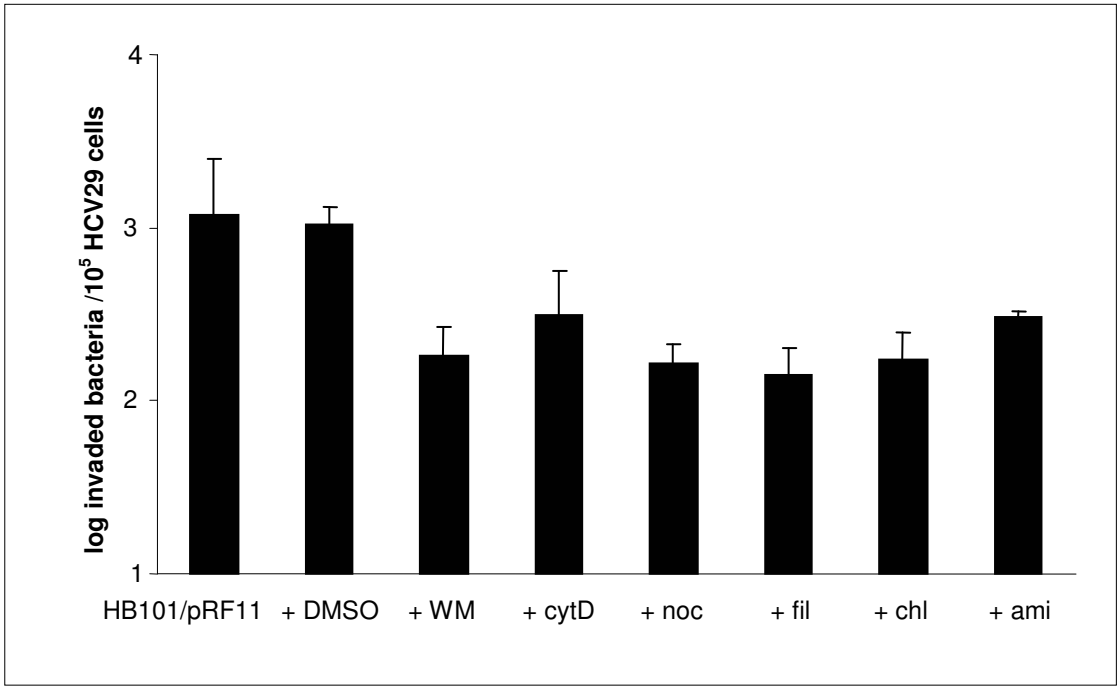

Figure 20. Invasion level of HB101/pRF11 expressing *iroN* in absence or presence of distinct inhibitors. DMSO served as negative control and had no effect on the invasive phenotype of HB101/pRF11, whereas every single inhibitor slightly decreased the invasion rate. Therefore, we suggested that IroN mediated invasion was not due to one of these specific pathways, rather than IroN supports unspecific uptake of bacteria into host cells. WM= wortmannin, cytD: cytochalasinD, noc: nocodazole, fil: filipin, chl: chlorpromazine, ami: amiloride.

As shown in Figure 20, a moderate decrease of the invasion rate of HB101/pRF11 expressing *iroN* compared to HB101/pRF11 alone could be detected in the presence of each of the respective inhibitor. However, this difference was not considered significant.

4.4. Outer membrane vesicles of ExPEC

4.4.1. OMV isolation and characterization

The localization of FyuA and IroN in the outer membrane prompted us to investigate if these siderophore receptors are possibly included into ExPEC vesicles that are constitutively shed by the outer membrane of Gram-negative bacteria. For this, vesicles were isolated from the cell-free culture supernatant of 536 and NU14 strains. In a first approach we characterized by electron microscopy (EM) and by atomic force microscopy (AFM). Electron micrographs of purified vesicle samples of strains 536 and NU14 after growth in LB medium indicated that both ExPEC strains produce apparently the same amount of OMVs and that the vesicles of both strains are similar in their size and structure (Figure 21A). Studies of purified vesicles using AFM confirmed these observations, but demonstrated the presence of flagellar fragments in vesicle preparations of strain NU14 (Figure 21B). These fragments are most likely contaminations resulting from disrupted flagellae present in the supernatant. This assumption was further supported by the observation that bacteria of the strain NU14 produced much more flagella than strain 536 as determined by AFM (Figure 21C).

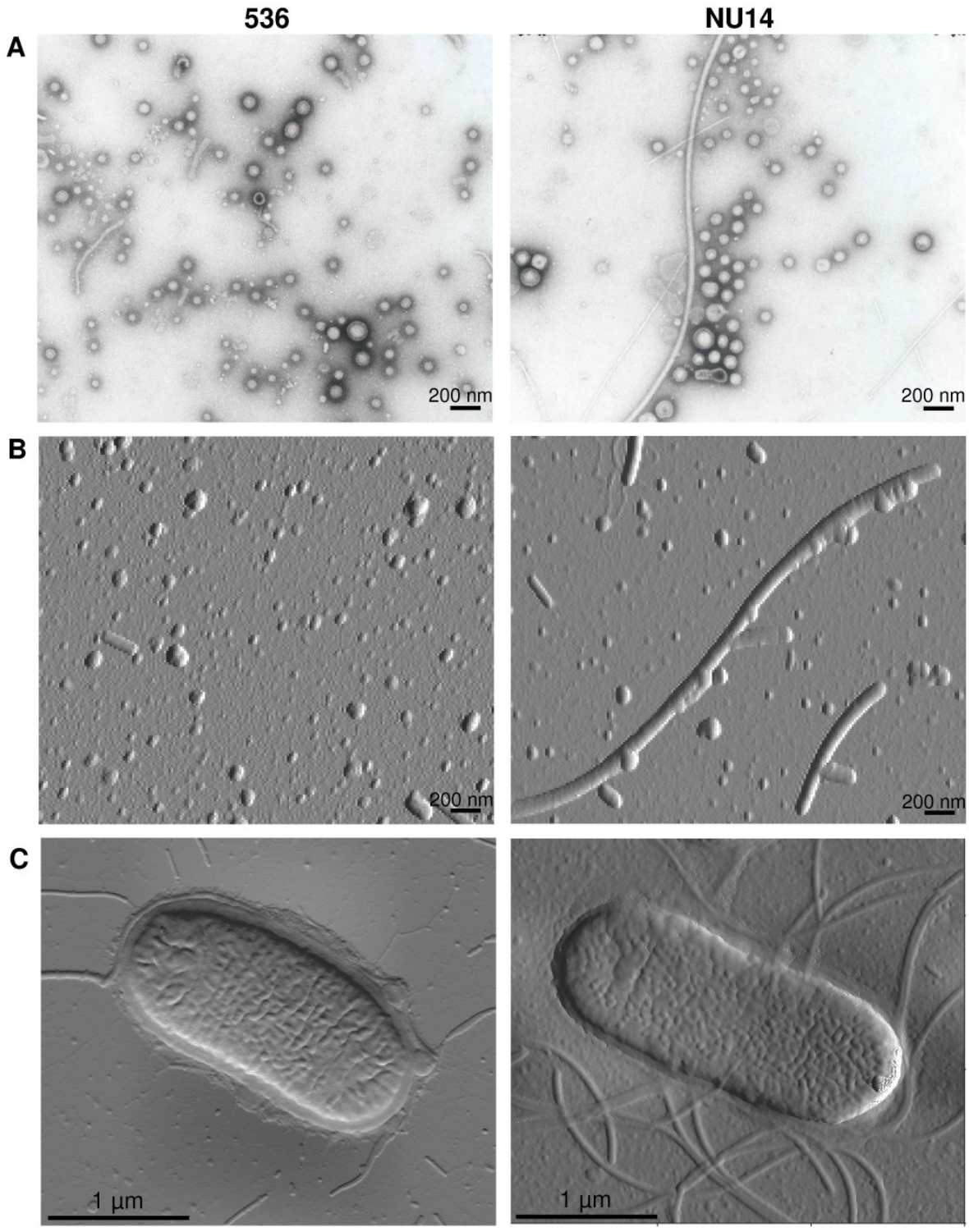

Figure 21. Ultrastructural analysis. A: Electron microscopy of OMVs isolated from ExPEC strains 536 (left) and NU14 (right). B: Atomic force microscopy of the same samples C: Atomic force microscopy of bacterial strains 536 (left) and NU14 (right).

Further characterization of 536 and NU14 vesicles by electrophoresis and Coomassie Blue staining of the SDS-gels showed a similar band pattern revealing three proteins in the approximated size of 35, 37 and 40 kDa, respectively (Figure 19A). However, OMV preparations of NU14 revealed an additional characteristic band in the size of approximately 60 kDa. In order to identify these proteins, the bands were cut from the gel and analysed by N-terminal peptide sequencing. The identified proteins were flagellin (60 kDa), which was only present in NU14 vesicle samples and the outer membrane proteins OmpA (37 kDa) and OmpC (40 kDa) present in vesicle preparations of both strains. Silver staining of the same samples demonstrated that much more proteins are present in vesicles, but that their amount was probably not sufficient to be visualized by Coomassie Blue staining (Figure 19B). Immunoblotting with an antibody raised against OmpA, the OMP that served as a marker for the amount of OMVs, revealed that the amount of vesicles was similar in both strains (Figure 19C). These data are consistent with the results from ultrastructural analyses by AFM and EM. As a control for vesicle purity, the cyclic AMP receptor protein (Crp), a cytosolic protein acting as a transcriptional regulator (Forsman et al., 1992), was used. Western blotting with anti-Crp antibodies showed the absence of cytoplasmic proteins in vesicle samples indicating that no contaminating proteins resulting from e.g. bacterial cell lyses were present in the samples.

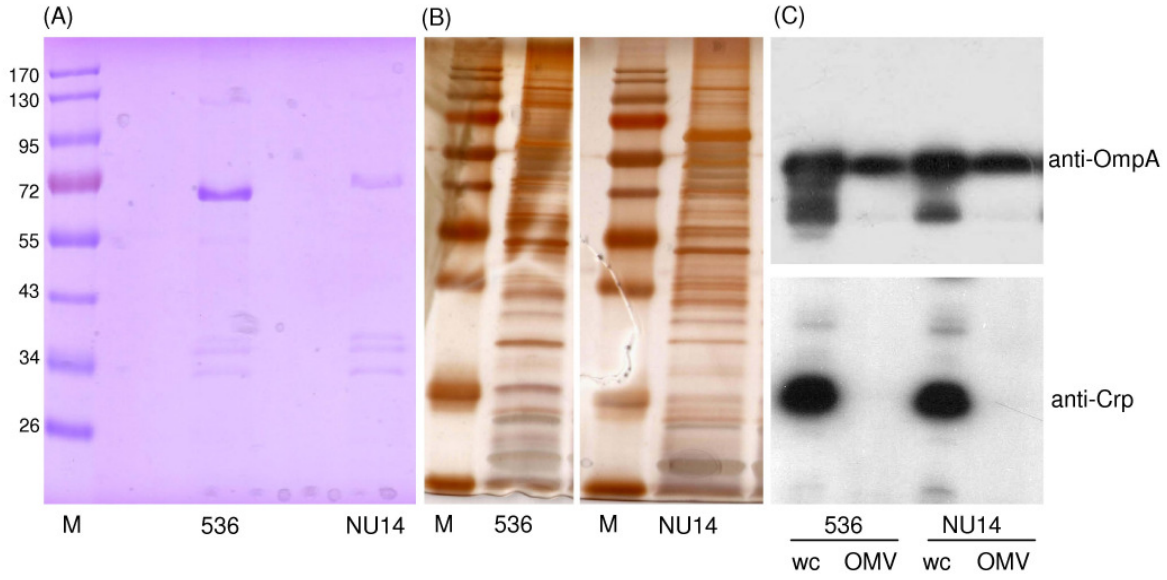

Figure 22. Electrophoretical analyses of vesicle samples isolated from strains 536 and NU14. Coomassie Blue (A) and silver stained (B) 10% SDS-PAGE. (C) Immunoblot analysis of whole cell extracts (wc) and vesicle samples (OMV) using the specific antisera as indicated. M: Marker, numbers indicate molecular weights in kDa.

In order to test if the siderophore receptors FyuA and IroN are present in ExPEC vesicles, Western blot analyses with polyclonal anti-IroN and anti-FyuA antibodies were carried out. Interestingly, we found that IroN was present in 536 and NU14 vesicles, whereas FyuA could be not detected (Figure 23). These observations are in agreement with the low expression level of *fyuA* in the ExPEC strains 536 and NU14. Consequentially, we suggest that FyuA might be present in OMV, but that the amount is not sufficient for detection and therefore the availability of FyuA in OMVs is probably not important for ExPEC virulence. In contrast, we assume that the presence of IroN in ExPEC vesicles might be important for ExPEC virulence.

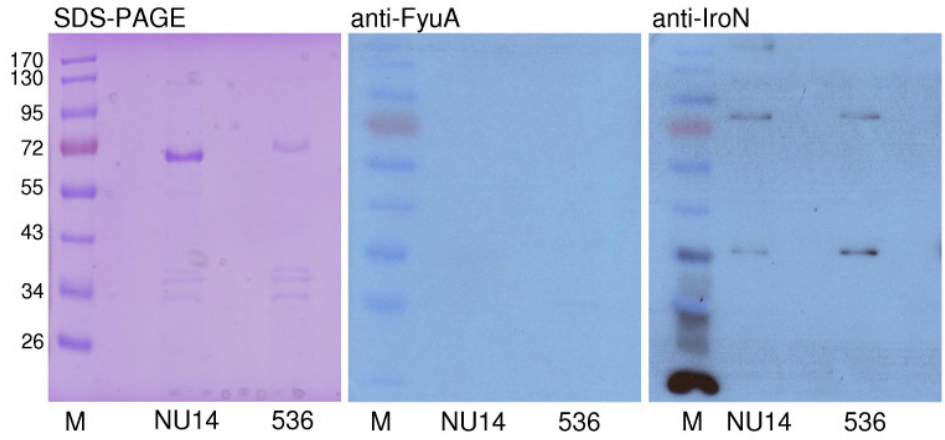

Figure 23. Immunoblot analyses of vesicle samples isolated from NU14 and 536 using anti-FyuA and anti-IroN antibodies. FyuA could be not detected, whereas IroN was present in vesicle samples of both strains. M: Marker, numbers indicate molecular weights in kDa.

4.4.2. IroN containing vesicles associate with urothelial cells

Recently, we found that IroN was involved into urothelial cell invasion. Therefore we supposed that IroN harbouring vesicles could possibly interact with host cells. To investigate if IroN containing vesicles interact with human urothelial HCV29 cells, fluorescence assays were performed. In order to exclude artefacts caused by any other virulence factor which could be present in ExPEC OMVs, we decided to use vesicles from the apathogenic *E. coli* strain HB101 carrying the recombinant plasmids for *iroN* (pRF11) or *fyuA* (pRF12), respectively. To investigate if these vesicles interact with HCV29 cells, cells were incubated with purified vesicles for 24 h at 37°C before immunostaining. Vesicles from HB101 carrying the vector pQE served as negative control. The amount of added vesicles was standardized by Western blotting with anti-*E. coli* antibody raised in rabbit (Figure 24).

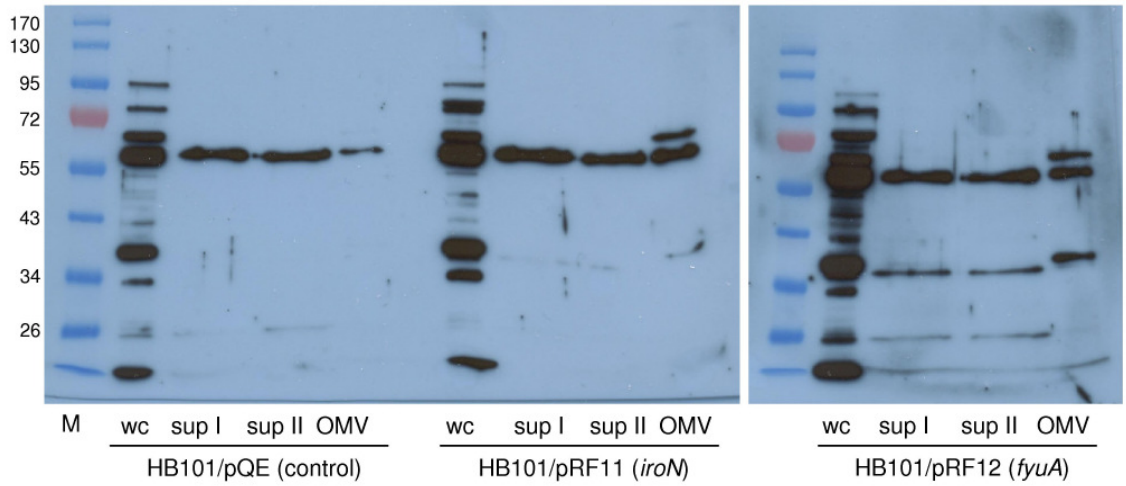

Figure 24. The amount of HB101 vesicles was standardized by Western blots using an anti-*E. coli* antibody. wc: whole cell lysate, sup I: supernatant before and sup II: after ultrazentrifugation, OMV: vesicle samples. M: Marker, numbers indicate molecular weights in kDa.

Immunofluorescence staining was carried out using anti-*E. coli* antibody detected with FITC-labelled anti-rabbit antibody for visualization of vesicles. Nuclei and F-actin were stained with DAPI and phalloidin-rhodamine to examine possible morphological changes of host cells. Interestingly, HCV29 cells that had been incubated with IroN vesicles contained intensely green fluorescent punctate areas (Figure 25A).

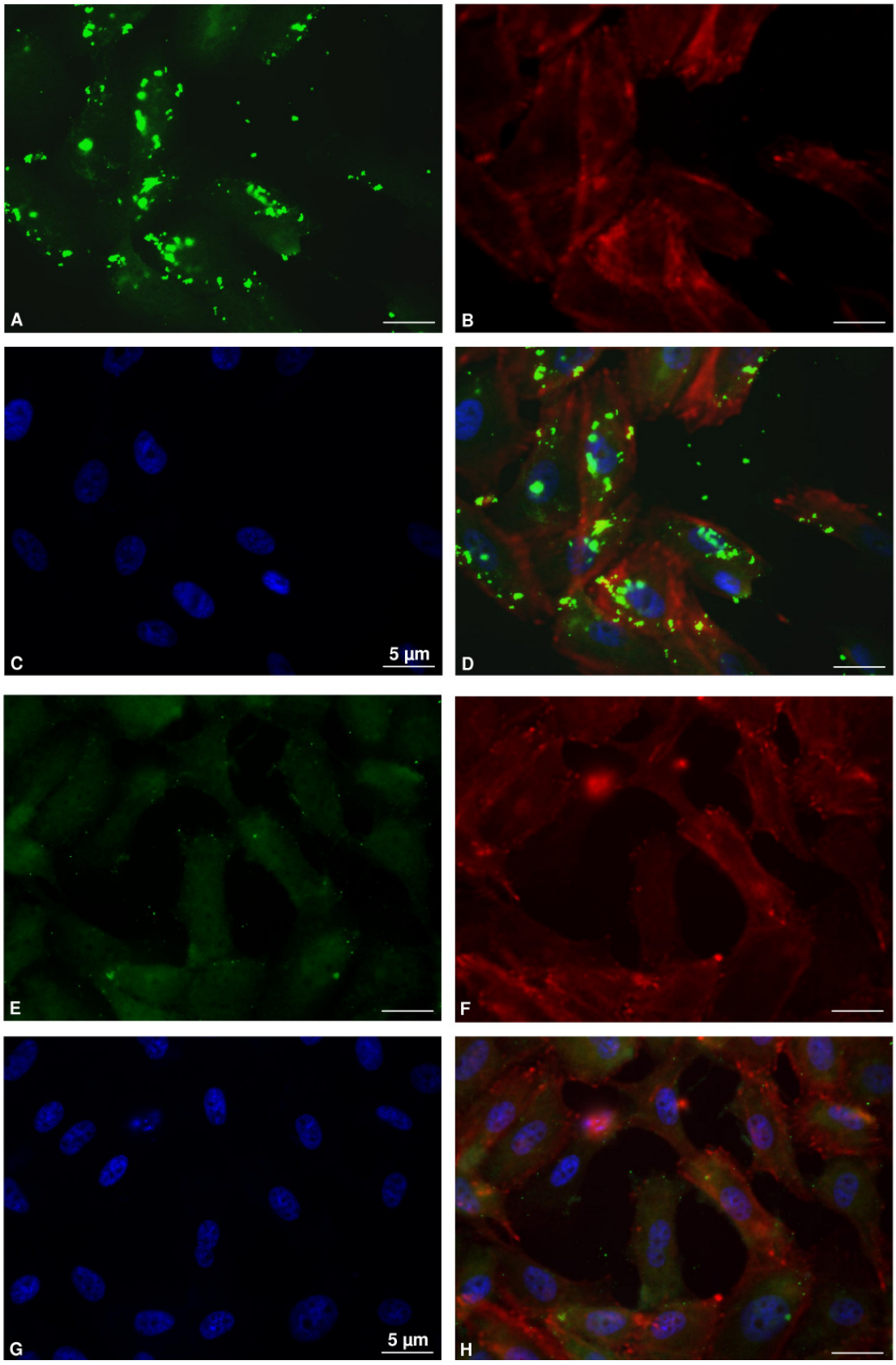

Figure 25. Vesicles from HB101/pRF12 (*iroN*) (A-D) and from HB101/pRF11 (*fyuA*) (E-H) were incubated with HCV29 cells for 24 h and treated with anti-*E. coli* antibody detected using FITC-labelled anti-rabbit antibody (green) (A,E). Phalloidin-rhodamine staining of F-actin (B,F), and DAPI staining (C,G). Merging of images A-C reveales image (D,H). IroN-, but not FyuA-carrying vesicles, were found to associate with HCV29 cells.

Comparison of the localization of green fluorescence spots representing IroN vesicles with the localization of the rhodamine labelled cytoskeleton of host cells (red) (Figure 25A-D), indicated that IroN vesicles were mostly associated with host cells, but were rarely found in the cell free background. This observation let us to suggest, that the fluorescence intensity was caused by IroN vesicles that were bound to host cells. The intensity of fluorescence associated with vesicles increased if the incubation time of host cells with vesicles was increased (data not shown). To test, if the association of OMVs to HCV29 cells was caused by the vesicles per se or by the high expression level of recombinant protein, we incubated HCV29 cells with FyuA-containing vesicles, isolated from HB101/pRF12 (*fyuA*). Interestingly, cells incubated with FyuA-carrying vesicles contained only a low level of dispersed fluorescence (Figure 25E-H). The fluorescence spots were rarely found and spread throughout the background, so that we concluded that FyuA vesicles were not associated with host cells like IroN vesicles. These data indicated that the presence of IroN in vesicles specifically mediated association of vesicles and host cells.

We further investigated the fate of IroN vesicles by examining whether the emergence of punctate fluorescence was caused by attachment of IroN vesicles to host cell or by internalization of these vesicles by the host cell. To distinguish between these two possibilities, inside-outside staining of HCV29 cells incubated with IroN vesicle was performed. Externally applied antibodies are unable to detect internalized antigens and therefore can also be used as probes to indicate internalization. For this, two different labelled secondary antibodies, a Texas Red (TR)-labelled antibody for outside and a FITC-labelled antibody for inside staining were used to visualize extracellular or internalized vesicles, respectively. At a first glance, most of the vesicle spots from different staining co-localised, visible as yellow spots in the overlay picture (Figure 26). However, focusing on special areas, tiny green fluorescent spots which represent most probably intracellular vesicles (marked with an arrow) could be detected. These fluorescent spots were only present in the inside staining (green) but were lacking in the outside staining (red). Taken together, we could show that IroN-carrying vesicles bind to host cells and that a fraction of these vesicles were most likely internalized by the host cell. Furthermore, we observed a ringlike fluorescence around the nucleus, which may indicate accumulation of vesicles in this area. This could be a hint for vesicle trafficking inside the host cells and we therefore suggested that internalized vesicles may enrich in special cell compartments.

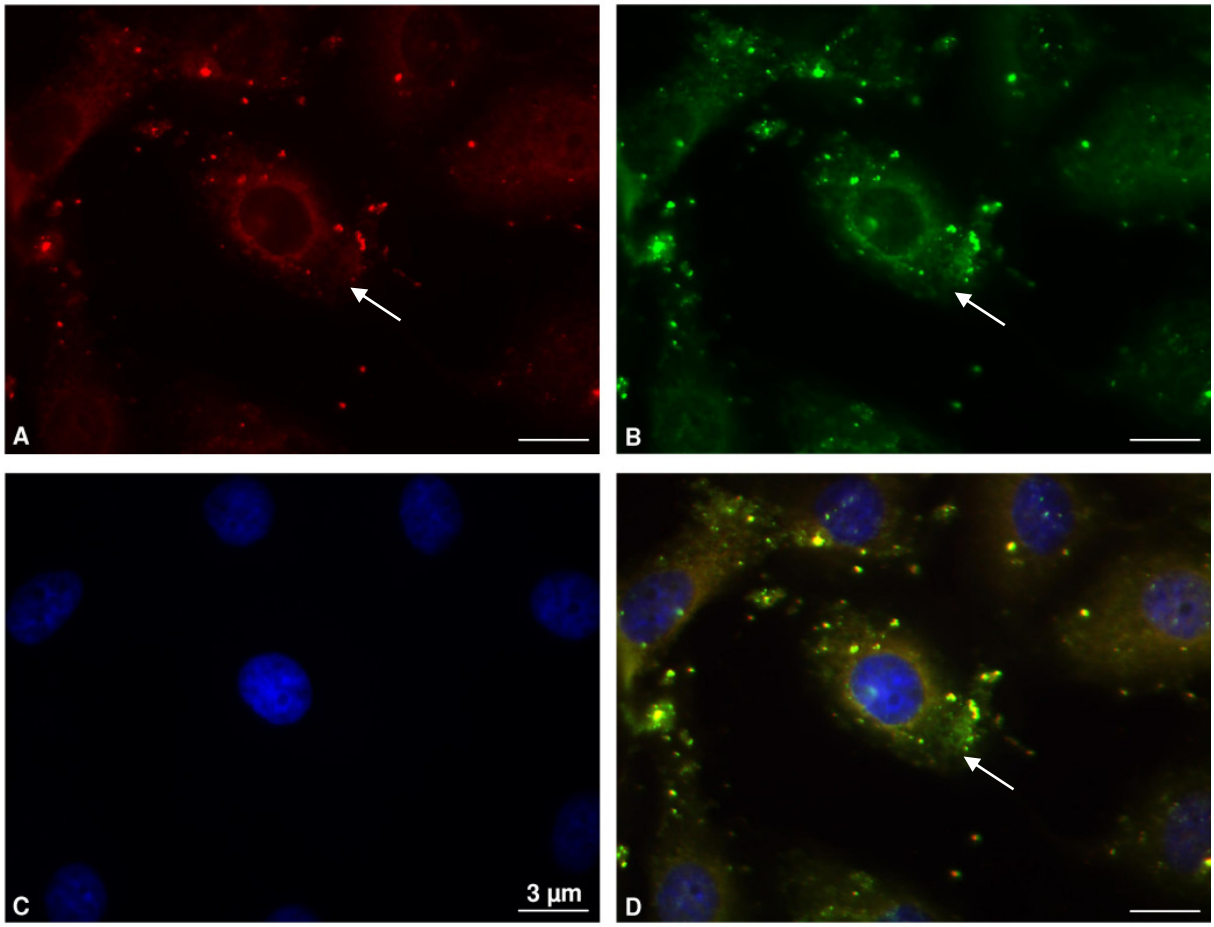

Figure 26. Inside-outside staining of IroN-carrying vesicles and HCV29 cells. Extracellular vesicles (red) were labelled with TR (A) and internalized vesicles (green) were stained with FITC (B), DAPI staining is shown in (C). Merging of images A-C revealed image (D) with yellow spot indicating extracellular vesicles, whereas tiny green spots represent intracellular vesicles (marked with an arrow).

Bacteria and bacterial products can be internalized by host cells via distinct uptake pathways such as endocytosis. To test if IroN-containing vesicles were possibly endocytosed, we used anti-lamp-1 antibody as a marker for late endosomes. IroN vesicles were incubated with HCV29 cells as described above and stained with FITC for vesicles (green) and TR for endosomes (red). As shown in Figure 27, the punctuate staining of vesicles (green) was frequently co-localised with the fluorescent spots derived from labeling with anti-lamp-1 antibody (red) which appeared yellow in the merged images. These data suggest that IroN vesicles or at least vesicle components were endocytosed by the host cell.

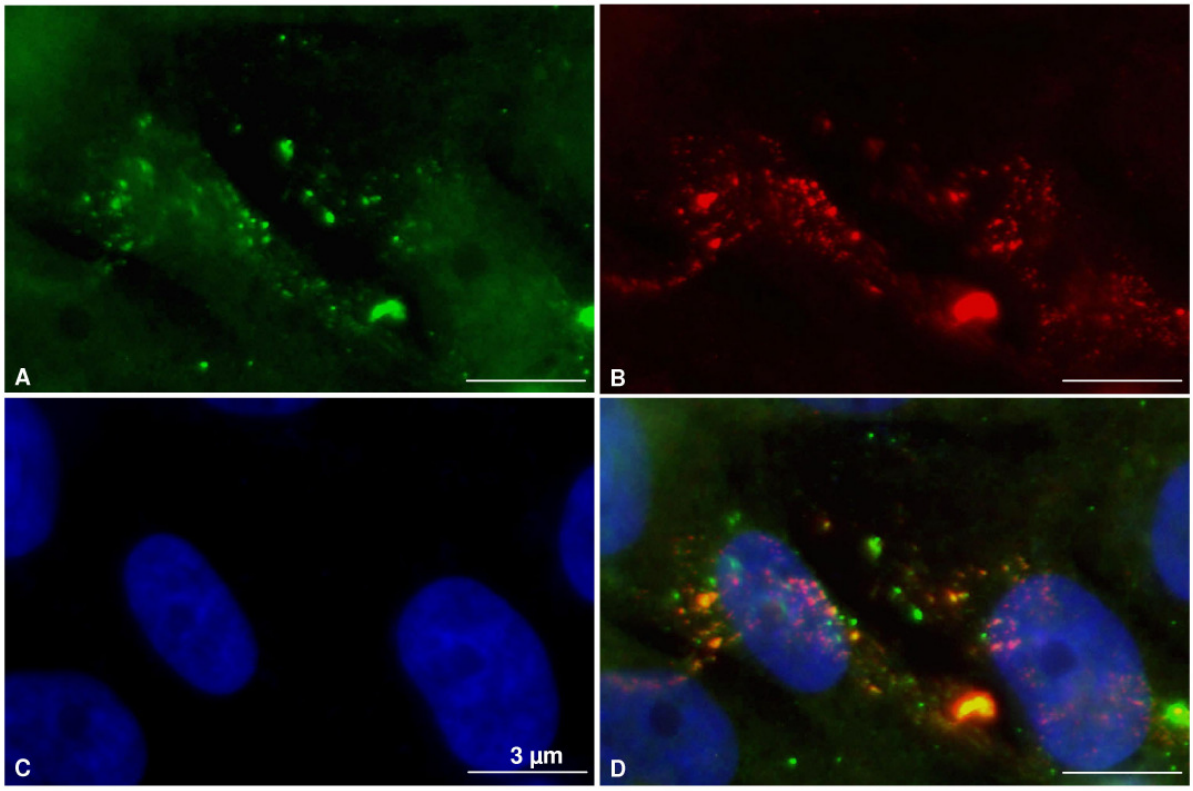

Figure 27. Subcellular localization of IroN-carrying OMVs within late endosomes. HCV29 cells were incubated for 24 h with IroN vesicles (green) (A). Endosomes were labelled with anti-lamp-1 (red) (B) and nuclei were stained with DAPI (C). Merging of images A-C (image D) indicates co-localization of IroN vesicles inside endosomal structures (yellow).

To further examine the subcellular localization of IroN vesicles, golgin 97 was used as eukaryotic marker for the endoplasmatic reticulum (ER) and trans-golgi networks (TGN). IroN vesicles were incubated with HCV29 cells for 20 or 30 h at 37°C before immunostaining with anti-*E. coli* antibody for IroN vesicles (green) and anti-golgin 97 for TGN (red). As demonstrated by confocal microscopy (Figure 28), IroN vesicles co-localised with the TGN. After 20 h, vesicles and TGN were found at cell poles and after 30 h vesicles co-localized with TGN at the site of the endoplasmatic reticulum around the nucleus of host cells (Figure 28C,F). These data indicate that vesicles were apparently intracellularly transported from the host cell membrane to the nucleus in a time dependent manner. These observations support the assumption that IroN vesicles were internalized by host cells and accumulate in an intracellular compartment distinct from the plasma membrane. IroN vesicles bind to host cells, trigger endocytosis and vesicles or vesicle components were then subsequently intracellular trafficked from the host cell membrane to the nucleus, where they were enriched in compartments of the endoplasmatic reticulum.

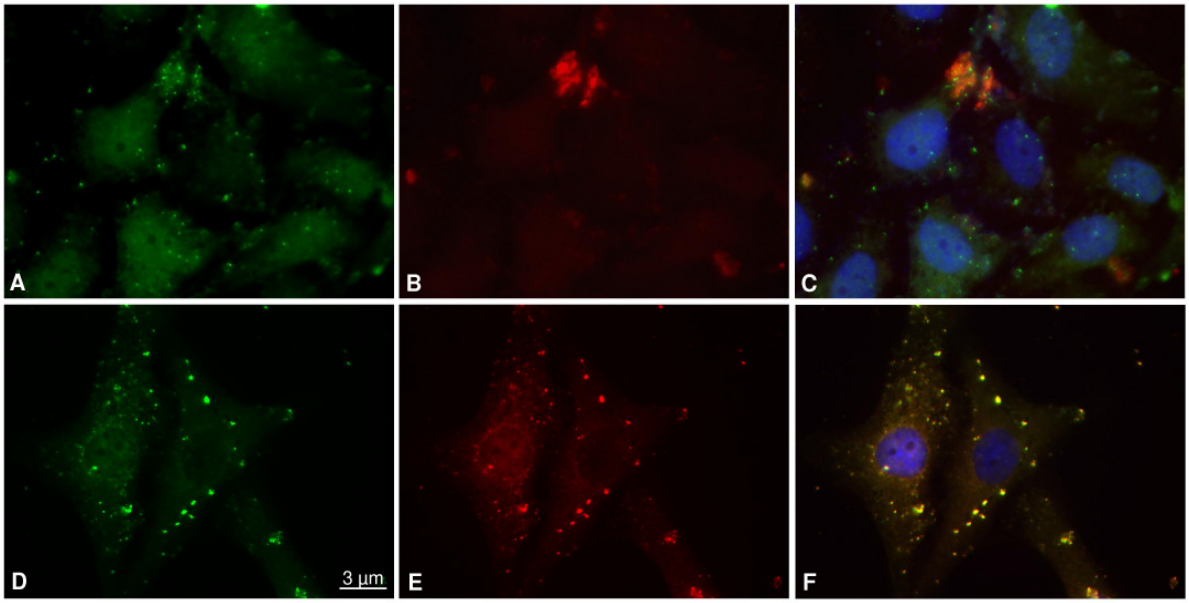

Figure 28. IroN vesicles co-localise with trans-golgi networks of HCV29 cells. Vesicles were labelled with FITC (A,D) and TGN were stained with anti-golgin 97 detected with TR-labelled anti-mouse antibody (B,E), merged images (C, F) indicate co-localization of vesicles and TGN (yellow). After 20 h (upper panel) TGN (red) and vesicles (green) are localized at the cell poles but after 30 h they could be detected near the nucleus (lower panel) indicating time dependent intracellular trafficking of vesicles.

4.5. Hemolytical active α-hemolysin is present in ExPEC vesicles

4.5.1. OMV isolation and characterization of the protein composition

As described in a previous section, OMVs isolated from 536 and NU14 Δ*fyuA* and Δ*iroN* mutant strains were used as a control in Western blot analyses with anti-FyuA and anti-IroN antibodies. Interestingly, comparing the protein pattern of isolated vesicles by electrophoresis a prominent band with an approximated molecular weight of 110 kDa could be detected in the vesicle samples of the 536Δ*fyuA* and 536Δ*iroN* mutant strains, that was absent from 536 wt vesicles (Figure 29A). This band was also not visible in any vesicle preparations of the NU14 strains (data not shown). As ExPEC strains are known to produce α-hemolysin, a secreted exotoxin with cytolytic activity, we speculated if the detected 110 kDa sized protein may corresponds to α-hemolysin (HlyA). To prove this, Western blot analyses with a specific polyclonal anti-HlyA antibody were performed with vesicle samples of 536 wt as well as Δ*fyuA* and Δ*iroN* mutant strains. As suggested, the specific antibodies reacted with a 110 kDa protein that was present in high amounts in 536Δ*fyuA* and 536Δ*iroN* strains and in a lower amount in 536 wt vesicle samples (Figure 29B). This observation confirmed our suggestion that the detected protein band represents α-hemolysin.

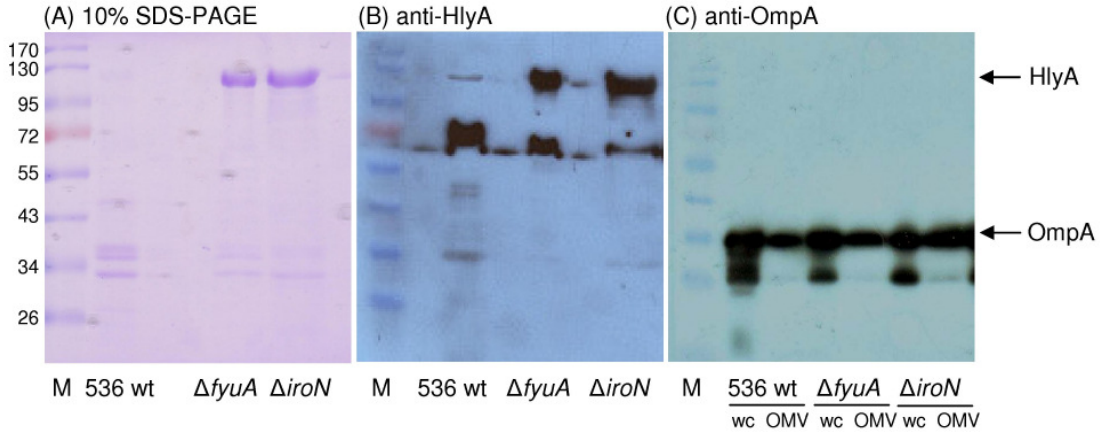

Figure 29. Electrophoretical analysis of vesicle samples isolated from 536 wt, 536Δ*fyuA* and 536Δ*iroN* strains. Corresponding Coomassie Blue stained 10% SDS-PAGE (A), immunoblot with anti-HlyA (B) and anti-OmpA (C) antibodies as indicated. M: Marker, numbers indicate molecular weights in kDa. wc: whole cell samples, OMV: vesicle samples.

We further suggested that the high amount of α-hemolysin was possibly caused by increased vesicle production by the mutant strains. However, immunoblotting of all vesicle samples with an anti-OmpA antibody indicated that all strains produced apparently the same amount

of OMVs (Figure 29C). Further, we could exclude contamination of vesicle samples with cytoplasmic proteins, as Western blot analysis with anti-Crp antibodies verified the absence of the cytoplasmic protein Crp in vesicle samples (data not shown).

The α-hemolysin is an exotoxin that is frequently associated with strains isolated from extraintestinal infections. Therefore, we wondered if the ExPEC wt strains 536 and NU14 did not secrete α-hemolysin or if the toxin was not included into vesicles. This is of particular interest, as both strains showed hemolysis when grown on blood agar plates. To investigate this, Western blotting with anti-HlyA antibody of whole cell extracts, supernatant and vesicle samples of 536 and NU14 wild-type strains were carried out. Interestingly, we could observe that both strains secreted HlyA, but that only low amounts of α-hemolysin were detectable in the vesicle samples of both wild-type strains (data not shown). Taken together, we found that α-hemolysin was present in ExPEC 536 vesicles and NU14 vesicles, but that the level of α-hemolysin was significantly higher in vesicles isolated from 536Δ*fyuA* and 536Δ*iroN* mutant strains than in the 536 wt vesicles.

To investigate if the increased association of HlyA with mutant vesicles was directly caused by mutation of the siderophore receptor genes *fyuA* and *iroN*, the mutant strains were complemented by transformation of plasmids carrying the respective target gene under the control of its native promoter. Immunoblotting of vesicles samples isolated from mutant and complemented strains with anti-HlyA antibodies showed a similar, α-hemolysin reduced, phenotype in vesicles of complemented and wild-type strains. These data confirmed that the mutations of *iroN* and *fyuA* were responsible for the abundant presence of α-hemolysin in the mutant vesicles. Next, we examined if overexpression of IroN or FyuA in the 536 wt strain possibly reduced the level of vesicle-associated α-hemolysin. Therefore, the amount of the toxin in vesicles from wild-type and recombinant 536 strains (536/p*fyuA* and 536/p*iroN*) was determined by Western blotting. However, no difference in the amount of vesicle-associated HlyA could be detected between wild-type and recombinant strains (data not shown).

4.5.2. The α-hemolysin is tightly associated with vesicles

The above results could mean that the α-hemolysin was either physically associated with mutant vesicles or copurified together with these vesicles. To distinguish between these two possibilities the binding pattern of α-hemolysin to 536 mutant and wild-type vesicles was studied by means of a dissociation assay. For this, vesicle preparations were incubated in absence or presence of NaCl, Na_2CO_3 or urea to detach loosely bound α-hemolysin or treated with Triton X-100 to disrupt the vesicle membrane. The results indicated that treatment of vesicles with high salt concentration (either 1 M NaCl or 0.1 M Na_2CO_3) did not extract the α-hemolysin from the vesicles to any greater extent than that observed with buffer alone (Figure 30). Moreover, dissociation assays with increasing concentrations of urea (0.8, 1.5 and 8 M) did also not affect association of α-hemolysin to mutant vesicles showing that the interaction of the α-hemolysin with the vesicles was highly resistant to the urea treatment. We conclude from this, that protein aggregation could not be the reason for the presence of the α-hemolysin in mutant vesicles. However, treatment with non-ionic detergents (0.5% Triton X-100) liberated all the α-hemolysin to the supernatant as a result of vesicle membrane disruption (Figure 30). Control experiments using soluble α-hemolysin did rule out the possibility that the α-hemolysin simply precipitated in presence of the high concentration of salts and thereby was recovered together with the vesicles (data not shown). We could therefore exclude the possibility that the abundant presence of α-hemolysin in the mutant vesicles was caused by copurification of free α-hemolysin from the supernatant. Rather α-hemolysin appeared to be tightly associated with the mutant vesicles and the toxin only appeared in soluble form when the membrane structure was disrupted.

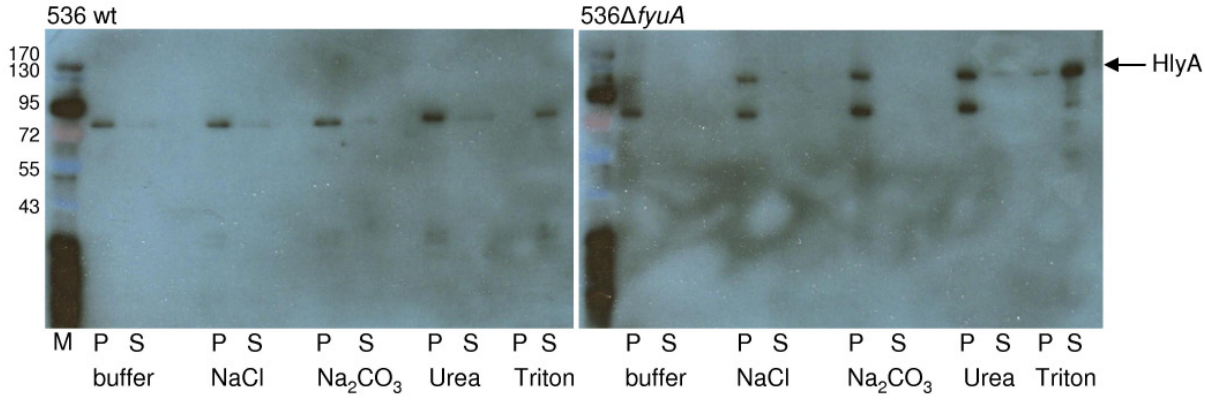

Figure 30. Western blot analyses with anti-HlyA antibodies of samples derived from the dissociation assay. α-hemolysin was tightly associated with vesicles as only treatment with non-ionic detergents, but not high salt concentration could liberate the toxin from the vesicle membrane to the supernatant in the mutant vesicle samples. P: vesicle pellet, S: supernatant. M: Marker, numbers indicate molecular weights in kDa.

4.5.3. Secretion level of HlyA

A possible explanation for the abundant presence of HlyA in the mutant vesicles was the possibility, that the mutant strains secreted higher amounts of α-hemolysin than the 536 wt strain. To investigate this, we compared the secretion level of α-hemolysin in bacterial whole cell extracts, supernatant and vesicle samples of wt and mutant strains. For this, the total amount of secreted HlyA in the supernatant before ultracentrifugation (sup I) and the level of remaining HlyA in the supernatant after ultracentrifugation (sup II) were compared with that in vesicle samples by immunoblot analyses using anti-HlyA antibodies (Figure 31). The 536Δ*hlyA* mutant strain served as negative control for the immunoblot with anti-HlyA. As expected, the total amount of secreted α-hemolysin appeared to be similar in wild-type and mutant strains (Figure 31, sup I). In the case of both mutant strains, most of the secreted HlyA was associated with vesicles (85%) and a lower percentage (15%) was found in the vesicle-free supernatant (sup II). In the 536 wt strain however, most of the secreted α-hemolysin was present as free soluble α-hemolysin in the supernatant (sup II), while only low amounts of vesicle associated HlyA (OMV) could be detected. Taken together, the secretion level of α-hemolysin seemed to be not increased in the mutant strains, as the amount of secreted HlyA in the supernatant before ultracentrifugation (sup I) was comparable in all strains. This is in agreement with the finding that the presence of the channel forming protein TolC was not increased in the mutant strains.

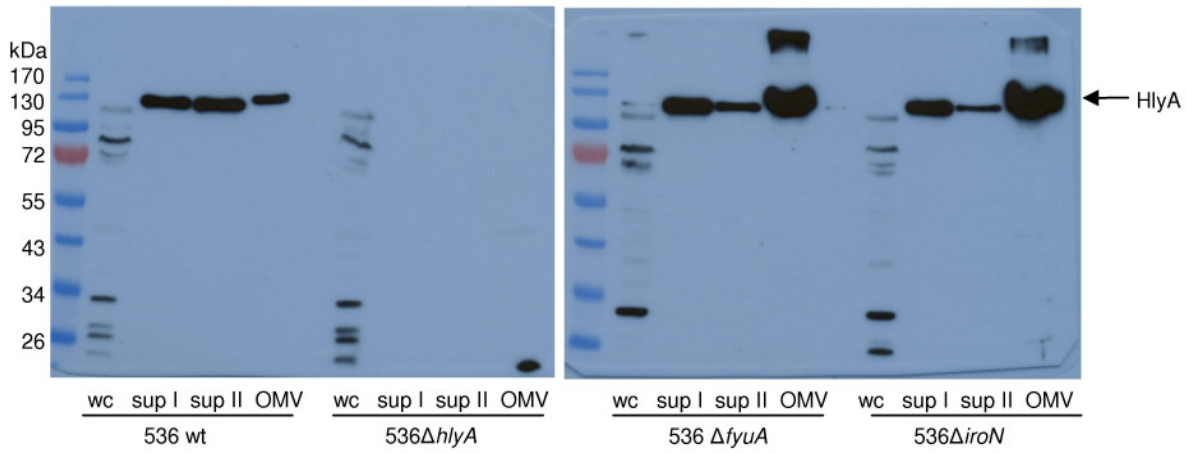

Figure 31. Secretion level of HlyA. Immunoblot analysis with anti-HlyA antibodies and whole cell extract (wc), supernatant before (sup I) and after (sup II) ultracentrifugation and vesicle samples (OMV) revealed similar secretion level (lane II) in wt and *fyuA* and *iroN* mutant strain. However, the amount of HlyA in OMV samples (lane III) was significant higher in the mutants than in the wt strain. M: Marker, numbers indicate molecular weights in kDa.

4.5.4. Free α-hemolysin can reassociate with OMV

The results described above prompted us to investigate if free α-hemolysin was possibly able to reassociate with mutant vesicles after vesicle shedding. Therefore, reassociation assays with culture supernatant of 536Δ*fyuA* and 536Δ*iroN* mutant strains containing high amounts of free α-hemolysin and vesicle samples were performed. As shown in Figure 32, free soluble α-hemolysin was present in the supernatant after ultracentrifugation (sup II) of Δ*fyuA* and Δ*iroN* and to a less amount also in 536 wt supernatant samples (Figure 32A). After incubation of 536 wt vesicles with the different supernatants, HlyA was reassociated from Δ*fyuA*, Δ*iroN* and wt supernatant to 536 wt vesicles (Figure 32B). In contrast, HlyA from Δ*fyuA* and Δ*iroN*, but not from wt supernatant was found to reassociate with Δ*hlyA* vesicles (Figure 32C). We conclude that α-hemolysin is not necessarily linked with vesicles during OMV formation, but rather α-hemolysin can be spontaneously reassociate with existing vesicles in the extracellular milieu. Interestingly, α-hemolysin, which was rarely present in the wild-type supernatant, could bind to 536 wt but not to Δ*hlyA* mutant vesicles. As α-hemolysin has a high affinity to aggregate with other α-hemolysin molecules, we suggested that attachment of free α-hemolysin to OMVs is advantaged when α-hemolysin is already present in vesicles. This observation was supported by the AFM pictures, where HlyA-containing 536Δ*fyuA* vesicles seemed to be more aggregated than HlyA-depleted 536 wt vesicles (Figure 34, p.112).

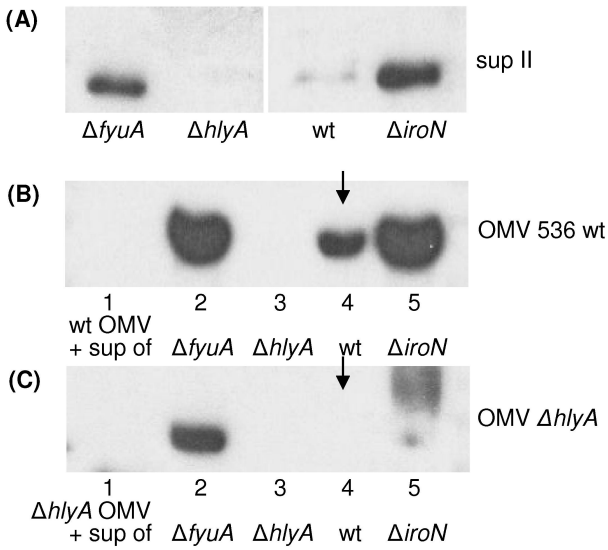

Figure 32. Reassociation of α-hemolysin to vesicle samples was detected by immunoblotting with anti-HlyA antibodies. (A) Presence of free HlyA in the supernatant after ultracentrifugation (sup II). (B) HlyA from supernatant of Δ*fyuA* and Δ*iroN* and wt reassociates to 536 wt vesicles. (C) HlyA from Δf*yuA* and Δ*iroN* but not of wt supernatant reassociates to Δ*hlyA* vesicles.

4.5.5. HlyA associated with OMVs is hemolytically active

α-hemolysin is one of the most important virulence factors involved in the pathogenesis of ExPEC, as the secreted exotoxin has cytolytic and/or cytotoxic activity against a wide range of mammalian cell types (Lally et al., 1999). To test if the α-hemolysin found in ExPEC 536 vesicles had hemolytic activity we assayed vesicle samples from all strains in a hemolytic contact assay. In presence of $CaCl_2$ which is necessary for hemolytic activity, OMVs isolated from Δ*iroN* and Δ*fyuA* mutant strains showed a significant higher hemolytic activity than 536 wt vesicles (Figure 33). In detail, lyses of erythrocytes were directly related to the amount of HlyA detected by immunoblotting. Furthermore, vesicles isolated from NU14 strains did not show hemolytic activity (data not shown).

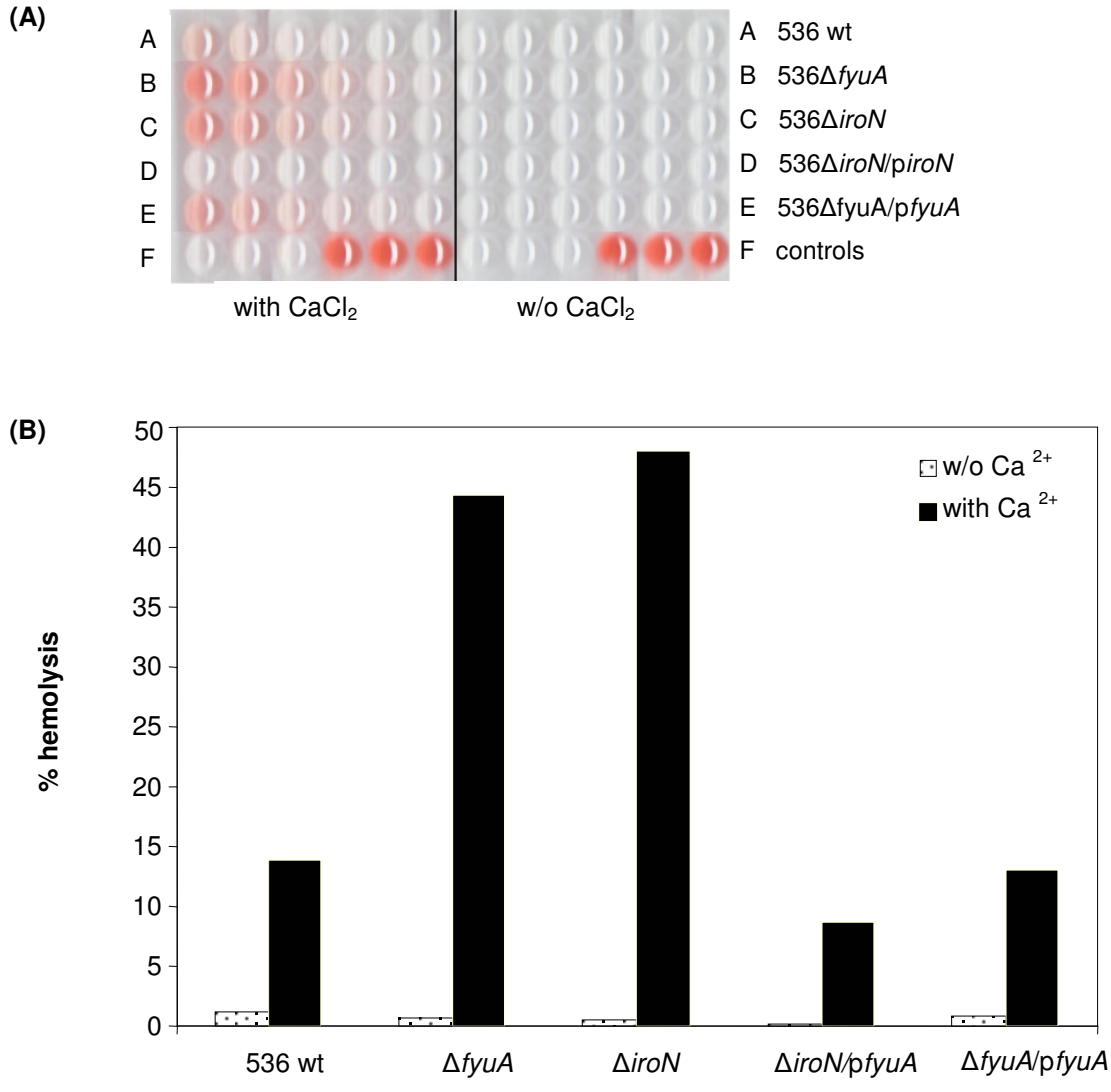

Figure 33. (A) Hemolytic activity assay with vesicle samples of 536 wild-type, mutant and complemented strains. (B) Hemolytic activity was measured as OD_{540} after 1 h incubation at 37°C in either absence (dotted bars) or presence (black bars) of 10 mM $CaCl_2$.

The activity of the α-hemolysin associated with OMVs was also tested on nucleated cells by monitoring the induction of cell detachment using HCV29 cells monolayers. When testing either the bacterial cultures or the cell-free supernatants (including OMVs) from the different 536 strains we observed massive loss of the HCV29 cell monolayer with more than 50% of the cells being detached after 90 min (data not shown). A similar level of cell monolayer disruption was observed when OMVs from these strains were tested. However, there was no significant difference detectable between vesicles, supernatant or bacterial between the different strains. Similar results were obtained by measuring the cytolytic activity detectable as the release of cytoplasmic lactate dehydrogenase (LDH) from infected HCV29 cells after 6 h incubation using the CytoTox96 assay (data not shown). Taken together, these results could not clearly confirm that the α-hemolysin present in OMVs was the main cause of cell detachment or cytotoxicity in these non-lytic assay systems, but rather there are possibly other factors present in the tested samples which disturbed the monolayers.

4.5.6. Altered protein composition and morphology in α-hemolysin containing vesicles

The selective presence of α-hemolysin in the mutant vesicles, but not in wild-type vesicles prompted us to investigate if vesicles isolated from the different strains had possibly a different morphology. Therefore, ultrastructural analyses were performed by electron microscopy (EM) and atomic force microscopy (AFM) (Figure 34). Interestingly, electron microscopic characterization of α-hemolysin containing vesicles isolated from mutant strains revealed two different subpopulations: smaller vesicles and vesicles with a bigger size and a thicker margin, whereas the wild-type vesicles lacking α-hemolysin were more homogenous in both, size and morphology (Figure 34B). These different appearances prompted us to hypothesize that the larger vesicle structures, which were more common in mutant strains possibly contain α-hemolysin. In fact, at higher magnification we observed a small ring-like structure with a central electron-dense core in these mutant vesicles. These structures were suspected to represent HlyA protein assemblies similar to the structures found in vesicles containing the pore-forming cytotoxin ClyA (Wai et al., 2003a). Moreover, AFM pictures revealed that the α-hemolysin containing mutant vesicles seemed to be more aggregated than the wild-type vesicles (Figure 34A), possibly due to the higher amount of α-hemolysin present in those vesicles.

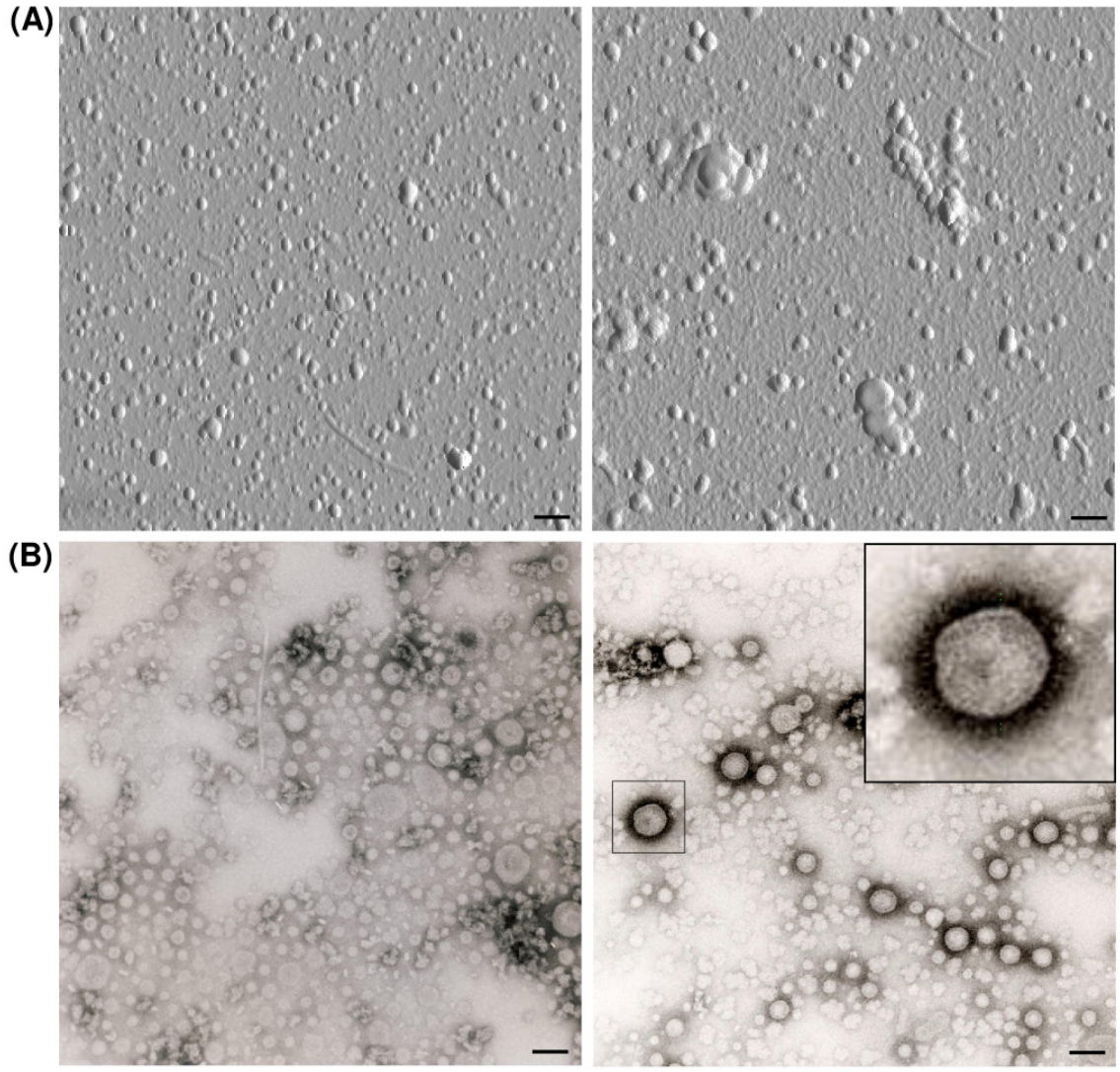

Figure 34. Ultrastructural analysis of vesicle samples. A: Atomic force microscopy picture of OMVs from *E. coli* 536 wild-type (left) and the 536Δ*fyuA* mutant strain (right). B: Electron microscopy picture of the same samples. Bar equals to 200 nm.

In order to further characterize the different subpopulation of vesicles, vesicle preparations from wild-type and mutant strains were fractionated by density gradient centrifugation. After centrifugation, 20 different fractions were removed and further analysed by immunoblotting (Figure 35). In general, the fractions 7-17 contained the highest amount of vesicles as determined by the presence of OmpA. Furthermore, α-hemolysin was mostly present in the fractions 8 to 10, and mutant vesicles contained higher amounts of α-hemolysin than wild-type vesicles (Figure 35A). Consistently, the fractions with the highest amount of α-hemolysin were the most hemolytically active fractions (Figure 35B).

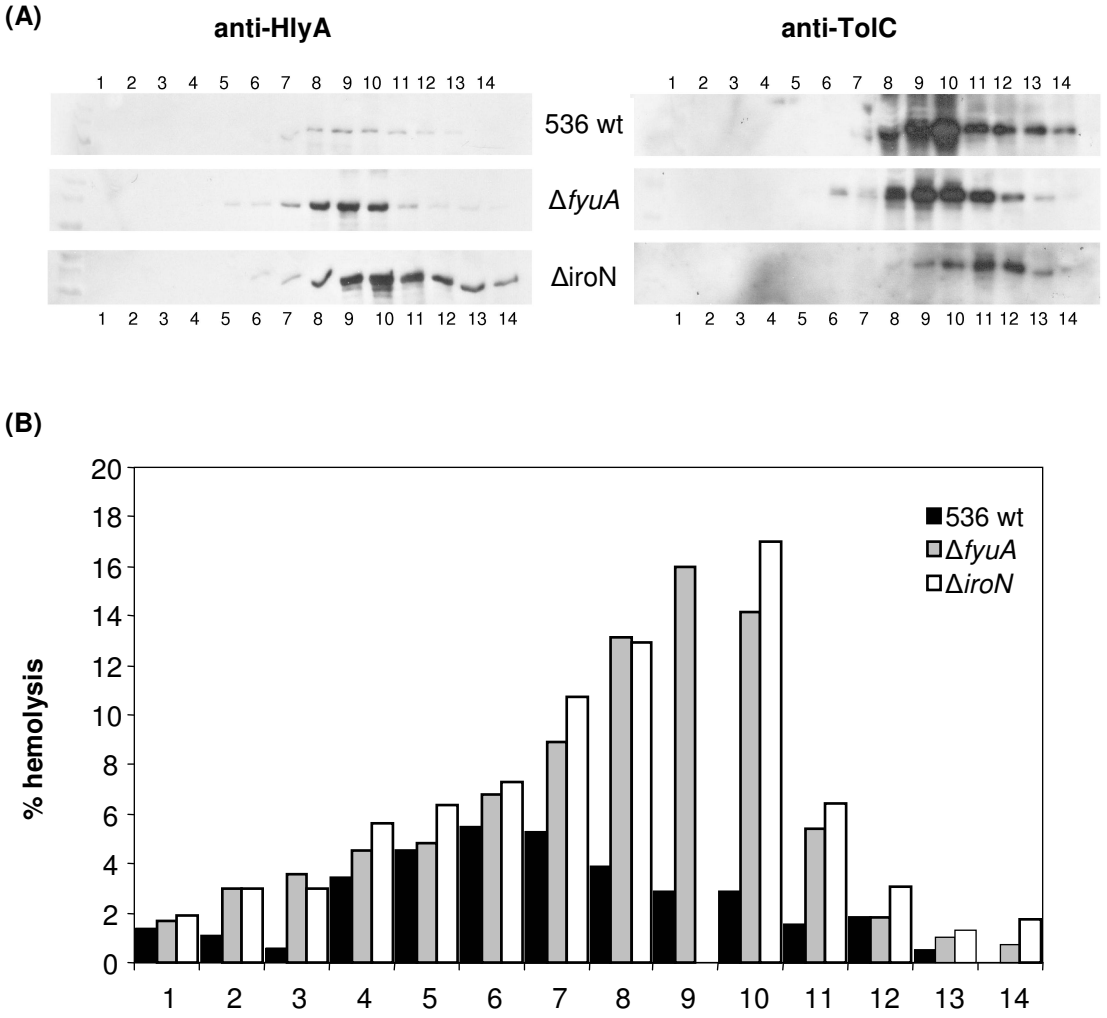

Figure 35. Vesicle fractionation. (A) Immunblot analysis of fractions 1-14 after gradient centrifugation of vesicle samples derived from 536 wt and mutant strains using anti-HlyA and anti-TolC antibodies. (B) Corresponding hemolytic activity of the same vesicle samples as shown in A.

Interestingly, the channel forming protein of the type I secretion system, TolC, could be detected in the same fractions containing the α-hemolysin. However, the amount of TolC was notably higher in 536 wt vesicles than in vesicles from Δ*iroN* and Δ*fyuA* mutant strains (Figure 35A). Taken together, we found that α-hemolysin containing mutant vesicles contained less TolC, whereas α-hemolysin reduced wild-type vesicles comprised higher amounts of TolC. Further Western blot experiments with anti-TolC antibody revealed that less TolC was present in mutant vesicles, whereas the amount of TolC was comparable in whole cell and outer membrane preparations of mutant and wild-type strains (Figure 36). In conclusion, we found that the amount of TolC was similar in bacterial strains, but differed in vesicles produced by these stains: wt vesicles contained less HlyA, but a normal amount of TolC, whereas mutant vesicles contained abundant amounts of HlyA and less TolC.

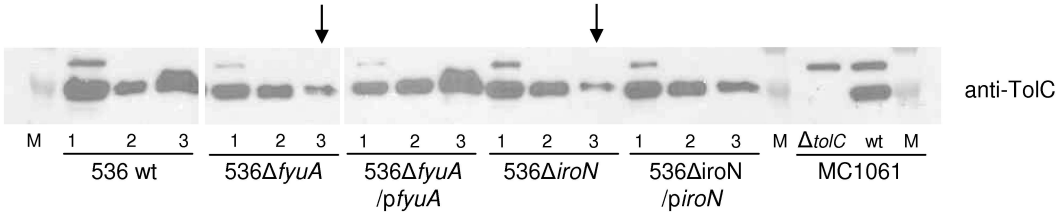

Figure 36. The amount of TolC was determined by immunoblot analysis of whole cell extracts (1), OMP (2) and OMV samples (3) using anti-TolC antibodies. The *fyuA* and *iroN* mutant vesicles (marked with an arrow) contained less TolC compared to wt vesicles, whereas the amount of TolC was similar in whole cell and OMP samples of all strains.

4.5.7. α-hemolysin was significantly more associated with mutant vesicles - but why?

In order to find an explanation for the improved association of α-hemolysin with the mutant vesicles, we proved several possibilities. First, the transcriptional level of *hlyA* was examined by means of TaqMan analyses. For this, total RNA was isolated from *E. coli* 536 wild-type and all mutant strains and subsequently treated with DNase to remove contaminating DNA. Total RNA was reversely transcribed and the resulting cDNA was then used as template for TaqMan analyses with specific *hlyA* primers. The results indicated that the transcriptional level of *hlyA* was not influenced by mutation of the *fyuA* or *iroN* gene respectively, as the *hlyA* level were comparable in 536 wild-type, mutant strains and complemented strains (Figure 37).

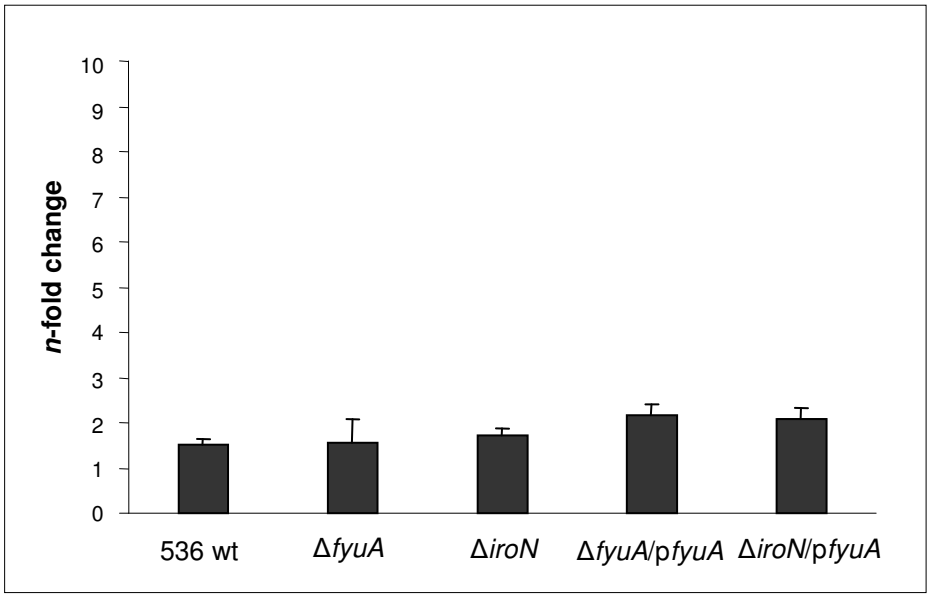

Figure 37. Transcriptional level of *hlyA* revealed by Taqman analyses indicated comparable *hlyA* level in wt, mutant and complemented strains.

Next, we wondered if a mutation of the *iroN* or *fyuA* gene possibly affected other outer membrane proteins and this in turn could influence the association of HlyA to mutant vesicles. To test this, outer membrane proteins were isolated and their protein pattern was compared by electrophoresis followed by silver staining of the gels. However, no significant differences in the outer membrane pattern between wild-type and mutant strains could be detected (data not shown). Furthermore, no significant differences in the outer membrane profile were visible when we compared wild-type and mutant OMPs in 2D gels (data not shown). Even vesicle samples of the distinct strains revealed almost identical protein pattern,

except for the amount of α-hemolysin when they were analysed by silver stained 1D or 2D gels.

Recently, it was described that HlyA has a high affinity to LPS (Horstman et al., 2004). As OMVs represent a subset of the outer membrane and contain therefore high amounts of LPS, it could be possible that free α-hemolysin was associated with vesicles by binding to vesicle LPS. To investigate if mutations of *fyuA* or *iroN* might affect the LPS composition of OMVs, LPS was isolated from OMVs as well as from bacterial strains, separated by electrophoresis and silver stained. However, there was no visible alteration in the LPS pattern, neither in the different bacterial nor in vesicle LPS samples. Immunoblot experiments using specific antibodies directed against different O/K-serotypes indicated a slightly higher amount of oligonucleotide side chains in the LPS samples prepared from the Δ*fyuA* mutant strain (Figure 38).

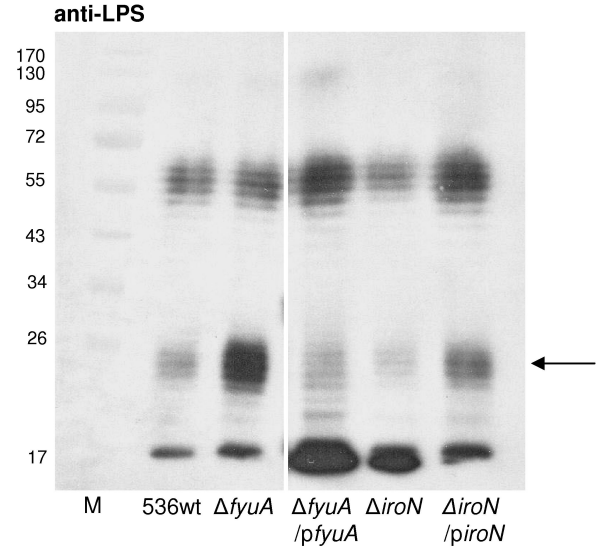

Figure 38. Immunoblot analysis of LPS preparations from OMVs using anti-O/K serotype antibodies indicated a slightly different amount of the oligosaccharide side chains (marked with an arrow). M: Marker, numbers indicate molecular weights in kDa.

Interestingly, LPS isolated from different vesicle fractions separated by gradient centrifugation showed a similar LPS profile: the oligosaccharide part was stronger present in the HlyA-containing 536Δ*fyuA* than HlyA-depleted 536 wt vesicle fractions (data not shown).

In order to further confirm the different expression of oligosaccharide side chains we decided to perform TaqMan analyses with primers specific for several LPS biosynthesis genes (*waaL*, *waaA*, *waaV*). These genes are present on the LPS biosynthesis gene cluster *waa* of the ExPEC strain 536 (Figure 39).

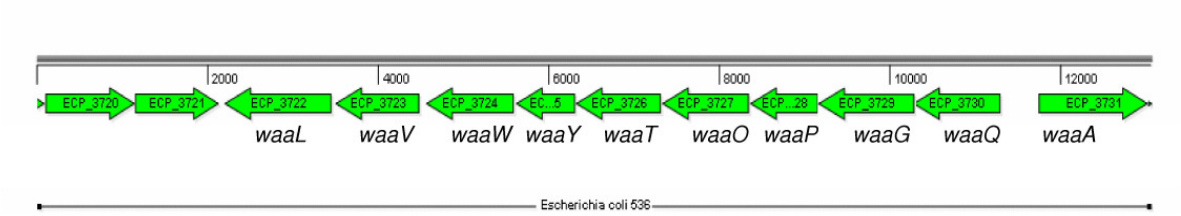

Figure 39. LPS biosythesis gene cluster (*waa*). Three genes were selected as target genes for TaqMan analyses. These genes are *waaL* encoding for a lipid A-core surface polymer ligase, *waaV* for a β 1-3 glycosyltransferase and *waaA* for a 3-deoxy-D-mannose-octulosonic-acid transferase.

Interestingly, the transcription profile of some of these genes was also found to be affected by *fyuA* or *iroN* mutation (Figure 40). In detail, the transcriptional levels of *waaV* and *waaL* were almost identical and signifcantly higher than that of *waaA*. Further, the level of *waaV* and *waaL* transcripts was about 5-fold decreased in the 536Δ*fyuA* mutant strain compared to the wild-type. In contrast, the level of these genes was up to 2-fold increased in the 536Δ*iroN* mutant strain. However, complementation of the mutant strains with plasmids carrying the respective target genes (p*fyuA* and p*iroN*) did not equalize the transcript levels to that of the 536 wt strain.

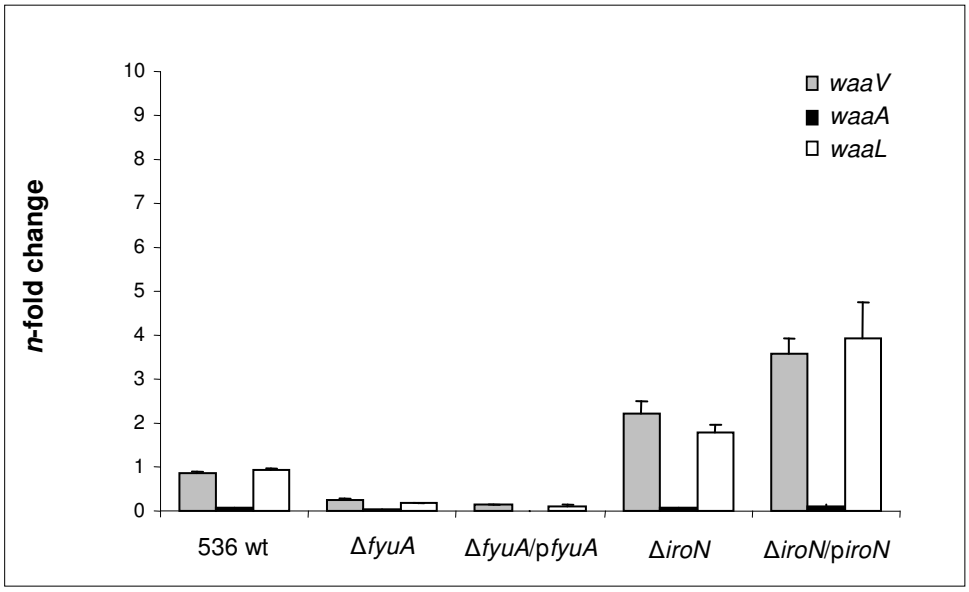

Figure 40. Different transcriptional level of *waaL*, *waaA* and *waaV* could be detected by TaqMan analysis.

These data suggest that the LPS profile was probably different in wild-type and mutant strains, as some parts of the oligosaccharide side chains were higher expressed in the 536Δ*fyuA* mutant strain and the transcription levels of some biosynthetic genes differed between wild-type and mutant strains. However, we could not provide evidence about a significant distinct LPS phenotype in these strains.

4.6. Influence of ExPEC vesicles on the cytokine profile of urothelial cells

Recently, it has been reported that epithelial cells produce IL-8 in response to *H. pylori* and *P. aeruginosa* vesicles (Bauman and Kuehn, 2006; Ismail et al., 2003). Furthermore, it is well known that bacterial LPS stimulate the production of a large array of immunomodulators from human monocytes. HCV29 human urothelial cells exemplify a cell type that ExPEC strains may encounter *in vivo*. However, as the cytokine repertoire of epithelial cells lining the human UT is limited (Hang et al., 1998), we decided to investigate the cytokine profil of vesicle stimulated murine J774 macrophages.

For this, J774 cells were incubated with vesicle samples of HlyA-containing (Δ*iroN* and Δ*fyuA*) and HlyA-deficient (wt and Δ*hlyA*) 536 strains. The amounts of added vesicle samples were standardized by Western blotting using an anti-*E. coli* antibody and the amount of HlyA present in OMV samples was determined by Western blotting using anti-HlyA antibody (Figure 41).

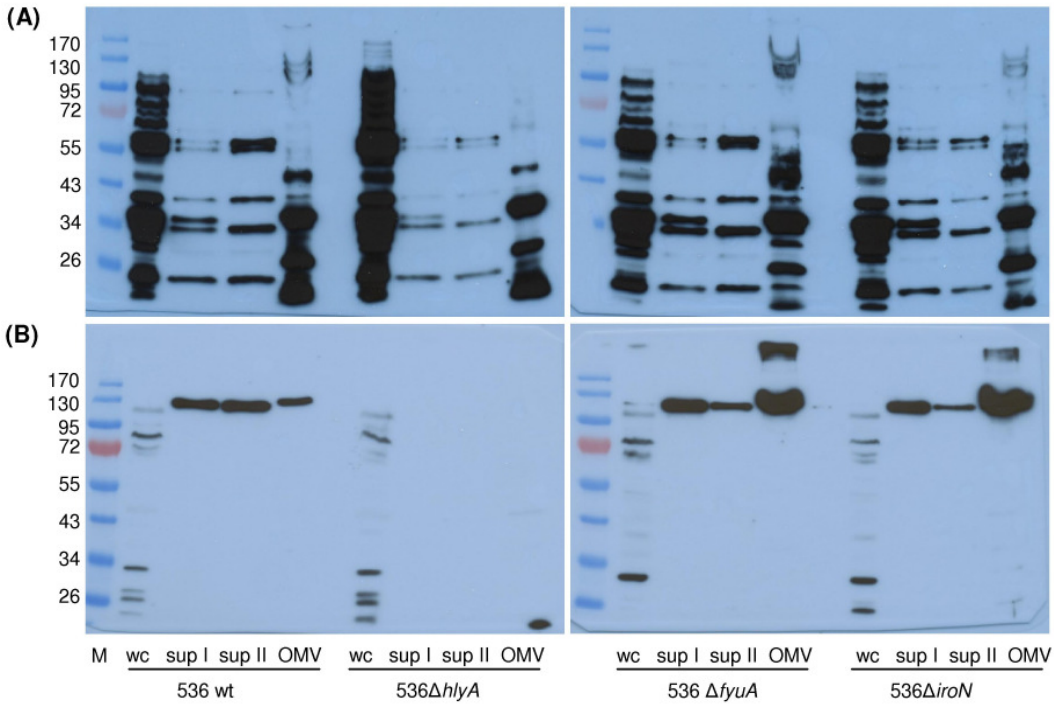

Figure 41. Immunoblot analysis of vesicles isolated from 536 strains with anti- *E. coli* antibody (A) indicated similar amounts of vesicle samples. (B) Western blot with anti-HlyA indicated the level of HlyA present in the supernatant (sup I) or vesicle samples. Whole cell extract (wc), supernatant before (sup I) and after (sup II) ultracentrifugation and vesicle samples (OMV). Vesicles isolated from Δ*fyuA* and Δ*iroN* mutant strains contained higher amounts of HlyA compared to wt or Δ*hlyA* vesicles.

After designated time points the cell culture supernatants were removed and analysed by cytokine ELISAs or by FACS analyses. As a control, J774 cells were stimulated with LPS (1 µg/ml) resulting in elevated levels of pro-inflammatory mediators (data not shown).

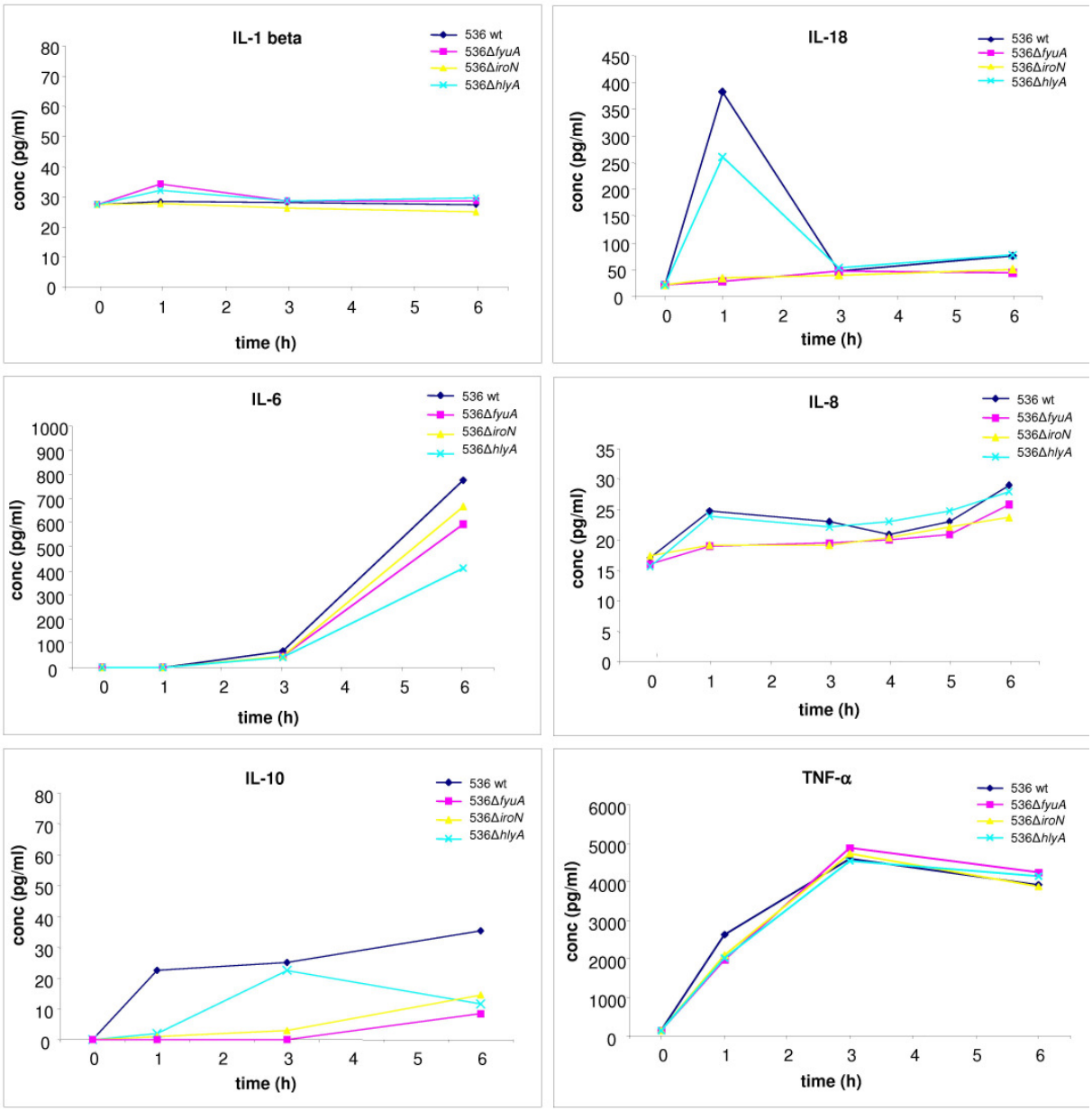

Figure 42. Cytokine profile of J774 macrophages after stimulation with vesicle samples showed that ExPEC OMVs were able to induce secretion of cytokines, but that the cytokine levels were dependent on vesicle LPS and protein composition.

As shown in Figure 42, vesicles induced no or moderate levels of IL-1β, IL-8 and IL-10, whereas a strong IL-18, IL-6 and TNF-α secretion could be detected. Production of IL-6, -8, -10 and TNF-α was increased with continued stimulation time and reached a maximum 3-6 h after stimulation. In contrast, the highest amount of IL-18 could be measured 1h after

stimulation. Interestingly, the elevated IL-10 and IL-18 level differed between vesicles of distinct strains, as only stimulation with HlyA-lacking 536 wt and Δ*hlyA* vesicles, but not with HlyA-harbouring Δ*fyuA* and Δ*iroN* led to a significant peak in the amount of secreted interleukine. The elevated IL-18 level was decreased to a level which was comparable to that obtained by stimulation with Δ*fyuA* and Δ*iroN* vesicles. The IL-18 response induced by OMVs exceeded that obtained by LPS, indicating that either the amount of LPS differed between OMV samples or that the presence of α-hemolysin possibly depressed IL-10 and IL-18 secretion. Taken together, we could show that ExPEC OMVs were able to induce secretion of cytokines, but that the cytokine levels were dependent on vesicle LPS and vesicle protein composition.

5. DISCUSSION

5.1. Expression and subcellular localization of FyuA and IroN

Recently, a high prevalence of the siderophore receptors IroN and FyuA in ExPEC isolates has been described (Johnson et al., 2005b; Schubert et al., 1998). However, the expression level of different siderophore receptors may vary depending on the strain and on the environmental condition at the site of infection. Accordingly, siderophore receptors are known to be up-regulated when grown under iron starvation conditions such as in urine or inside the host indicating the importance of effective iron uptake for urovirulence (Alteri and Mobley, 2007; Snyder et al., 2004).

The ExPEC strains 536 and NU14 used in this study produce several siderophores including the catecholate siderophore salmochelin and the mixed-type siderophore yersiniabactin, which are specifically recognized and transported into the bacterial cell by the siderophore receptors IroN and FyuA, respectively. In this work, we used quantitative RT-PCR techniques (TaqMan) and immunoblotting to determine quantitative changes in the transcription and/or expression of *fyuA* and *iroN* in response to normal or iron limitation conditions. These investigations demonstrated that the expression levels of both genes were induced under iron depletion conditions, but that the level of *iroN* was higher than that of *fyuA* under both conditions. Growth in NBD medium simulated an iron-limiting environment and resulted in a maximal expression of both siderophore receptors at pH 7. These results are in agreement with a recent study indicating that yersiniabactin and salmochelin were highly produced and most stable under neutral to alkaline conditions (pH 7.0 and 7.6, respectively) (Valdebenito et al., 2006). In general, immunoblotting methods are not sufficient for quantification of expression levels. However, the transcription levels revealed by TaqMan analyses were comparable with the expression levels found by immunoblotting, confirming our findings.

We can only speculate why *iroN* is stronger expressed compared to *fyuA* under the conditions used. Recently, it was described that siderocalin, a protein which is secreted by epithelial cells in the early stages of inflammation as part of the innate immune system, binds with high affinity to catecholate siderophores such as enterobactin (Fischbach et al., 2006). As a consequence, the iron supply and growth of the siderophore-producing bacteria is disturbed. However, it has been shown that glycosylation of enterobactin, resulting in salmochelin, is a mechanism by which these bacteria evade trapping of enterobactin by siderochalin (Valdebenito et al., 2007). The presence of the *iroA* gene cluster allows bacteria to evade this

component of the innate immune system, thus playing an important role in their virulence. It is further considered that different siderophores are produced in response to different environmental conditions, such as pH, temperature, and carbon source, during the development of an ExPEC infection. Yersiniabactin and salmochelin were maximally produced and most stable under neutral to alkaline conditions (pH 7.0 and 7.6, respectively), whereas aerobactin was produced at a slightly more acidic pH (pH 5.6), depending on the higher complex stability of hydroxamates at acidic pH values compared to the catecholates (Valdebenito et al., 2006). The production of several siderophores may help UPEC strains to compete with other *E. coli* strains and they may need the multitude of siderophores to adapt to the pH of urine, which varies between pH 4.6 and 8.0.

An increasing body of data indicates that siderophore receptors are important for urovirulence. Alteri and Mobley (2007) identified lately several known receptors for iron compounds (ChuA, IutA, IroN, IreA, Iha, FhuA and FepA) which were up-regulated during growth in human urine. By up-regulation of these siderophore receptors in this iron limiting environment, ExPEC compensates for these conditions by enriching its outer membrane with receptors for iron compounds. The concurrent production of numerous iron compound receptors by ExPEC may represent a fundamental strategy for the ability of this pathogen to colonize the human urinary tract.

5.2. Functional relevance of FyuA and IroN

The functional redundancy of five and more iron uptake systems is a unique feature of ExPECs. As discussed before, the maintenance of multiple functional siderophore systems maximizes the chance for successful iron procurement conferring a selective advantage. Further, the possession of different systems may afford the organism "insurance" in the event that one system becomes dysfunctional. However, the presence of several iron uptake systems could also indicate that siderophore receptors fulfil additional tasks in the pathogenicity of ExPECs.

In this regard, we performed invasion studies comparing recombinant IroN- and FyuA-carrying strains *in vitro*. We found that the salmochelin receptor IroN contributed to invasion of HCV29 urothelial cells *in vitro*. In contrast, the yersiniabactin receptor FyuA did not appear to have any direct affect on the invasion. This IroN mediated invasion was not unique to HCV29 cells, as similar invasion results were obtained using different urothelial cell lines. We could exclude, that IroN mediated invasion was influenced by other *E. coli* virulence traits such as fimbriae or adhesins, as also latex beads coated with the recombinant IroN protein were internalized by HCV29 host cells. Both, the uptake of IroN-coated beads or the internalization of IroN-carrying strains could be inhibited by pre-incubation with a polyclonal antibody raised against IroN in a concentration dependent manner. This further corroborated that the observed invasion phenotype was solely mediated by IroN.

The role of IroN in invasion was additionally confirmed under native expression conditions as well as in an ExPEC background, excluding artefacts caused by the high expression of recombinant IroN. Interestingly, not even the entire IroN protein was necessary for invasion, as only the C-terminal, but not the N-terminal part of the IroN protein was sufficient to mediate host cell invasion. These data suggest that not the functional protein, but rather special structures or domains are important for invasion. The predicted structure of FhuA, a protein that showed a high sequence identity compared to IroN, consists of several transmembrane domains that are connected by loops present on the surface of the bacterial outer membrane (Figure 43) (Braun and Endriss, 2007). Loops (L) 3, 4, and 5 are the largest and the most exposed ones, suggesting that these extracellular domains might interact with other components such as special receptors on the host cell side. As the structures of all siderophore receptors are highly similar, we assume that L 3 and 4 are present in the N-terminal part of IroN ($IroN_{1-387}$), whereas L 5 is present in the C-terminal part ($IroN_{372-726}$). The observation, that the C-terminus was sufficient to entail invasion, prompted us to

hypothesize, that the loop 5 of IroN is probably necessary for the contact between bacteria and host cells, resulting in invasion.

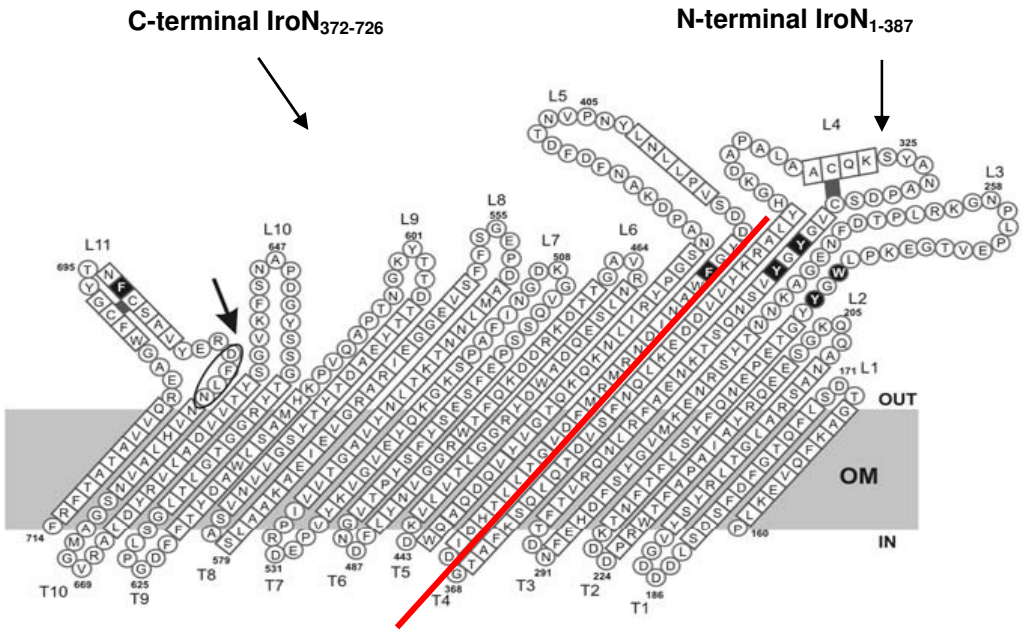

Figure 43. Transmembrane arrangement of the IroN homologous protein FhuA. Similar to this schematic draft, the N-terminal part of $IroN_{1-387}$ and $IroN_{372-726}$ were constructed. $IroN_{1-387}$ comprises L1-4, the C-terminal $IroN_{372-726}$ contains transmembrane domains including L5 as the biggest loop presented to the host cell (Braun and Endriss, 2007).

However, this assumption needs further investigations for conformation, e.g. cloning of the single loop. Especially, as we cannot be sure that the recombinant C-terminal part without a signal sequence was incorporated into the outer membrane in a similar conformation as the complete IroN protein.

Interestingly, expression of IroN did not increase bacterial adherence to host cells, suggesting that IroN mediated invasion was not due to enhanced adherence. This is in agreement with results presented by a recent study (Leveille et al., 2006). The authors observed that the siderophore receptor Iha specifically increased adherence whereas other siderophore receptors such as FepA, IrgA and IroN did not. Instead, we assume that IroN may directly trigger uptake of ExPEC into host cells, presumably by interaction of the prominent loop 5 with the host cell. Although we could exclude β1 integrin mediated endocytosis as internalization pathway, we could not decipher the exact mechanism of IroN mediated invasion. We can only assume that IroN mediated invasion probably depicts a rather unspecific uptake process of bacteria into host cells, as IroN mediated invasion could not be significantly reduced by the presence of any of the tested inhibitors (e.g. wortmannin, cytochalasin D or filipin).

An increasing body of epidemiological and experimental evidence supported the contention that ExPEC virulence is a combination of different virulence factors and that there is not a cardinal virulence determinant as in the case of intestinal pathogenic *E. coli* (Bower et al., 2005). Thus, it is likely that IroN does not represent the sole cause promoting invasion into host cells but contributes to full urovirulence in concert with other virulence factors. In this regard, siderophore receptors may function as additional factors mediating invasion. These suggestions may also explain why mutants affecting the single gene *iroN* did not exhibit a significant phenotype in the performed invasion assays. This was most likely due to the coincidental presence of several redundant factors, including siderophore receptors and other virulence determinants, which possibly account for the invasive phenotype of ExPEC strains.
Recently, it was reported that UPECs are able to form intracellular biofilm-like pods which could explain how bladder infections can persist in the face of robust host defences (Anderson et al., 2003). Thereby, the initial binding events to superficial bladder epithelial cells are mediated by the adhesive tip protein FimH of type 1 pili. However, several additional UPEC factors including the salmochelin receptor IroN are described to influence disease progression (Anderson et al., 2004). However, our study provided first evidence for the implication of the siderophore receptor IroN in ExPEC host cell interaction. Several other studies proposed that siderophore receptors may fulfil additional tasks in the pathogenicity of ExPECs. The outer membrane receptors for heme (ChuA) and aerobactin transport (IutA) of the extraintestinal pathogenic strain CFT073 have been shown to be required for full virulence in mice (Torres et al., 2001). Moreover, the recently identified siderophore receptor IreA (iron-responsive element) has been demonstrated to function both as siderophore receptor and as an adhesin in UTIs (Russo et al., 2001). However, *ireA* has a lower prevalence in ExPEC strains than *iroN* (Johnson et al., 2005b). Likewise, a recent study characterized the outer membrane protein Iha as both catecholate siderophore receptor and adherence factor of ExPEC strains (Leveille et al., 2006). Previous epidemiological studies revealed a high prevalence of IroN in ExPEC strains (Duriez et al., 2001; Johnson et al., 2005b). There is also accumulating experimental evidence that the IroN protein is involved in the infection process of ExPECs (Negre et al., 2004; Russo et al., 2002; Russo et al., 2003). However, all these studies failed to provide evidence for the direct involvement of IroN in invasion of urothelial cells. The present work demonstrated for the first time, that the catecholate receptor IroN contributes to invasion of urothelial cells by ExPEC *in vitro*. The findings presented here corroborate the hypothesis that siderophore receptors exert a dual role in the pathogenesis of ExPEC, as they facilitate both metabolic functions and host cell interaction. Further evaluation of the mechanisms how

ExPECs enter the host cell and how they replicate inside the cell is likely to provide a better understanding of intracellular bacterial pathogenesis.

5.3. IroN vesicles associate with urothelial cells

In this study, we described that the salmochelin receptor IroN was present in outer membrane vesicles produced by the ExPEC strains 536 and NU14. In contrast, the yersiniabactin receptor FyuA could not be detected in these vesicles. OMVs are constitutively shed from the outer membrane of Gram-negative bacteria, containing characteristics of the bacterial outer membrane. Both proteins, FyuA and IroN are transmembrane proteins located in the bacterial outer membrane, so that they theoretically could be incorporated into vesicles during their formation. The observed absence of FyuA is probably caused by its low expression level. The transcriptional regulation and the expression of siderophore systems depends on the iron-binding repressor Fur (ferric uptake regulation) (Wee et al., 1988). The lack of iron serves as an environmental signal to induce expression of FyuA and IroN. However, we found that *iroN* was stronger transcribed and expressed under normal as well as under iron depletion conditions compared to *fyuA*. These findings may explain why only IroN but not FyuA could be detected in ExPEC vesicles. Furthermore, these data corroborate the assumption that several regulation mechanisms exist for the expression regulation of individual siderophore receptors (see section 5.1.). Previously, two proteomic studies identified several iron accumulation components including ChuA, FhuA, FepA and IroN, but not FyuA, that are present in *E. coli* vesicles (Berlanda et al., 2007; Lee et al., 2007). However, these studies did not discuss the role of these siderophore receptors within vesicles.

Here, we describe for the first time, that IroN-harbouring vesicles associate with urothelial cells *in vitro*. In contrast, control vesicles did not accumulate with host cells, indicating that the contact between vesicles and host cells was solely mediated by IroN and not by other vesicle properties. The presence of IroN in vesicles entailed not only attachment but also internalization of OMVs into urothelial cells. These findings are in agreement with the previously described implication of IroN in urothelial cell invasion by ExPEC (see section 5.2.). Interestingly, fluorescence spots representing IroN vesicles accumulated around the nucleus indicating that vesicles were trafficked inside the host cells.

Bacteria and bacterial products can be internalized by host cells via endocytic pathways including phagocytosis (cell-eating) and pinocytosis (cell-drinking) and is often dependent on a specific receptor on the host cell side (clathrin-coated pits or caveolae-mediated endocytosis (lipid rafts) (Figure 44).

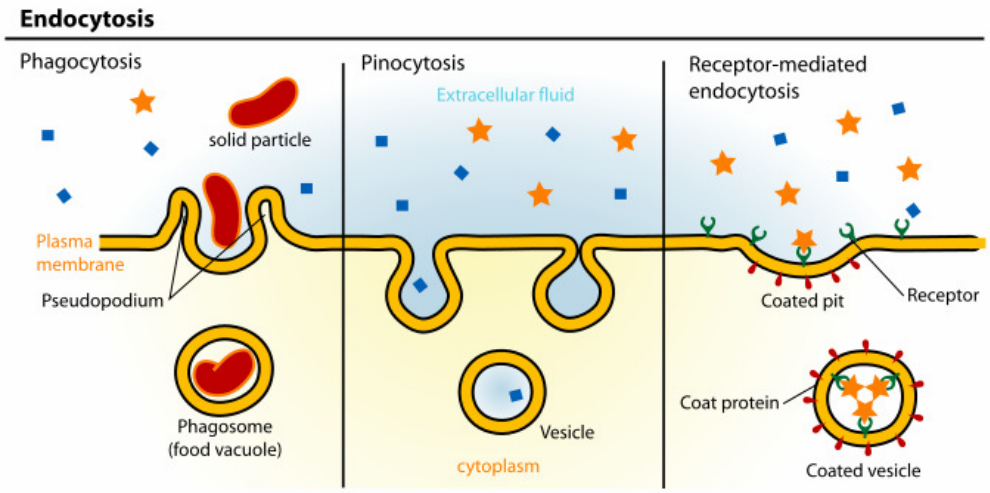

Figure 44. The term endocytosis includes phagocytosis (cell-eating) and pinocytosis (cell-drinking) and is often mediated by a specific receptor on the host cell side (e.g. clathrin-coated pits or caveolae-mediated endocytosis).

In fact, we found IroN vesicles co-localized with anti-lamp-1 antibody, which specifically recognizes late endosomes. Furthermore, we were able to visualize the co-localization of IroN vesicles with an anti-golgin 97 antibody, which served as eukaryotic marker for the endoplasmatic reticulum (ER) and trans-golgi networks (TGN). The formation of these networks was visible at polar sites of the host cells after 20h and was then probably transported towards the nuclei, as these vesicle-TGN structures could be detected as ring-like structures near the nucleus after 30h incubation time. These data support the hypothesis that IroN binds to host cells via a yet unknown mechanism, which in turn led to endocytosis of IroN-carrying vesicles. These vesicles or at least vesicle components were subsequently transported presumably within the endosome by intracellular trafficking from the host cell membrane to the nucleus, where they were enriched in compartments of the endoplasmatic reticulum. These results demonstrate for the first time that OMVs isolated from non-pathogenic strains are not only associated with host cells, but a fraction of these vesicles was internalized and intracellular trafficked by host cells, dependent on the presence of the outer membrane receptor IroN. However, the exact internalization pathway including specific receptors needs further elucidation.

In the past, several studies provided evidence for toxin delivery by pathogen-derived vesicles. For instance, ETEC vesicles not only target toxin delivery into mammalian cells, but also mediate LT-directed vesicle-host cell association (Kesty et al., 2004; Wai et al., 1995). Likewise, the vacuolating toxin VacA of *H. pylori* was associated with vesicles and suggested

to play a role in adhesion and internalization of *H. pylori* vesicles (Fiocca et al., 1999). In addition, CNF 1 delivered by UPEC vesicles attenuates polymorphonuclear leukocyte antimicrobial activity and chemotaxis (Davis et al., 2006; Kouokam et al., 2006). However, it was assumed that vesicle host cell interactions are not limited to toxin mediated binding and internalization as also vesicles lacking externally localized toxins were believed to interact with eukaryotic cells via other external components, such as outer membrane proteins. For instance, OmpA, a major vesicle component for several *E. coli* strains, binds to a receptor on human brain endothelial cells and triggers lipid raft-dependent internalization (Prasadarao, 2002). This study, however, only described the interaction of OmpA and its receptor, but did not provide evidence for a possible interaction of OmpA-carrying vesicles with host cells.

In this regard, the present study described for the first time the localization of IroN in ExPEC vesicles and the association with and internalization of these vesicles into host cells. We propose these data as a further hint for the implication of IroN in pathogen host cell interaction. Vesicle mediated adhesion and endocytosis may play an important role in pathogenesis, since these bacterial vesicles could deliver active luminal toxins and endotoxins into the eukaryotic cell. However, IroN-containing vesicles could also serve as a precursor to prime the host cells for following invasion by bacteria. Furthermore, the presence of vesicles or components of those inside endosomes may also have the purpose to drive evasion of the immune system.

In summary, we demonstrated that IroN-carrying vesicles mediate binding and internalization of vesicles into urothelial cells *in vitro*. We propose that *in vivo*, ExPECs utilize vesicles as vehicles to transport toxins and other virulence factors to the host mucosal layer, both locally and away from the site of infection. We further suggest that vesicles from bacterial pathogens are a direct conduit for toxin delivery, providing a protective environment for the transmission of lumenal proteins and enzymes from the bacterial periplasm into the host cell. Thereby, the binding specificity of surface adhesive factors on vesicles, such as IroN, dictates which host cells or tissues the vesicles target. Further study of intracellular events following vesicle internalization, and of the role of bacterial vesicles in immunomodulation, are necessary to clarify virulence attributes of vesicles and their role in ExPEC diseases.

5.4. ExPEC vesicles and α-hemolysin

In the previous chapter we discussed the presence of IroN in outer membrane vesicles as an internalization factor, responsible for vesicle binding, endocytosis and intracellular trafficking of vesicles or vesicle components inside the host cells. We further propose that these vesicles could act as vehicles for the delivery of virulence factors, such as active toxins inside the cell. This is of special interest, as we found that vesicles derived from the ExPEC strain 536 contain huge amounts of hemolytically active α-hemolysin.

The α-hemolysin is an exotoxin that is frequently associated with strains isolated from extraintestinal infections. The ExPEC strains NU14 and 536 examined in this study also secrete HlyA. Interestingly, α-hemolysin was not only detected as free soluble HlyA in the extracellular milieu, but a fraction of HlyA was also detectable in vesicle samples of strain 536. However, the level of α-hemolysin differed between 536 wt and mutant strains. Vesicles isolated from Δ*fyuA* and Δ*iroN* mutant strains were associated with high amounts of α-hemolysin, whereas this toxin was rarely found in the wt vesicles. The increased association of HlyA with mutant vesicles did not result from improved vesicle formation as the amount of produced vesicle was similar in mutant and wt strains. Also the secretion level of HlyA did not differ between the strains. Analyses of HlyA-containing vesicles by dissociation assays showed that the α-hemolysin was tightly associated with the OMVs and was not copurified from the culture supernatant.

The α-hemolysin is one of the most important virulence factors involved in the pathogenesis of ExPEC, as the secreted exotoxin has cytolytic and/or cytotoxic activity against a wide range of mammalian cell types (Lally et al., 1999). A recent study reported that α-hemolysin was associated with vesicles isolated from several ExPEC strains (Balsalobre et al., 2006). Here, we found that the α-hemolysin-containing 536 vesicle led to hemolysis of erythrocytes depending on the amount of vesicle-associated HlyA. However, we could not clearly confirm cytotoxic activity of α-hemolysin containing OMVs in non-lytic assay systems. Rather, we assumed that the presence of other factors present in vesicles samples disturbed the monolayers, as we could not detect significant differences in the cytotoxicity of HlyA-containing vesicles compared to HlyA-lacking vesicles.

Ultrastructural analyses of vesicle samples discovered morphological different subpopulation of mutant vesicles, whereas wild-type vesicles were homogenous in size and morphology. OMV samples that contained α-hemolysin are characterized by their larger size and thicker margin when compared with wt vesicles containing less α-hemolysin. Therefore, we

hypothesized that the larger vesicle structures, which were more common in mutant strains contain α-hemolysin. Higher resolution of these vesicles visualized a ring-like accumulation of pore-shaped structures. These structures were suspected to represent HlyA protein assemblies similar to that found in vesicles containing the pore-forming cytotoxin ClyA (Wai et al., 2003a). Gradient centrifugation of wt and mutant vesicle samples separated different vesicles subpopulations with distinct protein composition: the larger vesicles of mutant strains contained α-hemolysin and showed the highest hemolytic activity. In contrast, wild-type vesicles harboured less amounts of HlyA. However, our results showed that a type I machinery component, the TolC protein, was mainly present in wt vesicles and to a lesser extent in the α-hemolysin enriched mutant OMVs. Interestingly, the transmembrane channel forming protein TolC could be detected in similar amounts in whole cell and outer membrane samples and only differed in the vesicle samples: TolC appeared to be less present in HlyA-containing mutant vesicles than in wt vesicles. The simultaneous presence of HlyA, but absence of TolC in mutant vesicles ruled out the possibility that vesicles were shed from type 1 secretion system located areas of the outer membrane including HlyA as luminal cargo into OMVs. Rather, the HlyA is spontaneously associated to vesicles after blebbing. This suggestion could be further corroborated by the finding that free soluble HlyA from culture supernatant was able to reassociate to existing vesicles. Although, the transcriptional levels and the secretion of HlyA were similar in mutant and wt strains, free α-hemolysin was reassociated with a higher affinity to mutant vesicles than to the wt vesicles. Horstman et al. (2002; 2004) reported that α-hemolysin has a high binding capacity to LPS. Therefore, we supposed that reassociation of HlyA from the supernatant was favored by the presence of LPS in OMVs and that presumably the LPS profile in the mutant vesicles was influenced by mutation of *fyuA* and *iroN*. It should be noted that we could detect a slightly higher amount of oligosaccharide side chains in the LPS preparations of Δ*fyuA* mutant vesicles by immunoblotting. Further, the transcription level of LPS biosynthetic genes in the Δ*fyuA* and Δ*iroN* mutant strains was different from that of 536 wt. However, we could not provide evidence that the LPS pattern of mutant strains and vesicles was affected by mutation of *fyuA* and *iroN*. Furthermore, no significant differences in the outer membrane profile of wt and mutant strains could be detected. It is still puzzling why α-hemolysin was associated mainly with mutant vesicles, whereas it was only rarely found in wild-type vesicles.

Taken all the presented results together, a model of different vesicle blebbing in 536 wild-type and mutant strains is proposed:

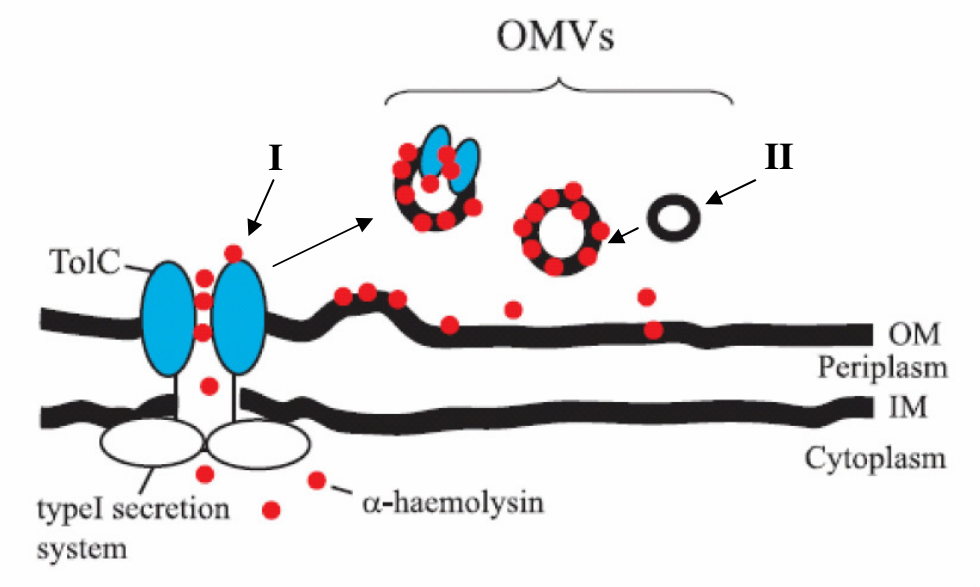

Figure 45. Model of α-hemolysin secretion and distinct OMVs blebbing in 536 wt (I) and Δ*fyuA* and Δ*iroN* mutant (II) strains resulted in mutant vesicles (II) that contain more HlyA, but less TolC than wt OMVs (I). This sketch was modified from Balsalobre et al. (2006).

As shown in Figure 45, α-hemolysin is secreted by the type 1 secretion system (T1SS), a *sec*-independent system, where the proteins are translocated in a one-step process to the extracellular media in a soluble form, without a periplasmic intermediate. Once in the extracellular milieu, HlyA reassociates to the outer membrane or to existing outer membrane vesicles, probably based on the high affinity of HlyA molecules to LPS. We assume that 536 wt vesicles containing normal amounts of TolC but less HlyA were mainly shed at sites of the T1SS (possibility I). In contrast, vesicles from Δ*fyuA* and Δ*iroN* mutant strains containing less TolC but high amounts of HlyA were predominantly blebbed from T1SS lacking areas (possibility II). In a next step, HlyA is reassociated to the mutant vesicles presumably due to a modified LPS profile of these vesicles. Alternatively, HlyA is preferentially bound to the special LPS pattern of the outer membrane of mutant strains directly after secretion. Vesicle budding from these areas originates vesicles which are characterized by their high amounts of HlyA, but less TolC.

In summary, we could show that hemolytically active α-hemolysin is present in vesicles of the ExPEC strain 536, especially in those of 536Δ*fyuA* and 536Δ*iroN* mutant strains. The association of α-hemolysin with OMVs may play an important role in the pathogenicity of extraintestinal α-hemolysin-producing *E. coli* isolates. *In vivo* experiments have shown the relevant role of α-hemolysin in the pathogenicity of UPEC (Hacker et al., 1983; Welch, 1991). Moreover, *in vitro* studies indicated that the α-hemolysin has cytotoxic and/or cytolytic effects against a wide variety of cell types, including erythrocytes, neutrophils, granulocytes and epithelial cells (Lally et al., 1999). Based on the presented finding that HlyA-containing vesicles have hemolytic activity, OMVs are suggested to play a role in dissemination of α-hemolysin to host cells during extraintestinal *E. coli* infections. To date, the molecular events involved in the delivery of α-hemolysin from OMVs to eukaryotic cells are unknown. However, we could show that IroN-containing vesicles bind to host cells and trigger endocytosis of vesicles or vesicle components. Presumably, IroN present in ExPEC vesicles serves as an internalization factor to address the delivery of α-hemolysin present in ExPEC vesicles into host cells. This assumption emphasizes the implication of ExPEC vesicles in host pathogen interaction.

5.5. Immunological role of ExPEC vesicles

Recently, it has been reported that in response to *H. pylori* and *P. aeruginosa* vesicles epithelial cells produce IL-8, a cytokine important for neutrophil and monocyte recruitment *in vivo*, (Bauman and Kuehn, 2006; Ismail et al., 2003). Furthermore, it has been shown that vesicles isolated from *Legionella pneumophila* specifically modulate host cells regarding their cytokine response (increase of IL-6, 7, 8, 13 as well as G-CS, IFN-γ, MCP-1) (Galka et al., 2008). OMVs contain various compounds (LPS, lipoproteins, proteins) that are recognized by eukaryotic cells and which are known to influence the release of cytokines. Our cytokine profiling experiments indeed revealed that OMVs isolated from 536 strains induce a specific cytokine secretion profile in murine J774 macrophages. These cells were used for cytokine profiling studies, as the cytokine repertoire of epithelial cells lining the human UT, such as HCV29 cells, is limited (Hang et al., 1998). Vesicles isolated from 536 strains specifically stimulated the release of IL-6, IL-8, IL-10, IL-18, and TNF-α, whereas no or only a moderate amount of IL-1β could be detected after stimulation with ExPEC vesicles. These data indicated that ExPEC OMVs contribute to inflammation by triggering secretion of pro-inflammatory mediators. It is well known that bacterial LPS stimulates the production of a large array of immunomodulators from human monocytes. In this regard, we first assumed that vesicle induced cytokine response was due to vesicle LPS. However, the induced cytokine level differed depending on the protein composition of vesicles, as stimulation with HlyA-reduced 536 wt and HlyA-lacking Δ*hlyA* vesicles resulted in higher IL-18 and IL-10 cytokine levels compared to stimulation with HlyA-harbouring Δ*fyuA* and Δ*iroN* vesicles. We concluded from this, that the presence of α-hemolysin could possibly reduce the secretion of these cytokines. Alternatively, either the amount of LPS or the LPS pattern could be different between OMV samples. This assumption was supported by the finding that the LPS pattern of Δ*fyuA* and Δ*iroN* mutant vesicles was different from that of 536 wt. Another possibility is that HlyA molecules which have a high affinity to LPS block the LPS residues by binding to vesicle LPS. However, the level of TNF-α was elevated by all tested vesicles in a similar manner, indicating that vesicles presumably possess a comparable LPS profile. Therefore, we hypothesize that the presence of α-hemolysin may depress cytokine secretion, especially that of IL-10 and IL-18 from macrophages. This assumption is in agreement with a previous report, where the authors described, that α-hemolysin, at non-toxic concentrations, depressed the spontaneous as well as *E. coli* induced cytokine release (Konig et al., 1994).

The role of vesicles stimulated cytokine secretion for the pathogenesis is not clear. IL-10 was described as suppressive cytokine that is able to limit the immune response and to prevent host damage. In contrast, all other cytokines have rather pro-inflammatory functions. These conflicting findings clearly indicate the need for further evaluation of the role of OMVs in the pathogenesis as well as in stimulation of the host's immune response.

Taken together, we found that ExPEC vesicles contribute to inflammation by triggering the release of cytokines. Production and secretion of cytokines provides the basis for a rapid host response and is an important host defence mechanism against mucosal attack by microbes or toxic agents. However, we observed that HlyA-containing vesicles depressed secretion of IL-10 and IL-18. This finding demonstrated that OMVs serve as potential virulence vehicle carrying α-hemolysin and that these vesicles have the potential to modulate and overcome host response in order to benefit a bacterial infection.

But there is another aspect in the light of OMV stimulated immune response:

OMV induced cytokine response reflects the ability of vescicles to stimulate the immune system. In this regard, it should be noted that a recently developed meningococcal outer membrane vesicle vaccine (MeZNB) provided protective antibody responses against systemic serogroup B *Neisseria meningitidis* disease in humans (Oster et al., 2005; Thornton et al., 2006). Furthermore, *Neisseria* OMVs given intranasally elicit not only immune responses but also act as an effective mucosal adjuvant for the hepatitis B surface antigen (HBsAg) in mice (Sardinas et al., 2006).

In a recent publication, possible target antigens of ExPECs were identified that elicit protective immune responses specific to ExPEC strains demonstrating a preventive subunit vaccine against diseases caused by ExPECs (Durant et al., 2007). One of these target proteins was the salmochelin receptor IroN as it fulfils the two main criteria for antigen selection: Firstly, IroN is located at the cell surface and thus presented to the host cell. Secondly, IroN has a high prevalence in ExPEC, but a low prevalence in commensal strains, so that the immune system should specifically recognize ExPECs after immunization with the respective IroN-vaccine.

In the present study, further evidence was provided for the importance of IroN as virulence factor as it is involved in iron uptake as well as in host pathogen interaction. In addition, we could detect IroN as a component of ExPEC vesicles and demonstrated the involvement of IroN-carrying vesicles in binding to and delivering virulence factors into the host cell. Furthermore, we could show that ExPEC vesicles are able to stimulate the host immune response. Taken all these observations together, we propose IroN-containing vesicles as potential target for the development of new prevention strategies: ExPEC outer membrane vesicles could serve as adjuvant and IroN present in these vesicles may display with its potential immunogenic character an excellent vaccine candidate.

6. SUMMARY

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are responsible for the majority of community-acquired infections outside the gastrointestinal tract. These infections range from asymptomatic urinary tract infections (UTIs) to life threatening diseases such as pyelonephritis or sepsis. The concurrent production of numerous iron compound receptors is a unique feature of ExPEC strains and represents a fundamental strategy for the ability of these pathogens to colonize the human urinary tract. The possession of different iron uptake systems may afford the organism "insurance" in the event that one system becomes dysfunctional. On the other hand, the presence of several iron uptake systems could also indicate that siderophore receptors fulfil additional tasks in the pathogenicity of ExPECs. Some siderophore receptors including the yersiniabactin receptor FyuA and the salmochelin receptor IroN have been shown to be more prevalent in ExPEC than in commensal strains. Thus, the present study was focused on the ExPEC siderophore receptors FyuA and IroN and their implication in host pathogen interaction.

Analyzing the transcription and expression of *fyuA* and *iroN* by quantitative RT-PCR techniques (TaqMan) and immunoblotting, *iroN* showed a higher transcription and expression level than *fyuA* under normal and under iron depletion conditions. Furthermore, IroN but not FyuA was involved in urothelial cell invasion. For IroN mediated invasion, not the entire, functional IroN protein was necessary, as the C-terminal part of IroN alone was sufficient to mediate invasion. IroN mediated invasion was not influenced by the high expression of recombinant proteins as comparable invasion levels were observed with natively expressed IroN and with IroN-positive ExPEC strains. Although, several internalization pathway could be excluded, it could be not enlightened how IroN-expressing *E. coli* strains were internalized by urothelial cells. However, the adherence levels were not increased by IroN-carrying strains, suggesting that IroN mediated invasion is a more direct effect, which could be supported by the interaction of the dominant extracellular loop 5 of the IroN protein with the host cell. A further hint for the implication of IroN in host pathogen interactions was the finding that IroN was present in outer membrane vesicles produced by ExPEC strains. These vesicles were further shown to harbour hemolytically active α-hemolysin, a toxin that is frequently associated with ExPEC isolates. The expression of IroN in the apathogenic strain HB101 conferred vesicles, which were isolated from this strain, the ability to associate to urothelial cells *in vitro*. IroN-containing vesicles bind to urothelial cells in a time dependent

manner, trigger endocytosis and were then transported presumably inside the endosome by intracellular trafficking to membrane compartments of the endoplasmatic reticulum. Therefore, IroN in ExPEC vesicles was proposed to serve as an internalization signal to address the delivery of biological active α-hemolysin, which was associated to ExPEC vesicles, into host cells.

These findings support the implication of the siderophore receptor IroN in the pathogenesis of ExPEC: IroN functions as iron uptake receptor and serves as internalization signal to promote bacterial cell invasion and to transport active virulence factors such as toxins via outer membrane vesicles into host cells. Furthermore, we could show that ExPEC vesicles are able to stimulate the host immune response by secretion of cytokines. Taken all these observations together, we propose IroN-containing vesicles as potential target structures for the development of new prevention strategies against ExPEC diseases.

7. ZUSAMMENFASSUNG

Extraintestinal pathogene *Escherichia coli* (ExPEC) können beim Menschen eine Vielzahl von Erkrankungen hervorrufen, zu denen die harmlose Cystitis, aber auch die schwer verlaufende Pyelonephritis mit bleibenden Nierenschäden oder sogar Meningitis und Sepsis zählen. ExPEC-Stämme besitzen eine Vielzahl von Siderophor-Systemen, die durch Siderophor-Rezeptoren in der äußeren Membran von Bakterien gekennzeichnet sind. Diese häufig als hochgradig Virulenz-assoziiert beschriebenen Rezeptoren tragen maßgeblich zu der Fähigkeit der ExPECs, den menschlichen Harnwegstrakt zu besiedeln, bei. Einerseits kann das gleichzeitige Vorhandensein von mehreren Siderophor-Rezeptoren als eine Art „Versicherung“ für den Ausfall eines dieser Systeme betrachtet werden, andererseits könnte es auch darauf hinweisen, dass Siderophor-Rezeptoren andere Funktionen als die der Eisenaufnahme übernehmen können. Darüber hinaus haben einige dieser Siderophor-Rezeptoren, wie z.B. der Yersiniabactin-Rezeptor FyuA oder der Salmochelin-Rezeptor IroN, eine besonders hohe Prävalenz in ExPEC-, nicht aber in kommensalen Stämmen.

Diese Arbeit ist auf die Siderophor-Rezeptoren IroN and FyuA und deren Beteiligung an der Interaktion zwischen Wirtszelle und Pathogen fokussiert. Zunächst wurde mittels quantitativer RT-PCR und Immunoblot-Technik die Transkription und Expression von *iroN* und *fyuA* untersucht. Dabei konnte eine erhöhte Expression von *iroN* verglichen mit *fyuA* sowohl unter normalen als auch unter Eisenmangelbedingungen festgestellt werden. Diese Ergebnisse weisen darauf hin, dass *iroN* eine wichtige Rolle während der Entwicklung von Harnwegsinfekten spielt. Es konnte gezeigt werden, dass IroN, nicht aber FyuA, an der Invasion von humanen Urothelzellen beteiligt ist. Dafür war nicht das vollständige, funktionelle IroN-Protein notwendig, sondern der C-terminale Teil alleine war ausreichend für die Invasion in Wirtszellen. Diese IroN-vermittelte Invasion wurde nicht durch das hohe Expressionsniveau von rekombinantem IroN beeinflusst, da sowohl nativ exprimiertes IroN als auch IroN-positive ExPEC-Stämmen vergleichbare Invasionseigenschaften aufzeigten. Allerdings konnte der genaue Internalisierungsprozess IroN-exprimierender *E. coli*-Stämme nicht entschlüsselt werden. Da jedoch die Adhärenz der IroN-exprimierenden Stämme nicht erhöht war, ist die IroN-vermittelte Invasion wahrscheinlich eher ein direkter Effekt. Die Interaktion zwischen Wirtszelle und Bakterien wird möglicherweise durch eine extrazellulär ausgeprägte Struktur (Loop 5) des Transmembran-Proteins IroN verursacht. Ein weiterer Hinweis für die Beteiligung von IroN an der Interaktion von Wirtszelle und Pathogen ist der Nachweis von IroN in sog. Außenmembranvesikeln, die von ExPEC-Stämmen produziert

werden. In diesen Vesikeln konnte darüber hinaus hämolytisch aktives α-Hämolysin, ein Toxin, das häufig mit ExPEC-Isolaten assoziiert ist, nachgewiesen werden. Die Expression von IroN in dem apathogenen *E. coli* Stamm HB101 führte zu IroN-tragenden Vesikeln, die sich *in vitro* an Urothelzellen anlagern. Diese IroN-Vesikel binden abhängig von der Inkubationszeit an Urothelzellen und bewirken ihre Endozytose. Innerhalb der Wirtszelle werden die Vesikel, bzw. Vesikelbestandteile, vermutlich auf endosomalem Weg zum Zellkern oder zum Endoplasmatischen Retikulum transportiert. Dabei dient IroN, das auf der Oberfläche von ExPEC-Vesikel nachgewiesen wurde, vermutlich als Internalisierungssignal für den Transport von biologisch aktivem, mit ExPEC-Vesikeln assoziiertem α-Hämolysin in Wirtszellen. Diese Beobachtungen weisen auf eine bislang nicht bekannte Beteiligung des Siderophor-Rezeptors IroN an der Interaktion zwischen Wirtszelle und Pathogen hin: IroN agiert einerseits als Eisenaufnahme-Rezeptor und dient andererseits als Internalisierungssignal sowohl für bakterielle Zellinvasion als auch für den Transport von aktiven Virulenzfaktoren, wie z.B. α-Hämolysin, mittels Außenmembranvesikel in die Wirtszelle. Im Hinblick auf die Notwendigkeit neuartiger Präventionsmöglichkeiten gegen ExPEC-assoziierte Krankheiten spielt das beschriebene Außenmembranprotein IroN eine besondere Rolle. Da IroN sowohl an der bakteriellen Oberfläche präsentiert wird als auch an Virulenzmechanismen, wie Eisenaufnahme und Invasion, beteiligt ist, stellt es ein potentielles Kandidaten-Antigen für die Entwicklung neuartiger Impfstoffe dar.

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9. APPENDIX

9.1. List of original publications

This project includes some data which are already published in the original publications listed below.

Feldmann F, Sorsa LJ, Hildinger K, Schubert S
The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic *Escherichia coli* in vitro.
Infect Immun. 2007 Jun;75(6):3183-7.

Feldmann F, Wai SN, Magistro G, Yoshimitsu M and Schubert S
The salmochelin siderophore receptor IroN present in outer membrane vesicles of extraintestinal pathogenic *Escherichia coli* serves as internalization signal for urothelial cells
PLoS Pathog., submission in preparation

Sorsa LJ, Müller T, **Feldmann F**, Hildinger K and Schubert S
Development and validation of a diagnostic DNA microarray specific for extraintestinal pathogenic *Escherichia coli*.
Manuscript in preparation

Sorsa LJ, **Feldmann F,** Hildinger K, Dufke S and Schubert S
Characterization of four novel genomic regions of uropathogenic *Escherichia coli* highly associated with the extraintestinal virulent phenotype: a jigsaw puzzle of genetic moduls.
Int J Med Microbiol. 2007 Apr;297(2):83-95.

9.2. Oral presentation

Feldmann, F., Magistro G., Sorsa L.J., Wai S.N., Schubert, S.
Implication of extraintestinal pathogenic *Escherichia coli* siderophore receptors in host pathogen interaction.
EPG 3rd Student's Meeting of the NoE-EPG (Network of Excellence EuroPathoGenomics) Graduate Academy, February 24- 27, 2008 in Innsbruck, Austria

Feldmann, F., Sorsa, L.J., Hildinger K., Schubert, S.
The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic *Escherichia coli* in vitro.
24th Annual Meeting of the DGHM, Sept. 30- Oct. 4, 2007, Göttingen, Germany

Feldmann, F., Sorsa, L.J., Messmer K., Schubert, S.
Identification of virulence factors in extraintestinal pathogenic *Escherichia coli* (ExPEC) implicated in host-pathogen interaction.
Final colloquium of the Graduate Academy "Infection and Immunity", Oct.13-17, 2005, Venice, Italy

9.3. Poster presentations

Feldmann, F., Sorsa, L.J., Hildinger K., Schubert, S.
Impact of siderophore receptors of extraintestinal pathogenic *Escherichia coli* on urothelial cell invasion.
Interact, 1st Life science PhD symposium, Dec. 7, 2007, Munich, Germany

Feldmann, F., Sorsa, L.J., Hildinger K., Schubert, S.
The Salmochelin siderophore receptor IroN promotes invasion of extraintestinal pathogenic *Escherichia coli* into urothelial cells.
FEMS (Federation of European Microbiological Society) 2006, 2nd Congress of European Microbiologists, Jul. 4 - 8, 2006, Madrid, Spain.

Feldmann, F., Sorsa, L.J., Messmer K., Schubert, S.
Identification of virulence factors in extraintestinal pathogenic *Escherichia coli* (ExPECs) implicated in host pathogen interaction.
1st Student's Meeting of the NoE EPG (Network of Excellence EuroPathoGenomics) Graduate Academy, Dec. 2 - 4, 2005, Würzburg, Germany

Sorsa, L.J., **Feldmann, F**., Messmer K., Schubert, S.
Molecular pathotyping and functional genomics in extraintestinal pathogenic *Escherichia coli* (ExPEC).
PROKAGEN 2005 - 2nd European Conference on Prokaryotic Genomes, Sept. 23 - 26, 2005, Göttingen, Germany

9.4. Curriculum vitae

Personal details

First and last name	Friederike Feldmann
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Professional Experience

Jul. 2004 – Mar. 2008	Ph.D. thesis at the Max von Pettenkofer-Institute, Ludwig-Maximilians-University (LMU) Munich, Germany Supervisors: Sören Schubert /Karl-Heinz Schleifer Title: "Implication of extraintestinal pathogenic *Escherichia coli* siderophore receptors of in host-pathogen interaction" During this time stay abroad (2 month) in the group of S. N. Wai, Department of Molecular Biology, Umeå, Sweden, within the scope of the Network of Excellence "EuroPathoGenomics" (NoE EPG) Graduate Academy

Education

Apr. 2004	Undergraduate studentship in the group of L. Eberl, Department of Microbiology, Technical University Munich (TUM)
Apr. 2004	MSc (German university diploma) in Biology Grade 1.1 (on a scale of 1 to 4, with 1 being the highest) "passed with distinction"; Major subject Microbiology, minor subjects Limnology and Virology
Apr. 2003 – Mar. 2004	Diploma thesis in the working group of L. Eberl, Department for Microbiology, TUM, Grade 1.0 Title: "Identification of novel virulence factors of *Burkholderia cenocepacia* H111 in the *Caenorhabditis elegans* pathogenicity model"

May – Jun. 2002	Undergraduate studentship (2 month) in the group of W.Ludwig, Department for Microbiology, TUM
Aug. 2000 – Aug. 2002	Practical trainings in Microbiology (6 month), Virology (1 month), Limnology (1 month) and animal Physiology and Morphology (1 month)
Sep. 1989 – Apr. 2004	Biology studies at the Technical University Munich (TUM)
1989 – 1998	A-levels (German Abitur), Homburg/Saar (Germany), Grade 1.8

References

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Skills

Molecularbiology techniques

DNA / RNA isolation and purification techniques

PCR techniques

Reverse transcription

TaqMan analyses

Cloning techniques

*in vitro m*utagenesis of prokaryotes

Expression of recombinant proteins in different *E. coli* expression systems

Protein purification and SDS-PAGES

2DE gel electrophoresis

Polyclonal antibody production and purification

Southern and Western blot hybridization

ELISA

Immunofluorescence staining

Microbiological and cellbiological techniques

Cultivation of diverse microorganisms

Physiological characterization and phenotypic investigations of microorganisms

Cell fractionation and isolation of different membrane fractions

Centrifugation techniques (gradient and ultracentrifugation)

Cultivation of *Caenorhabditis elegans*

C. elegans pathogenicity model

Cultivation of eukaryotic cell lines

Adherence and invasion assays

Animal studies (mouse model of ascending urinary tract infections)

Epifluorescence microscopy/ Laserscanning microscopy / Atomic force microscopy

Teaching activity

Supervision of several biological undergraduate, medical graduate students and medical technical assistants

PC skills

MS-Office (Word, Excel, Power Point)

Adobe Photoshop

Lasergene

DNA and protein sequence analyses using BLAST

Languages

German (native)

English (fluent, written and spoken)

French (understand written and spoken)

Hobbies

Nature, Travelling, Hiking, Climbing, Snowboarding, Reading, Music

9.5. Acknowledgements

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