Studies
on the architecture and on transferability of pathogenicity islands
of uropathogenic *Escherichia coli* strain 536

Untersuchungen
zur genetischen Struktur und Übertragbarkeit von Pathogenitätsinseln
des uropathogenen *Escherichia coli* Stammes 536

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe.
Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.
Des weiteren erkläre ich, dass ich früher weder akademische Grade erworben noch zu erwerben versucht habe.

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I.
SUMMARY

The establishment of genomic approaches including the sequence determination of complete bacterial genomes started a new era in microbiological research. Since then more than two hundred prokaryotic and eukaryotic genomes have been completely sequenced, and there are additional complete genome projects including different bacterial species and strains in progress (http://www.tigr.org, http://www.sanger.ac.uk). The continously growing amount of bacterial DNA sequence information gives us also the possibility to gain deeper insight into bacterial pathogenesis. With the help of comparative genomics, microbiological research can focus on those DNA sequences that are present in pathogenic bacteria but are absent in non-pathogenic strains. With this knowledge and with the help of molecular biological methods such as PCR, DNA-chip technology, subtractive hybridisation, transcriptomics and proteomics we can analyse in detail what makes a particular bacterial strain pathogenic. This knowledge also gives us the possibility to develop new vaccines, therapeutic approaches or diagnostic tools.

The aim of this work was the structural and functional analysis of DNA regions of uropathogenic *Escherichia c*oli strain 536 that belong to the flexible *E. coli* gene pool. The first part of this thesis focused on the identification and structural characterisation of pathogenicity island V of strain 536 (PAI V_{536}). PAI V_{536} is integrated at the *pheV* tRNA gene at 64 minutes of the *E. coli* K-12 chromosome. In addition to the intact *pheV* tRNA gene, a truncated copy ('*pheV*) that represents the last 22 bp of this gene's 3'-end was identified 49 kb downstream of *pheV* on PAI V_{536}. The analysis of the DNA sequence flanked by *pheV* and *'pheV* revealed characteristics that are typical of PAIs. This DNA region exhibits homology to IS-elements and prophages and also comprises determinants coding for the Pix fimbriae, a phosphoglycerate transport system, an autotransporter, as well as for hypothetical proteins. Downstream of '*pheV*, the K15 capsule determinant (kps_{K15}) of this strain is located. Structural analysis of the 20-kb kps_{K15} locus revealed a so far unknown genetic organisation indicative of recombination events between a group 2 and group 3 capsule gene cluster. Downstream of the capsule determinant, the genes encoding a type II secretion system (general secretion pathway -GSP) are located on PAI V_{536}. The K15 capsule locus was functionally characterized. Specific inactivation of each of the regions 1 to 3 of the kps_{K15} gene cluster, and the use of a K15 capsule-specific antiserum demonstrated that this determinant is the functional K15 capsule locus of strain 536. It has been shown in an experimental murine model of ascending urinary tract infection with suckling mice that the K15 capsule contributes to urovirulence. Interestingly, the K15 capsule is not involved in

serum resistance of strain 536. Inactivation of the PAI V_{536}-encoded type II secretion system excluded a role of this general secretion pathway for capsule biosynthesis and virulence of strain 536 in the murine ascending urinary tract infection model.

In the second part of the thesis, the transferability of PAIs was further investigated. Using PAI II_{536} as a model, mobilisation of this island from strain 536 into suitable recipient strains was investigated. For this purpose, an antibiotic resistance cassette, the R6K origin of replication as well as plasmid pGP704 carrying the mobilisation region of plasmid RP4 have been inserted into PAI II_{536}. Transformation with the helper plasmid RP4, resulted a derivative of strain 536 that was used as a donor for conjugation experiments, while for recipient the *pir* + laboratory strain SY327 was used. After deletion the circularised PAI II_{536} was mobilised with the help of the conjugative helper plasmid (RP4) into the recipient laboratory strain SY327. The frequency of this event was about 10^{-8}. It was also demonstrated that in the transconjugant strains the mobilized PAI II_{536} could be permanently present as a circular form and also can be integrated into the chromosome at the same chromosomal insertion site (*leuX*) as in the donor strain 536. Furthermore, after mobilisation and chromosomal integration of PAI II_{536} it was possible to remobilise this PAI back to a PAI II_{536}-negative derivative of strain 536.
The results obtained in this thesis increase our knowledge of the structure and function of a pathogenicity island of uropathogenic *E. coli* strain 536 and shed some light on the mechanisms contributing to genome plasticity and evolution of pathogenic *E. coli* variants.

II. ZUSAMMENFASSUNG

Mit der Einführung von Genomanalytik einschließlich der Sequenzbestimmung bakterieller Genome, startete eine neue Ära in der mikrobiologischen Forschung. Seit Beginn dieser Ära sind mehr als 200 prokaryotische und eukaryotische Genome komplett sequenziert worden. Weitere Genomanalysen über verschiedene Bakterienspezies und Stämme sind in Arbeit (http://www.tigr.org, http://www.sanger.ac.uk). Durch die stetig anwachsende Menge an Informationen über bakterielle DNA Sequenzen, sind wir in der Lage, einen tieferen Einblick in die bakterielle Pathogenität zu bekommen. Mittels vergleichender Genomanalyse kann sich die mikrobiologische Forschung auf bestimmte DNA Sequenzen konzentrieren, welche in pathogenen Stämmen vorhanden sind, in apathogenen Stämmen aber fehlen. Mit diesem Wissen und durch molekularbiologische Methoden wie Polymerase Kettenreaktion, DNA-Chip-Technologie, Subtraktiver Hybridisierung, Transkriptom- und Proteom-Analyse können wir im Detail untersuchen, welche Faktoren für die Pathogenität eines speziellen Bakterienstammes verantwortlich sind. Diese Erkenntnisse geben uns auch die Möglichkeit neue Impfstoffe, therapeutische Verfahren und diagnostische Werkzeuge zu entwickeln.

Ziel dieser Arbeit war die Untersuchung der Struktur und Funktion von DNA-Regionen des uropathogenen *Escherichia coli* Stammes 536, welche zum flexiblen Genpool von *E. coli* gehören. Der erste Teil dieser Arbeit konzentriert sich auf die Identifizierung und strukturelle Charakterisierung der Pathogenitätsinsel V des *E. coli* Stammes 536 (PAI V_{536}). Die Integrationsstelle von PAI V_{536} liegt im *E. coli* K-12 Chromosom beim tRNA Gen *pheV* bei 64 Minuten. Zusätzlich zum intakten *pheV* tRNA Gen wurde auf der PAI V_{536} eine verkürzte Kopie des Gens (*´pheV*) 49 kb stromabwärts von *pheV* identifiziert. Diese Kopie repräsentiert die letzten 22 bp des 3´-Endes von *pheV*. Eine Analyse der von *pheV* und *´pheV* eingeschlossenen DNA-Sequenz zeigte typische Eigenschaften einer Pathogenitätsinsel. Die untersuchte DNA-Region besitzt Homologie zu IS-Elementen und Prophagen, außerdem beinhaltet sie Determinaten, die für Pix Fimbrien, ein Phosphoglycerat-Transportsystem, einen Autotransporter sowie für unbekannte Proteine kodieren können. Stromabwärts von *´pheV* liegt die K15 Kapsel Determinante (kps_{K15}) des *E. coli* Stammes 536. Eine strukturelle Analyse des 20-kb kps_{K15} Lokus zeigte eine bislang unbekannte genetische Anordnung, welche auf ein Rekombinationsereignis zwischen einem Gruppe 2 und einem Gruppe 3 Kapsel Gencluster hinweist. Stromabwärts der Kapsel Determinante sind auf der PAI V Gene lokalisiert, welche für ein Typ II Sekretionssystem („General Secretion Pathway“) kodieren. Der K15 Kapsel Lokus

wurde funktional charakterisiert. Die spezifische Inaktivierung der Regionen 1 bis 3 des kps_{K15} Genclusters und die Verwendung eines K15 Kapsel-spezifischen Antiserums zeigten, daß es sich tatsächlich um den funktionalen K15 Kapsellokus des *E. coli* Stammes 536 handelt. Im Tiermodel einer aufsteigenden Harnwegsinfektion bei neugeborenen und säugenden Mäusen, konnte gezeigt werden, daß die K15 Kapsel zur Urovirulenz beiträgt. Interessanterweise trägt die K15 Kapsel des *E. coli* Stammes 536 nicht zur Serumresistenz bei. Die Inaktivierung des in der PAI V_{536} kodierten Typ II Sekretionssystems schließt eine Rolle des „General Secretion Pathways" bei der Kapsel Biosynthese und bei der Virulenz des *E. coli* Stammes 536 im Mausinfektionsmodel einer aufsteigenden Harnwegsinfektion aus.

Im zweiten Teil dieser Arbeit wurde die Mobilisierung von Pathogenitätsinseln untersucht. PAI II_{536} wurde als Model genutzt, um den Transfer einer PAI von *E. coli* 536 in einen geeigneten Rezipientenstamm zu zeigen. Zu diesem Zweck wurde eine Antibiotikaresistenzkassette, der Replikationsstartpunkt RK6 und das Plasmid pGP704, das die Mobilisierungsregion des Plamides RP4 besitzt, in die PAI II_{536} inseriert. Nach Transformation des Helferplasmids RP4 wurde das daraus resultierende Derivat des *E. coli* Stammes 536 als Donor für Konjugationsexperimente mit dem Rezipientenstamm SY327 eingesetzt. Diese Mobilisierungsexperimente zeigten, daß die gesamte PAI II_{536} mit einer Frequenz von ungefähr 10^{-8} in einen Rezipientenstamm übertragen werden kann. In den Transkonjuganten konnte PAI II_{536} an derselben chromosomalen Insertionsstelle (*leuX*) wie im Donorstamm *E. coli* 536 inserieren. Weiterhin war es möglich PAI II_{536}, nach Mobilisierung und chromosomaler Integration in einem Rezipientenstamm, wieder zurück in ein PAI II_{536} negatives Derivat von *E. coli* 536 zu transferieren.
Die Ergebnisse dieser Arbeit erweitern unser Wissen hinsichtlich der Struktur und Funktion einer Pathogenitätsinsel des uropathogenen *E. coli* Stammes 536 und geben Aufschluß über den Mechanismus, der zur Genomplastizität und Evolution pathogener *E. coli* Varianten beiträgt.

III.
INTRODUCTION

1 EPIDEMIOLOGY OF URINARY TRACT INFECTIONS

The urinary tract is the second most common site of bacterial infection in humans (Warren, 1996). It is accepted that most uropathogens originate from the host microbial flora, and most commonly from the faecal flora, but they may also represent 'exogenous' bacteria, that can be introduced during diagnostic or therapeutic instrumentation of the urinary tract (Schaeffer, 1997). Uncomplicated urinary tract infections (UTI) are most commonly caused by uropathogenic *Escherichia coli* (UPEC) strains which are responsible for more than 80 % of the cases. It is a general rule that the more complicated the background in which a urinary tract infection occurs is, the lower is the probability that this UTI is caused by *E. coli* (Schaeffer, 1997). Only a few species can initiate infection of the normal urinary tract but a wide range of organisms may cause infection in patients with structural or functional abnormalities of the urinary tract or in immunocompromised patients. Some of the aetiological agents of UTI beside *E. coli* (90 %) are *Proteus* spp. (5-8 %), *Klebsiella* spp. (1-2 %), *Enterococcus faecalis* (<1 %), *Streptococcus* spp. (<1 %), *Staphylococcus aureus* (<1 %), and *Pseudomonas aeruginosa* (10-15 % of the nosocomial UTI cases) (Topley and Wilson, 1999). UTIs caused by other unusual organisms, such as *Acinetobacter* spp. (Ng *et al.*, 1996) or group B streptococcus, (Munoz *et al.*, 1997) appear to be more frequently reported among patients with diabetes mellitus (DM). Fungal infections are also more common than in patients without DM, particularly those involving *Candida* species, occasionally causing dramatic clinical presentations with obstruction of the urinary tract through "fungus ball" formation (Patterson *et al.*, 1995).

Most urinary tract infections are caused by a smear infection with the host's resident flora. Consequently, these microorganisms can cause an ***ascending urinary tract infection***, during which they first colonize the urethra (urethritis) and bladder (cystitis). A common symptom of such infections is a burning sensation during urination. In some cases, the infection continues to ascend further through the ureters in to the kidneys where they cause pyelonephritis. Symptoms of this disease type are back pain and fever. Pyelonephritis is a more serious disease than cystitis and urethritis because more tissue damage can occur. The high vascularisation of kidneys opens a new possibility for bacteria: By passing the vascular barrier, bacteria have the opportunity to enter the blood stream and cause septicemia. Once bacteria entered the bloodstream, any organ may be infected, and conditions ranging from pneumonia to meningitis may develop.

Urinary tract infections are not always ascending infections. Bacteria in the bloodstream can seed the kidneys and cause kidney or bladder infection. Such an infection is called ***hematogenous*** or ***descending urinary tract infection***. While ascending UTIs are mainly caused by Gram-negative bacteria, descending UTIs almost always involve Gram-positive bacteria (Peddie *et al.*, 1978).

2 VIRULENCE FACTORS OF UROPATHOGENIC *E. COLI* STRAINS

The urinary tract represents an unusual niche for faecal *E. coli* strains. A long term colonisation requires bacterial adhesion, and multiplication as well as defense against the host‘s immune system.

The properties of urine as a bacterial growth medium were already recognized by Pasteur (1863). The capacity of urine to support bacterial growth depends on its chemical composition, pH and osmolarity. Whereas some chemical constituents, such as glucose and amino acids, serve as bacterial nutrients, others, such as urea and organic acids, act as growth inhibitors (Asscher *et al*., 1966). The glucose present in normal urine is the main energy source for the growth of urinary pathogens and its concentration is normally sufficient to support maximal bacterial growth rates (O'Sullivan *et al*., 1961). The amino acid composition of urine may vary considerably and affects bacterial growth (Roberts *et al*., 1968). Organic acids normally present in urine may be bacteriostatic according to the degree of their dissociation, which is pH-dependent. Urea in high concentration is bactericidal for uropathogens (Schlegel *et al*., 1961; Kaye, 1968) but its effect is mainly due to an increase in osmolality (Asscher *et al.,* 1968). The growth rate is optimal in a pH range of 6.0-7.0 and an osmolality below 200-1100 mosm kg-1 (Asscher *et al.,* 1966).

Tolerance and utilisation of the components of urine is only one side of the coin. On the other hand a urinary tract pathogen also has to have such factors that make it possible to successfully colonize the epithelial surface of the urinary tract. Not only adhesins are important in this process, but also those factors that help to protect it from those cellular responses that are provoked by colonization of the urothelium. Additional factors can help bacteria to invade uroepthelial cells, to get into the blood stream, to survive in blood and to possess an organotropism forexample to the brain. Those factors that enable bacteria to evoke a pathogenic process are called virulence factors:

Adhesins

Bacterial adherence to the epithelial surfaces of the urinary tract is an essential step during establishment of an UTI as constant or periodic flow of urine may contribute to clearing of the bacteria. Different adherence factors enable the attachment of bacteria to the uroepithelium (Reid and Sobel, 1987) and can be subdivided into two major types: fimbrial and afimbrial adhesins.

Fimbrial adhesins consist of a rod-shaped shaft of 6-7 nm in diameter comprising over a thousand major subunits and minor subunits. The adhesin is located at the very tip of the fimbriae, often connected with the shaft via a so-called adapter pilus. The role of the major subunits is yet unclear, although they have been proposed to be important for adherence onto mammalian extracellular matrix proteins (Korhonen, 2000).

According to the impeding effect of mannose, fimbrial adhesins are divided in to two groups.

In urinary tract infections in humans (Ofek *et al*., 1981; Pere *et al*., 1987) and in experimental animal models (van den Bosch *et al*., 1980) the presence of **mannose-resistant (MR) fimbriae** on *E. coli* correlates with virulence. A number of different MR fimbriae can be distinguished on the basis of the specific receptors to which they bind. The best studied of these are ***P fimbriae*** (*pap* – *p*yelonephritis *a*ssociated *p*ili) that bind to neutral globoseries glycolipids, including globotetraosylceramide and trihexosylceramide, which are antigens of the P blood group system (Leffler and Svanborg-Edén 1980). The minimal receptor to which these fimbriae bind is α-d-Gal-(1→4)-β-d-Gal (Gal-Gal). P fimbriae are expressed *in vivo* by *E. coli* in urinary tract infections (Kiselius *et al*., 1989) and the receptors of these fimbriae are widely distributed in the kidney and bladder (Johnson, 1997). A number of MR fimbriae that do not recognize the Gal-Gal receptor are known as X adhesins or non-P MR adhesins (Johnson, 1997). Thus, ***S fimbriae*** bind to sialyl glycosides (Hanisch *et al*., 1993) and ***G fimbriae*** bind to N-acetylglucosamine (Väisänen-Rhen *et al*., 1983). Binding sites for S fimbriae are also widely distributed in the urinary tract but S-fimbriated *E. coli* isolates are probably more important in neonatal meningitis than in urinary tract infection.

Many *E. coli* strains and other members of the Enterobacteriaceae possess fimbriae (e.g. type 1 fimbriae) whose function can be inhibited by mannose, and requires a special receptor structure (Khan *et al.*, 2000). The importance of these fimbriae in the uropathogenic process is not convincing but the facts that through binding to uromucoids and poor attachment to renal tissues support that they may have a role in the colonization of the urethra and bladder (Hagberg *et al*., 1983a, 1983b). Signature tagged mutagenesis and transcriptomics revealed that type 1 fimbriae

are expressed in vivo during UTI, so type 1 seems to be important. Ørskov and coworkers (1980) suggested that in the colon, where mucus is plentiful, type 1 fimbriae actively maintain colonization of *E. coli* including potentially uropathogenic strains, whereas in the urinary tract mucus plays a dual role. Organisms that produce only type 1 fimbriae are flushed out with the mucus, whereas those that produce P and other MR adhesins will colonize the epithelium. The on-off switches ('phase variation') that control fimbrial expression may be part of this process. It was recently shown that expression of the type 1 fimbria is regulated on transcriptional level by a promoter situated on an invertible element, which can exist in one of two different orientations. Orientation of the invertible element determines if transcription occurs or not. During the course of a urinary tract infection, it was observed that the infecting *E. coli* population alternates between fimbriated and nonfimbriated states that profoundly influences colonization of the bladder (Gunther *et al.*, 2001, 2002). Not only dynamic systems but also certain allelic variations correlate with urovirulence. Certain FimH variants that bind special mannose structures can help the uropathogenic process while others not (Sokurenko *et al.*, 1999). Beside these more or less known fimbrial adhesins some new fimbrial gene clusters were recently found and identified in uropathogenic *E. coli* strains (Buckles *et al*., 2004). Their role in the uropathogenic process is not always clear (Lügering *et al.*, 2003), and should be cleared.

Uropathogenic strains have also adhesins that are not pili. These **afimbrial adhesins** are uncommon and their significance in the pathogenesis of urinary infections is unknown. One interesting feature is that their receptors are different blood group antigens like N, M and Dr (Nowicki *et al.*, 1990).

Toxins

Many uropathogenic *E. coli* strains produce exotoxins. Strains without toxin production are less virulent (van den Bosch, Emödy and Kétyi, 1982). The first exotoxins that were found are called *haemolysins* (Ludwig and Goebel, 1997). They do not only lyse erythrocytes, but also kill other cell types. They belong to the RTX (*r*epeats in *to*x*i*n) haemolysins because they all contain a nine aminoacid tandem repeat. RTX toxins are poreforming toxins and they create pores in the membrane of eukaryotic cells. For this activity Ca^{++} is required (Welch, 1991). The *cytotoxic necrotizing factor* (CNF) belongs to a group of bacterial toxins that modify Rho, a subfamily of small GTP-binding proteins that regulate formation of the actin cytoskeleton (Aktories, 1997). High sequence homology can be observed between the two CNF variants (Lemichez *et al.*, 1997).

While the *cnf1* gene is located on the chromosome (Falbo *et al.*, 1992), the *cnf2* gene on a plasmid, which was identified as the Vir plasmid (Oswald *et al.*, 1989; Smith, 1974). CNF1 is mainly associated with extraintestinal *E. coli*, and was also reported from a PAI of an uropathogenic *E. coli* strain (Blum *et al*., 1995). CNF intoxicated eukaryotic cells become enlarged and multinucleated as the results of membrane ruffling, formation of focal actin stress fibers and absence of cell division (Lemichez *et al.*, 1997).

Cytolethal distending toxins (CDTs) also make their effect on cell division of the eukaryotic cells. They inhibit cellular proliferation by inducing an irreversible cell cycle block at the G_2/M transition (Comayras, 1997). These toxins that have a unique mode of action (Elwell and Dreyfus, 2000), were reported from a number of unrelated microbes including uropathogenic *E. coli* (Bakas, 1996); however their role in uroinfection is unclear.

Iron acquisition systems

Iron is an essential bacterial nutrient that in the human host is tightly bound to protein carriers, such as transferrin or is stored intracellularly. The normal urine contains sufficient iron, probably derived from the breakdown of various cells, to allow bacterial growth. During tissue-invasive processes iron availability becomes limiting and under these conditions two mechanisms for iron scavenging are available. Haemolysin releases haemoglobin from erythrocytes and bacterial **siderophores** (Griffiths, 1997) compete for iron with host iron-binding proteins. Siderophores are low molecular weight chelators that are secreted by *E. coli,* and are able to „steal“ Fe^{3+} ions from host carriers and – by recognizing specific receptors located in the bacterial outer membrane – transport it into the bacterial cell (Guerinot, 1994). The genes of siderophores are chromosomally or plasmid-encoded. Enterobactin is a wide-spread siderophore in Enterobacteria and has a conserved localisation in the *E. coli* core genome. Many extraintestinal pathogenic *E. coli* strains produce aerobactin, another siderophore which can be chromosomally or plasmid-encoded (Dobrindt *et al.*, 2003; Sorsa *et al.*, 2003). 75 % of uropathogens associated with septicaemia produce aerobactin (Montgomerie *et al*., 1984). In the last years, more siderophore systems were described in different bacteria like the ferric citrate system (*fec*) from *S. flexneri* and *E. coli* (Luck *et al*., 2001), *yersiniabactin* (*ybt*) from *Yersinia* species and other *Enterobacteriaceae*, hemin uptake system (*shu*/*chu*) from *S. flexneri* and pathogenic *E. coli* (Torres and Payne, 1997), and salmochelin (*iro*) from *Salmonella enterica* and *E. coli* isolates (Bäumler *et al*., 1996; Dobrindt *et al*., 2003; Sorsa *et al*., 2003).

Lipopolysaccharide (O antigen)

Lipopolysaccharide (LPS, endotoxin) is a key component of the outer membrane of Gram-negative bacteria that is responsible for the O antigenic properties. It was suggested before that only a restricted range of O serogroups of *E. coli* is responsible for urinary tract infections (e.g. O1, O4, O6, O18, O75) (Roberts *et al*., 1975). Later it became clear that O antigenic lipopolysaccharides are powerful virulence factors (Hull, 1997) directly involved in the pathogenesis of urinary tract infection. During pathogenesis they could have different effects. Besides inducing the shedding of uroepithelial cells (Aronson *et al*., 1988), interfering with ureteral motility (Thuselius and Araj, 1987) and causing renal damage by the activation of granulocytes (Steadman and Topley, 1997) its role in serum resistance is important mainly in invasive infections such as pyelonephritis and in urinary tract-derived septicaemia. Susceptibility of an organism to complement-dependent bacteriolysis is due to its surface characteristics and particularly its O antigen (Porat *et al.*, 1992).

LPS comprises three distinct regions: Lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide O antigen. The lipid portion (lipid A) is embedded in the outer membrane while the core and O antigen portions extend outward from the bacterial surface. In *E. coli*, five different core structures (K-12 and R1-R4) have been described (Amor *et al*., 2000; Holst, 1999; Rietschel *et al*., 1992). The O repeating units are highly polymorphic, and more than 190 serologically distinguished forms in *E. coli* are known today (Ørskov *et al*., 1992). The genes coding for LPS core synthesis are located at a conserved position on the *E. coli* K-12 chromosomal map (81-82 min) (Berlyn, 1998). The *wa** (formerly *rfa*) gene cluster is responsible for biosynthesis of the LPS core, whereas the genes required for the O side chain synthesis are encoded by the polymorphic *wb** (former *rfb*) genecluster.

Capsule (K antigen)

Production of an extracellular polysaccharide is a common feature of many bacteria (Whitfield, 1993). The capsule, which often constitutes the outermost layer of the cell, mediates the interaction between the bacterium and its immediate environment, and plays a crucial role in the survival of bacteria in hostile environments. These highly hydrated molecules are composed of repeating single units joined by glycosidic linkages. A striking feature of bacterial capsular polysaccharides is their diversity. In *E. coli* more than 80 chemically and serologically distinct

capsules were described (Jann, 1992). According to the organisation of the corresponding gene clusters, their regulation of expression and the biosynthetic mechanism four different groups of capsules types can be distinguished (group 1-4) (Figure 1) (Whitfield, 1999).

Group 1 and 4 capsules resemble those expressed by *Klebsiella* and *Erwinia,* and consist of colanic acid. A typical feature of group 4 capsules is that they also comprise a subset of amino-sugars. Although the characteristics of capsule biosynthesis in these groups are not completely understood it is known that their genetic information is located on a highly polymorphic region of the *E. coli* chromosome at 45 minutes position in which the biosynthetic genes of a number of other cell surface polysaccharides are also located (Berlyn, 1998).

The biochemistry and genetics of capsule production of group 2 and 3 capsules is relatively well understood. These capsules contain N-acetyl-neuraminic acid and they are structurally homologous to capsules of *N. meningitidis* and *H. influenzae*. Their gene cluster shows a conserved modular genetic

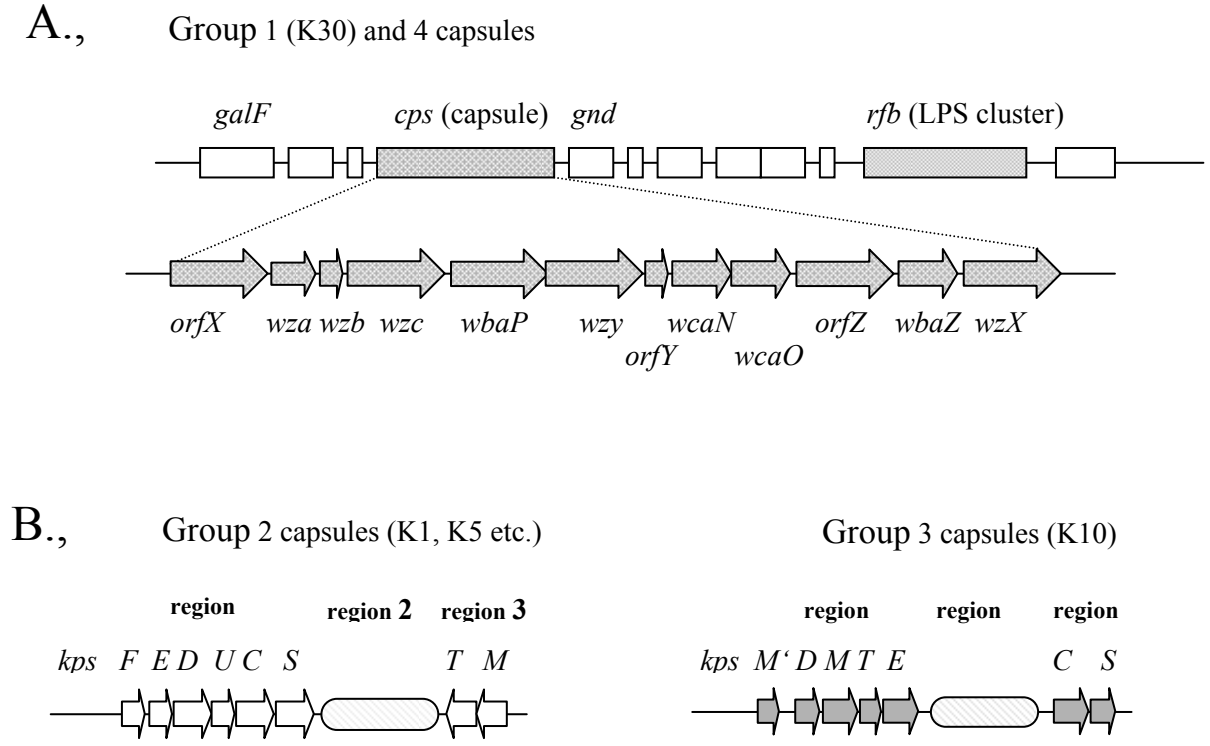


Fig. 1: Genetic organisation of group 1 to 4 capsule determinants of *E. coli*. **A**, Group 1 (K30) and 4 (K40) capsule determinants are associated with LPS and other cell surface polysaccharide gene clusters. **B,** The locus of group 2 (K5) and 3 (K10) capsule gene clusters is not associated with other surface polysaccharide genes and they exhibit a typical structure consisting of a variable region (region 2) which is flanked by two conserved regions (region 1 and 3).

organisation consisting of three regions. A highly variable antigen specific region (region 2) that encodes for the enzymes of capsule biosynthesis is flanked by two conserved regions (region 1 and 3) (Roberts, 1996). Translocation across the cytoplasmic membrane is mediated by the KpsC, M, S and T proteins, whereas translocation across the periplasm and the outer membrane involves the KpsD and E proteins (Bliss, 1996; Roberts, 1996; Whitfield, 1999). In case of the group 2 capsule, there are two additional genes, *kpsF* and *U*. The role of KpsF is unknown while KpsU is a CMP-3-keto-3-deoxy-manno-octulosonate (CMP-KDO) synthetase, which is a ligase necessary for the successful translocation through the inner membrane (Roberts, 1996).

Most of the known group 2 and 3 capsule gene clusters are located at position 64 minutes on the *E. coli* K-12 chromosome, and they are associated with the tRNA genes *serA* and *pheV* (Ørskov, 1974; Ørskov, 1976; Vimr, 1991).

Susceptibility factors

In addition to the above mentioned bacterial virulence-associated factors, there are host factors or certain conditions that facilitate colonization by uropathogens. A number of anatomical, physiological or pathological factors render individuals susceptible to urinary tract infection (Hooton, 2001), while others, for unknown reasons, hinder the pathogenic process.

3 PATHOGENICITY ISLANDS AND GENOMIC ISLANDS

In the genome of pathogenic bacteria specific genes encode virulence factors that are not present in the non-pathogenic variant of a species. In the early 1980s blocks of chromosomal regions were identified, which carry virulence genes that are exclusively associated with virulent strains of the species. It was also observed that these blocks can delete from the core genome which leads to the loss of the encoded virulence gene, and leads to a reduced virulence. These deletable large chromosomal regions are designated pathogenicity islands (PAIs) and were initially described in a uropathogenic *E. coli* strain (Blum *et al*., 1994). Since then, several new islands have been described from different organisms. The increasing information about PAIs revealed the characteristic features of these unstable chromosomal regions (Hacker and Kaper, 1999). PAIs:

- carry genes encoding one or more virulence-associated genes.
- are present in the genome of pathogenic bacteria and absent from the genome of non-pathogenic members of the same or closely related species.

- occupy relatively big genomic regions (10 < 200 kb).
- have a different G+C content compared to the core chromosome.
- represent distinct genetic elements, often flanked by "direct repeats" (DR).
- are often associated with tRNA genes.
- carry often kryptic or functional mobility genes like IS elements, transposases and integrases.
- Frequently represent unstable DNA regions.

Although PAIs have common features, they exist in the genomes of various pathogens with the capacity to cause different infections. The encoded virulence factors, the size, and the presence of different mobility genes make PAIs individual. Their mosaic-like structure is the result of continuous recombination events. With the help of mobility genes or simple recombination processes PAIs always renew themselves by loosing unnecessary genes and gaining new useful virulence associated genes. Traits of this dynamism became evident by comparing different PAIs (Rumer *et al.*, 2003).

There are also evidences that PAIs are able to cross the borders between species. The High Pathogenicity Island (HPI) of *Yersinia* is a good example for this. It means that there must be a process with which complete PAIs or at least big fragments can be transferred to another species by horizontal gene transfer.

PAIs not only carry virulence associated genes that are characteristic for different pathotypes, but also directly or indirectly have an effect on the fitness of bacteria. That is why in the last years the definition of PAIs were extended to the more general concept of "genomic islands" (GEIs). Such islands can be divided into subtypes according to the niche in which they increase the fitness of a bacterium. "Symbiosis island" of *Mesorhizobium loti* (Sullivan and Ronson, 1998) and the "ecological islands" of pollutant-degrading bacteria (van der Meer and Sentchilo, 2003) are good examples for the genomic island concept.

4 The uropathogenic *E. coli* strain 536

The uropathogenic *E. coli* strain 536 (O6:K15:H31) was isolated from a patient with an acute renal pelvis inflammation (Berger *et al.*, 1982). Since then, different virulence-associated factors have been identified from which several are associated with one of the four characterized PAIs of this strain. These include two α-hemolysin variants encoded on PAI I_{536}, and PAI II_{536}, P-related fimbriae (PAI II_{536}), S-fimbriae (PAI III_{536}), the yersiniabactin siderophore system (PAI IV_{536}) (Dobrindt *et al.*, 2002). At the time starting with this thesis there were also data available that

suggested the presence of a fifth PAI encompassing the K15 capsule determinant (Figure 2) (Dobrindt *et al.*, 2002; Janke *et al.*, 2001).

Beyond the PAI-encoded virulence genes, this strain carries other small integrated fragments coding for additional factors that are also important in the pathogenic process, like the hemin receptor (*chuA*), enterobactin and an LPS of the O6 serogroup (Berger *et al.*, 1982). There are a couple of virulence-associated genes and hypothetical proteins that are still uncharacterized. The completion of the strain 536 genome sequencing project will enable a more thorough analysis also at functional level.

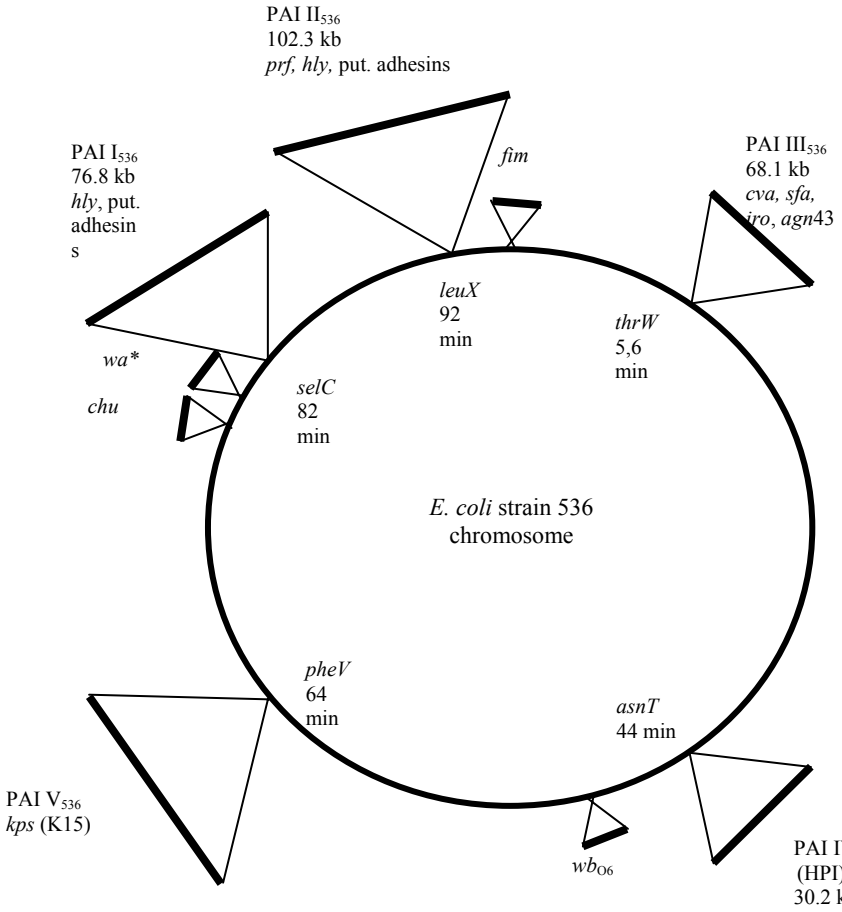


Fig. 2: Chromosomal map of the uropathogenic *E. coli* strain 536. This map is based on the chromosomal map of *E. coli* K-12 strain MG1655 and depicts the chromosomal localisation of the known PAIs and some other virulence-associated genes.

5 Horizontal gene transfer – genome plasticity – bacterial evolution

Since the early experiments of Griffiths (1928) we have known that bacteria do in fact exchange genetic information. We know already that this not only happens under artificial conditions but also in nature. There are three fundamentally distinct mechanisms by which such genetic transfer can occur. Isolated DNA molecules from the environment/medium can be taken up through transformation. DNA can also be transferred by a process in which a close cell to cell contact is required and which is called conjugation. The third form of DNA transfer is mediated by bacterial viruses (bacteriophages).

The best studied process is conjugation. Special genes (*tra*-transfer functions such as pili formation, origin of transfer (*oriT*), nick formation etc.) encode for proteins necessary for this process and are often carried by plasmids that can transfer themselves by conjugation. These **self-transmissible plasmids** are usually large (>25kb) and have a more or less broad host range. The RP4 plasmid that also carries three antibiotic resistance markers for example has a size of 60 kb (Pansegrau *et al*., 1994), and can mediate DNA transmission by conjugation also to Gram-positive bacteria and even to yeast (Bates *et al*., 1998; Heinemann *et al*., 1989; Trieu-Cuot *et al*., 1987). Another type of plasmids is not self-transmissible but by having one or two *mob* genes they are mobilizable. These **mobilisable plasmids** can be transferred in the presence of a self-transmissible helper plasmid (e.g. RP4), but not alone. They can acquire antibiotic resistance markers and they play an important role in spreading antibiotic resistance traits (Brunton *et al.*, 1979). In this process **conjugative transposons** can also take part. They can excise themselves from the chromosome of the donor strain by forming a covalently closed circle that does not replicate but integrates into the recipient's chromosome. These elements that can excise and integrate, such as prophages, and transfer by conjugation, such as plasmids, have been found in various bacteria (Burrus *et al.*, 2004). Specificity of the integration is determined by the integrase or recombinase that are encoded on these elements. There are such integrases that are really site-specific and are able to integrate into the core chromosome in certain sites. Others appear to be not so specific and can be integrated in several places of the host's genome. Site-specific excision and low specificity of integration in the same cell lead to the intracellular transposition of the element, and result in multiple copy number of this element (Umeda and Ohtsudo, 1989).

A common feature of all the forms of gene transfer between bacteria is the requirement for the transferred DNA to be inserted into the recipient chromosome by recombination. General or homologous recombination occurs between homologues regions of DNA, which must be highly

similar but do not have to be identical. Other recombination events that do not appear to require regions of homology are often referred to as non-homologous or illegitimate recombination. These events were shown to be due to the action of insertion sequences (Naas *et al.*, 1995).

Comparative studies of the structure of selected genes among those species where genetic exchange frequently occurs have shown that portions of the genes are markedly different from those of other members of the species. Such sequences have been referred to as mosaic genes (Brunder and Karch, 2000). Comparative analyses of multiple representatives of the genomes of particular species have also demonstrated that beside genes the whole bacterial genomes are relatively variable and fluid structures (Brunder and Karch, 2000). The plasticity is mainly the result of rearrangement of genes within the genome and acquisition of novel genes by horizontal gene transfer systems, e.g. plasmids, bacteriophages, transposons, gene cassettes or pathogenicity islands. Plasticity does not only mean of the acquisition of new genes (Figure 3) but also involves the process of gene loss, which also can contribute to virulence (Maurelli *et al*., 1998).

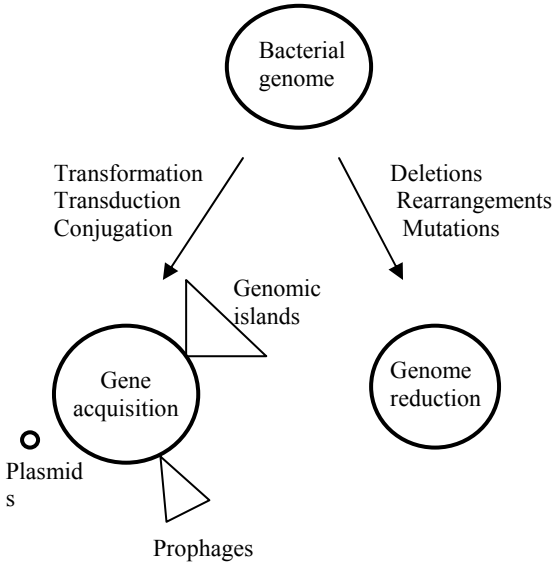


Fig. 3: Mechanisms involved in genome evolution (modified from Dobrindt and Hacker, 2001).

Aim of this work

One aim of this work was to reveal the detailed genetic organisation of the fifth PAI of the UPEC strain 536 in order to learn more about virulence factors of this strain and about the structure and diversity of PAIs among pathogenic *E. coli*.

The second aim was to get deeper insight into bacterial genome plasticity that is hypothetised to be also driven by pathogenicity islands. Using PAI II_{536} as a model, we wanted to verify the transferability of a complete PAI with conjugation.

IV.
MATERIALS and METHODS

1 BACTERIAL STRAINS, COSMIDS AND PLASMIDS

All the bacterial strains that were used in this work are listed in this chapter.

Table 1a: Laboratory *E. coli* strains used in this study

E. coli strain	Characteristics	Reference
MG1655	K12	Blattner *et al*., 1997
DH5α	F^-, *end*A 1, *hsdR*17(r_k^-, m_k^-, *supE*44, *thi*-1, *rec*A1, *gyrA*96, λ^-, Δ(*argF-lac*)U169 Φ80*dlacZ*ΔM15	Bethesda Research Laboratories, 1986
SY327 λ*pir*	F^-, *araD* Δ(*lac pro*), *argE*(Am), *recA56* Rif^R *gyrA* λ*pir*	Miller and Mekalanos, 1988
Sm10 λ*pir*	*thi*1, *thr*1, *leu*B6, *sup*E44, *ton*A21, *lac*Y1, *rec*A::RP4-2-Tc::Mu, Km^R, λ*pir*	Miller and Mekalanos, 1988
XL1-blue	*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*46, *thi, relA*1, *lac*$^-$, F^-(*proAB*$^+$, *lacI*^{q}, *lacZ* ΔM15, *Tn10* (Tet^R))	Bullock *et al*., 1987

Table 1.b: Wild type bacterial strains and their mutants used in this study

536	O6:K15:H31, *ent*$^+$, *chu*$^+$, *fec*$^-$, *hly*$^+$, *fim*$^+$, *sfa*$^+$, *prf*$^+$, *fla*$^+$, microcin$^-$, Sm^R	Berger *et al*., 1982
536*rfaH::cat*	536, *rfaH* inactivated by a *cat* cassette, Sm^R, Cm^R	Nagy *et al*., 2000
536Δ$kpsSC_{K10}$	536, $kpsSC_{K10}$ have been partially deleted and inactivated by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R	This study
536Δ$kpsC'_{K5}$	536, $kpsC'_{K5}$ has been partially deleted and inactivated by insertion of *cat* cassette, K15 capsule$^+$, Sm^R, Cm^R	This study
536Δ$kpsC_{K10}$	536, $kpsC_{K10}$ has been partially deleted and	This study

	inactivated by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R	
536ΔR2-1	536, region 2 of the kps_{K15} determinant has been partially deleted and inactivated by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R (serum resistant)	This study
536ΔR2-2	536, region 2 of the kps_{K15} determinant has been partially deleted and inactivated by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R	This study
536Δ*kpsMT*	536, *kpsMT* have been partially deleted and inactivated by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R	This study
536Δkps_{K15}	536, the complete kps_{K15} determinant has been deleted and replaced by insertion of *cat* cassette, K15 capsule$^-$, Sm^R, Cm^R	This study
536gsp::cat	536, genetic region between *gspD and L* has been deleted and inactivated by insertion of a *cat* cassette, Sm^R, Cm^R	This study
RZ532	O6:K$^+$:H31, *pap/prs*$^+$, *sfa/foc*$^+$, *fim*$^+$, *aer*$^-$, *hly*$^+$	Zingler *et al*., 1992
IMI402	ETEC strain, isolate 34344f (O6:K15:H_)	IMIB strain collection
IMI403	ETEC strain, isolate C9221a (O6:K15:H16)	IMIB strain collection
IMI415	*E. coli* isolate E642 (O6:K15)	IMIB strain collection
536-21	536, PAI I_{536}^-, PAI II_{536}^-, *hly*$^-$, *fim*$^+$, *sfa*$^+$, *prf*$^-$, *ent*$^+$, *fla*$^+$, serum sensitive, Sm^R	Hacker *et al*., 1983
536-114	536, PAI I_{536}^-	Knapp, 1982
536-225	536, PAI II_{536}^-	Knapp, 1982
SY327M23	SY327 containing PAI II_{536} integrated into *leuX*, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	This study
SY327M46	SY327 containing PAI II_{536} integrated into	This study

	leuX, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	
SY327M54	SY327 containing PAI II$_{536}$ integrated into *leuX*, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	This study
SY327M26	SY327 containing PAI II$_{536}$ in a circular form, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	This study
SY327M59	SY327 containing PAI II$_{536}$ in a circular form, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	This study
SY327M77	SY327 containing PAI II$_{536}$ in a circular form, *hly*$^+$, *prf*$^+$, Nal^R, Cm^R	This study
536mob_10	536, *pir* is integrated in the *λattB* site	This study
536-19mob	536, *pir*$_{\lambda att}$, mob$_{GP704}$ in PAI II$_{536}$ Sm^R, Ap^R, Cm^R	This study
536-19/1mob	536, *pir*$_{\lambda att}$, mob$_{GP704}$ inserted in PAI II$_{536}$, pRP4, Sm^R, Ap^R, Cm^R, Tc^R, Km^R	This study

Table 1c: Plasmids and cosmids used in this study

Designation	Description	Reference
pUC19	Ap^R, *ori*f1, *lacZ*	Yanisch-Perron *et al*., 1998
pACYC177	*ori* p15a, Ap^R, Km^R	New England Biolabs
pACYC184	ori p15a, Cm^R, Tc^R	New England Biolabs
SuperCos 1	Cosmid vector, Ap^R,	Stratagene
pBaleoBac11	*ori*S, *sop*A/B, *sop*C, *rep*E, Cm^R	New England Biolabs
pGEM®-T Easy	Cloning vector, Ap^R	Promega
pBluescript-II KS	Cloning vector, Ap^R	Stratagene
pLDR8	*int* gene expression vector, Km^R	Diederich *et al*., 1992

pLDR9	Cloning vector for integration into the λ-*attB*, Km^R	Diederich *et al*., 1992
pCVD442	*ori*R6K, *mob*RP4, *sac*B, Ap^R	Donnenberg and Kaper, 1991
pKD46	*oriR101, repA101 ts, araC-*P_{araB}, Red recombinase *γ, β, exo,* Ap^R	Datsenko and Wanner, 2000
pKD3	*ori*R6K, FRT site, Cm^R	Datsenko and Wanner, 2000
pKD4	*ori*R6K, FRT site, Km^R	Datsenko and Wanner, 2000
pCP20	Ap^R, Cm^R	Cherepanov *et al*., 1995
pGP704	*ori*R6K, *mob*RP4, M13 tg131, Ap^R	Mekalanos
pSG704	*ori*R6K, *mob*RP4, M13 tg131, Cm^R	This study
RP4	*ori*V, *trf*, Tra, *ori*T, *pri*, Ap^R, Kan^R, Tet^R	Pansegrau
pCos25/52	contains the first 3rd of PAI V_{536}, Ap^R	This study
pCos6b38	contains the second 3rd of PAI V_{536}, Ap^R	Janke *et al*., 2001
pCos24/23	contains the third 3rd of PAI V_{536}, Ap^R	This study
pCos2/13	contains a 7-kb insert downstream from Type-II GSP, Ap^R	This study
pBal-kompK15	pBeloBAC11, contains the complete kps_{K15} locus of strain 536, Cm^R	This study

2 OLIGONUCLEOTIDES

Oligonucleotides used in this study were ordered from Sigma ARK Genosys (Steinheim) or MWG Biotech (Ebersberg). Annealing temperatures were always calculated according to the following formula:

(G+C) / length of oligo x 41 + 69.3 - 650 / length of oligo .

The list of primers used in this study can be found in the appendix A/3.

3 CHEMICALS, EQUIPMENTS AND ENZYMES

Chemicals and enzymes used in this work were provided by the following companies:

GE Healthcare/Amersham Biosciences Europe (Freiburg), Clontech (Heidelberg), Difco (Augsburg), Gibco BRL (Eggenstein), Invitrogen (Karlsruhe), MBI Fermentas (St. Leon-Roth), Merck (Darmstadt), Molecular Probes (Poort Gebouw), New England Biolabs (Frankfurt am Main), Oxoid (Wesel), Promega (Heidelberg), Quiagen (Hilden), Roche Diagnostics (Mannheim), Sigma-Aldrich (Taufkirchen), Schleicher & Schuell (Dassel), Stratagene (Heidelberg), Dianova (Hamburg).

The following **kits** have been used in this study:

- "ECL™ Direct Nucleic Acid Labeling and Detection System", GE Healthcare/Amersham Biosciences Europe (Freiburg)
- "PRISM™ BigDyeTerminator-Cycle Sequenzierkit", PE Applied Biosystems (Weiterstadt)
- "Expand Long Template PCR System", Roche Diagnostics (Mannheim)
- "Readymix™ REDTaq™ PCR System", Sigma-Aldrich (Taufkirchen)
- "SuperScript™ II RNase H⁻ Reverse Transcriptase", Invitrogen (Karlsruhe)
- "pGEM® T-easy Vector System I", Promega (Mannheim)
- "QIAquick Gel Extraction Kit", Qiagen (Hilden)
- "QIAGEN Midi and Maxi Kit", Qiuagen (Hilden)

4 MEDIA AND BUFFERS

Distilled water was used for all media. All media were autoclaved for 20 minutes. For agar plates, 15 g bacteriological agar (Difco Laboratories, Detroit, MI, USA) per one liter of medium was added before autoclaving.

Luria-Bertani medium (LB) for 1000 ml (Sambrook *et al*., 1989)**:**

Trypton	10 g
Yeast Extract	5 g
NaCl	5 g

X-Gal medium:

After autoclaving LB-medium supplemented with the following additives:

IPTG (0.1 M)	0,5 ml/l
X-Gal (2 %, (w/v) in N,N`-dimethylformamide)	3 ml/l

Blood Agar plates (3-5%)**:**

Before plating out the autoclaved and pre-cooled LB agar medium, washed cow blood was added so that the end contcentration was 3-5 %. Blood was centrifuged at 5,000 rpm for 10 minutes, and upper phase (serum) was cautiously discarded. Blood cells were resuspended in sterile physiological NaCl solution. If serum was not clear enough this step was repeated.

M9 lactose medium:

800 ml dH_2O supplemented with 15 g agar was autoclaved and pre-cooled to 60-70 °C. 200 ml 5x M9 salt solution and 100 µl 1 M $CaCl_2$ were added that were separately autoclaved. Then the sterile filtered (0.22 µm) components of the medium like $MgCl_2$, casamino acids, lactose and thiamine were added as written below.

5 x M9 salts	
Na_2HPO_4 x 2 H_2O	60 g
KH_2PO_4	15 g
NaCl	2,3 g
NH_4Cl	5 g
dH_2O	1000 ml

Casamino acids (10 %)	30 ml
1 M $MgCl_2$	2 ml
1 M $CaCl_2$	0,1 ml
Lactose (20%)	10 ml
Thiamine (0.2 %)	1 ml
dH_2O	750 ml

Stock solutions of antibiotics and their working concentrations:

	mg/ml	µg/ml
Ampicillin	100	100
Chloramphenicol	20	20
Kanamycin	100	100

Streptomycin	10	10
Tetracycline	5	10

5 EQUIPMENTS

- Autoclave	Webeco
- Centrifuge	Heraeus Biofuge 13R
	Heraeus Megafuge 1.0R
	Eppendorf 5415 C
	Beckman J 2-21
- Clean bench	Nunc Inter Med
- Electrophoresis chamber	Bio Rad
- Electroporator	BioRad, Gene Pulser transfection
- X-ray films	Hyperfilm, ECL
- Gene linker	BioRad
- Hot Plate	Eppendorf Thermostat 5320
- Hybridisation oven	HybAid
- Ice machine	Scotman AF-20
- Incubator	Mammert Tv40b
	Heraeus B5050E
- Magnetic stirrer	GLW
- Micro pipettes	Gilson, Eppendorf
- Microwave oven	Moulinex
- Nylon membrane	Byodine, Nytran
	Optitran BA-S 85
- PCR Thermocycler	Eppendorf, Biometra
- pH-Meter	WTW pH523
- Photometer	Pharmacia Ultrospec 3000
- Platform Shaker	Bühler E55 swip
	Innova TM 4300
- Power Supply	BioRad 200mA, 500V
- Rotation mixer	eppendorf mixer 5432
- Sequence analyser	ABI Prism

- Shaker	GLW
	GFL Water bath
	Innova TM 4300
- Speedvac concentrator	INIVAPO 150H Uniequipe
- Sterile filter	Schleicher & Schuell 0.22µm
- Vacuum blotter	Pharmacia
- Vacuum oven	Heraeus instruments VTR 5036
- Vortexer	GLW
- Water bath	GLW 1083

6 METHODS

In cases where "rpm" values are given instead of "g" values centrifugation was carried out in Eppendorf centrifuge

6.1 Isolation of plasmid DNA

6.1.1 Rapid boiling plasmid isolation (with alkaline and heat lysis)

For screening plasmid clones containing the correct DNA insert after ligation, this fast plasmid isolation protocol was used. Cells from an agar plate or from O/N LB cultures were collected and resuspended in 150 µl lysis buffer containing 2 mg/ml lysozyme. After 5 minutes incubation at room temperature, samples were boiled at 100 ºC for 40 seconds and placed on ice for 10 minutes. After 10 min centrifugation (13,000 rpm) pellets were removed with a toothpick and 0.4 vol 5M NH_4-acetate (pH 7.5) and 1 vol of isopropanol was added to the supernatant in order to precipitate the DNA. Precpitated DNA was collected by centrifugation (15 min, 13,000 rpm) and washed with 70 % ethanol. After drying, the plasmid DNA was resuspended in 20 µl sterile dH_2O or in TE buffer.

Lysis buffer: 10 mM Tris-HCl (pH 8.0)
8 % (w/v) Sucrose
0,5 % (w/v) Triton X-100
50 mM EDTA

6.1.2 Plasmid mini and maxi preparation (QIAGEN Plasmid Purification System)

Bacteria were collected from 1.5 ml O/N LB culture by centrifugation (2 min, 13,000 rpm) and the pellet was resuspended in 150 µl Buffer I. After 10 minutes incubation at room temperature 150 µl Buffer II was added. After clearing up 150 µl Buffer III was added to the solution, and it was placed on ice for 10 min to precipitate the proteins. Following centrifugation (10 min, 13,000 rpm), the supernatant (~400 µl) was collected in another tube. From this, plasmid DNA can be precipitated or can be purified with diatomaceous earth or by phenol/chloroform extraction.

Buffer I	Buffer II	Buffer III
50 mM Tris-HCl (pH 7.5)	1 % (w/v) SDS	3 M Na-acetate
10 mM EDTA (pH 8.0)	0.2 M NaOH	
RNase 10 mg/ml		

For a maxi preparation, 50 ml O/N LB culture were used. After centrifugation (15 min, 6,000 rpm - Beckman J 2-21), the cells were suspended in 4 ml Buffer I and 100 µl lysozyme (100 mg/ml) was added. After 15 min incubation in room temperature, 4 ml Buffer II and then 4 ml Buffer III were added, and the mixture was placed on ice for 15 min. After centrifugation (30 min, 13,000 rpm), the supernatant was column filtered into another tube and the DNA was precipitated with 0.7 volume isopropanol. By thorough mixing and centrifugation, the DNA pellet was transferred to a 2 ml Eppendorf tube and washed with 70 % (v/v) ethanol. After centrifugation (10 min, 13,000 rpm), the pellet was resuspended in 500 µl dH_2O and 50 µl 5 M NH_4-acetate was added. Then, equal quantiy of phenol/chloroform was added. After shaking the tube, the solution was centrifuged (10 min, 13,000 rpm) and the supernatant was transferred to another tube where double quantity of 96 % (v/v) cold (-20 **°C)** ethanol was added to precipitate the DNA. After centrifugation (15 min, 13,000 rpm) the pellet was washed with 70 % (v/v) ethanol and centrifuged for 5 minutes. Alcohol was discarded and the pellet was dried. Plasmid DNA was solved in 100 µl water.

6.1.3 Plasmid isolation using diatomaceous earth

In order to obtain highly purified plasmid DNA (for sequence analysis), the following method was used. Plasmid prep was performed as described in (6.1.2). After precipitation the proteins with buffer three, the DNA solution was mixed with 900 µl L6 Buffer, and 50 µl diatomaceous earth suspension (20 % (m/v)) was added. It was shaked well and left for 1 min at room temperature to allow binding of the DNA to the diatomaceous earth. Then the suspension of the diatomaceous earth was filtered through a special filter and diatomaceous earth particles were washed with 2 ml washbuffer. DNA was eluted from the diatomaceous earth particles with 50 µl dH_2O by centrifugation (30 sec, 13,000 rpm in an Eppendorf centrifuge) into an Eppendorf tube.

L6 Buffer	Diatomic earth suspension	Washbuffer
100 ml 0,1 M Tris-HCl (pH 6.4)	10 g diatomaceous earth	10 ml 5 M NaCl
8.8 ml 0,5 M EDTA (pH 8)	50 ml dH_2O	5 ml 1 M Tris-HCl (pH 7.5)
13.2 ml dH_2O	500 µl ccHCl	2.5 ml 0,5 M EDTA (pH 8)
2.6 ml Triton X-100	250 ml dH_2O	
120 g Guanidin thiocyanate	250 ml EtOH	

6.2 Isolation of chromosomal DNA (Grimberg *et al*., 1989)

1 ml O/N LB culture was centrifuged (4 min, 8,000 rpm) and then washed with 1ml TNE buffer. The pellet was resuspended in 270 µl TNEX buffer containing 30 µl lysozyme (5 mg/ml) and incubated at 37 ºC for 30 min. Then, 15 µl proteinase K (20 mg/ml) was added and the mixture was incubated for 2 hours at 65 ºC. After the solution became clear, 15 µl 5 M NaCl and 500 µl EtOH were added and incubated on ice for 15 min to precipitate DNA. Precipitated DNA was collected by centrifugation (15 min, 13,000 rpm) and the pellet was washed twice with 80 % (v/v) ethanol. After moderate drying, the isolated nucleic acid was resuspended in 100 µl water.

TNE buffer	TNEX buffer
10 mM Tris (pH 8)	10 mM Tris (pH 8)
10 mM NaCl	10 mM NaCl
10 mM EDTA (pH 8)	10 mM EDTA (pH 8)
	1 % (v/v) Triton X-100

6.3 Phenol/Chloroform extraction

Protein contaminations were removed from DNA probes by phenol/chloroform extration. One volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) was added to the DNA sample and was carefully mixed. The sample was centrifuged for at least 2 minutes. Then, the upper aqueous phase was transferred to a new Eppendorf tube. The supernatant was extracted again with chloroform to remove traces of phenol from the aqueous DNA solution. The clear supernatant was then transferred to a new tube in order to precipitate the DNA as described below.

6.4 Precipitation of nucleic acids

In order to obtain pure and concentrated DNA samples, DNA was precipitated after a phenol/chloroform extraction by the addition of 0.1 volume 3 M Na-acetate (or 5 M NH_4-acetate) and 2 volumes ice-cold absolute ethanol. After incubation (15 min at –20 ºC), the DNA was pelleted by centrifugation (15 min, 13,000 rpm). After resuspension in dH_2O or TE buffer, the DNA concentration was assessed on an agarose gel or by optical density measurement.

6.5 Measuring the concentration of nucleic acids

The concentration of nucleic acids was measured as the absorption at a wavelength of 260 nm. Using a 1-cm wide quartz cuvette, the absorption of 1 (A_{260}=1) equals to nearly 50 µg/ml of double stranded DNA, 40 µg/ml of RNA or nearly 33 µg/ml of single stranded oligonucleotides. Accordingly, the DNA concentration can be calculated. The purity of the DNA samples can be measured at a wavelength of 280 nm due to the absortion by aromatic amino acids of proteins. The quotient of these two values (A_{260}/A_{280}) indicates the purity of the DNA sample. If the value is above 1.8, the DNA is very pure.

6.6 Digestion of DNA with restriction enzymes

Purified DNA samples that do not contain traces of phenol, can be specifically digested by restriction endonucleases. 1 unit (U) of enzyme is able to digest 1 µg DNA in 1 hour. The aqueous DNA solution, containing a suitable concentration of a reaction buffer, is incubated with the required quantity of the restriction enzyme form time periods ranging from 1 hour to O/N incubation. The incubation temperature is ususally 37 ºC. The restriction is usually stopped by heat inactivation (20 min, 80 ºC), phenol/chloroform extraction, the use of DNA purification kits or by the addition of 5x loading buffer.

General restriction buffer: 330 mM Tris-Acetate (pH 7.9)
660 mM K-acetate
100 mM Mg-acetate
5 mM DTT
1 mg/ml BSA
dH_2O

10 × Stop-Mix:	Bromphenol blue	0.25 %
	Xylene cyanol	0.25 %
	Ficoll type 400	25 %

6.7 Horizontal gelelectrophoresis

For the separation of DNA fragments, horizontal gelelectrophoresis was performed under non-denaturing conditions. Depending on the size of the DNA fragments to be separated, the agarose concentration varied between 0.8-2 % (w/v) in running buffer (1 × TAE or 1 × TBE). In order to have a visible running front and to prevent diffusion of the DNA, 0.2 vol Stop-Mix was added to

the samples before loading. The electrophoresis was carried out using a voltage in the range between 16-120 V. The gels were stained in ethidium bromide solution (10 mg/ml), then washed with water and photographed under an UV-transilluminator.

50 x TAE-buffer		10 x TBE-buffer	
Tris	242 g	Tris	108 g
Acetic acid	57.1 ml	Boric acid	55 g
0.5 M EDTA (pH 8)	100 ml	0.5 M EDTA (pH 8)	40 g
dH_2O	add to 1000 ml	dH_2O	add to 1000 ml

6.8 Isolation of DNA from agarose gel

After staining the gel in ethidium bromide, it was checked under UV light and the required DNA band was cut out of the gel and extracted with one of the two following methods.

6.8.1 DNA isolation from agarose gels using the QIAquick® Gel Extraction Kit (Qiagen)

The piece of the agarose gel containing the DNA fragment was placed in an Eppendorf tube and the required quantity of QG buffer (supplied by the manufacturer) was added. Upon incubation for 10 min at 50 ºC, the agarose was totally melted and the solution was pipetted onto a QIAquick® spin column. After centrifugation (30 sec, 13,000 rpm) the spin column was washed with 500 µl QG buffer to remove the remaining agarose traces (30 sec, 13,000 rpm) and then washed with 750 µl PE buffer. The column was then centrifuged again as described above to remove remaining traces of PE buffer. The column was transferred to a new Eppendorf tube and 20 µl water was added to elute the DNA. It was left for 2 minutes at RT and then centrifuged for 1 minute with maximum speed in an Eppendorf bench centrifuge.

6.8.2 DNA isolation from agarose gels using the Geneclean® kit (Qbiogene)

The cut out portion of the agarose gel was mixed in an Eppendorf tube with 3 vol NaI solution and melted at 50 ºC. Then, 5-7 µl glass milk (supplied by the manufacturer) was added and the mixture was incubated for 5 minutes at room temperature. The glass milk particles with adhering DNA were pelleted (20 seconds, full speed in an Eppendorf centrifuge) and washed with 1 ml washing solution. This step was repeated and the wash solution was removed from the pellet with a pipette. The pellet was then resuspended in 10 µl dH_2O and incubated at 50 ºC for 5 minutes. The eluted DNA was separated from the glassmilk by centrifugation (1 min, 13,000 rpm) and transferred into a new Eppendorf tube.

6.9 Dephosphorylation and phosphorylation of DNA fragments

If vector DNA is digested with one restriction enzyme, the ligation efficency can be increased by dephosphorylation of the ends of the linearized vector thereby preventing religation of the cut vector DNA. For this purpose, the digested DNA sample is incubated with alkaline phosphatase (isolated from shrimp (SAP) or the calf intestine (CIP)) which catalyses the removal of 5' phosphate groups from DNA, RNA and ribo- and deoxyribonucleoside triphosphates. Since CIP- or SAP-treated fragments lack the 5' phosphoryl termini required by DNA ligases, they can not re-ligate. Dephosphorylation requires a suitable reaction buffer and incubation for 60 min at 37 ºC. Inactivation of SAP is achieved by incubation for 20 min at 80 ºC, wheras for inactivation and removal of CIP a phenol/chloroform extraction is recommended.

To increase the ligation efficiency, DNA fragments cut out with one enzyme should be phosphorylated using the polynucleotide kinase (PNK). Phosphorylation of the DNA at the 5' hydroxyl termini of DNA hinders religation.

6.10 Ligation of DNA

The linearised vector and the insert DNA fragment can be ligated by a DNA ligase. Ligation efficiency is affected by the ratio of vector- and insert DNA molecules which should optimally be 1:3. “Sticky end” ligation was usually carried out O/N at 16 ºC or 4 ºC while blunt end ligations were carried out O/N at room temperature.

Ligation into the pGEM®-T Easy vector requires an ”adenine“ overhang at the 3' ends of the PCR product. Some DNA polymerases add an “A” to the 3'-end of the DNA strand. When necessary, a ”tailing“ reaction was carried out: 2-300 ng DNA fragment, 1 µl 10x PCR buffer, 0.3 µl $MgCl_2$, 1µl dATP (2 mM), 1 µl Taq was incubated for 30 minutes at 70 ºC. After incubation 2-3 µl from this mixture was directly used for ligation.

6.11 Polymerase Chain Reaction (Saiki *et al.*, 1988)

With this method it is possible to amplify a DNA region *in vitro*. Theoretically, one double stranded DNA moleculare can be amplified in 25 reaction cycles to more than 8 million copies. The key enzyme of this amplification by the so-called polymerase chain reaction (PCR) is the heat stable Taq DNA polymerase which functions at extremely high temperatures. PCR is used not only for DNA amplification but also for site-directed mutagenesis. The main components

required for PCR are: DNA (template), dNTPs (nucleotides), primers (oligos), reaction buffer, $MgCl_2$, Taq DNA polymerase and water.

For screening purposes requiring only amplification of selected DNA regions, the Quiagen Taq polymerase system and the REDMIX system (Sigma, Deisenhofen) was used. If necessary, e.g. for cloning experiments, enzyme systems providing a DNA polymerase with proof reading activity were used, e.g. the DAp Goldstar system (Eurogentec) and the Expand Long Template PCR System (Roche Diagnostics), to avoid incorporation of point mutations into the PCR products.

A conventional reaction mixture is composed as follows:

10 x Taq-polymerase buffer	10µl
20 mM dNTP-mix	4 µl
0.5 µg/µl primer 1	2 µl
0.5 µg/µl primer 2	2 µl
100 ng/µl template-DNA	1 µl
Taq DNA polymerase	1µl
25 mM $MgCl_2$	depends on the length of the PCR product
H_2O	add to 100 µl

This reaction mixture is kept for 2 minutes at 94 ºC. After this initial denaturation step, DNA amplification occurs during 25 reaction cycles. One cycle consists of three steps: 30 seconds DNA double strand denaturation (94 ºC), 30 seconds primer annealing to the DNA template, at a temperature which depends on the primer sequence and a third an primer elongation step (72 ºC), which depends on the length of the product.

6.12 Preparation of bacteria competent for DNA uptake by transformation

Genetic manipulation of bacteria requires that the target cell should be able to take up plasmids or DNA fragments. These bacteria should be competent for the uptake of naked DNA. This can be achieved by different methods.

6.12.1 Bacterial DNA uptake upon $CaCl_2$ treatment and heat shock

1 ml of an O/N LB culture was used for inoculation of a fresh 50 ml LB culture. Cells were then grown to an OD_{600} of 0.5 and incubated for 30 min on ice. The culture was centrifuged for 5 min

at 2000 × g at 4 °C and the pellet was resuspended in 17.5 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. After a second centrifugation step, the cells were resuspended in 2.5 ml ice-cold 0.1 M $CaCl_2$ solution and either used immediately, or ideally they were frozen at –80 °C after the addition of 50 % (v/v) glycerol.

For transformation, 50 µl of competent cells were thawn on ice and ca. 100 ng of plasmid DNA was added to the cell suspension. After incubation on ice for 30 min, the cells were incubated at 42 °C for 90 sec and then again placed immediately on ice for 5 min. 1 ml LB was added and the cell suspension was incubated at 37 °C for 1 h, allowing the expression of the selection marker before the mixture was plated on selective LB agar.

6.12.2 Transformation by electroporation

For transforming DNA into wild type strains, the more efficient electroporation was used. The efficiency of electroporation is affected by factors such as the voltage gradient, resistance and capacitance. The genetic background of the host cells, as well as the post-pulse treatment, the topological form and treatment of DNA samples also contribute to the electroporation efficiency observed (Zhu and Dean, 1999).

To prepare electro-competent bacterial cells, 50 ml of late exponential (OD_{600} 0.7-0.9) culture was used. The cells were pelleted (7 min, 5,000 rpm) and washed three times with decreasing volumes of sterile cold destilled water (50 ml, 25 ml and 10 ml). Then, the cells were resuspended in 5 ml cold sterile 10% (v/v) glycerol/water solution and centrifuged again. The cells were resuspended in 150 µl 10 % (v/v) glycerol/water solution and 50 µl aliquots of this suspension were frozen at –80 ºC or immediately used for electroporation.

For transformation, a 50 µl aliquot of cold electro-competent cells was mixed with DNA (0.1-3 ng) – dependending on the strain and DNA (plasmid or linear)), and transferred into a precooled electroporation cuvette. The dry surfaced cold cuvette was placed between the electrodes and they were discharged. Conditions of the electroporation depended on the bacterial host background. For a K-12 strain, electroporation with1.8 kV, 400 Ω and 25 µF is sufficient, but for electroporation of wild type strains (where capsules and complete LPS is present) the use of 2.0 kV with 800 Ω is more efficient. After the electric pulse, the cuvette was placed on ice for 1 minute and bacteria were washed out with LB, transferred into an Eppendorf tube and incubated at 37 ºC for 1 hour to allow the expression of the selection marker. This mixture was then plated out on selective media and incubated as required.

6.13 Conjugation

For conjugation experiments, late log phase cultures of the donor and the recipient strain were mixed in a ratio of 3:1 (or as indicated) and plated on LB agar plates. From the starting cultures, serial dilutions were made to determine their CFUs. After O/N incubation, the cells were resuspended from the plates with 1.5 ml physiological NaCl solution. The cell suspension was then diluted tenfold and samples (100 µl) were plated on selective media that should only allow growth of transconjugants. For the calculation of the rate of conjugation, samples were also plated selective media that only allow growth of the recipient strain. Next day, the colonies were counted and checked. To assess the efficiency of the conjugation, the *rate of conjugation* was calculated:

$$\text{rate of conjugation} = \text{number of conjugants} \ / \ \text{number of recipients}$$

6.14 Pulsed-field gel electrophoresis (PFGE)

High molecular weight DNA was prepared as follows: The bacteria were grown O/N in 10 ml LB medium at 37 °C. 4 ml of the culture was harvested by centrifugation and the pellet was washed two times with SE buffer. After resuspending the cells in 1 ml SE buffer, the OD_{600} was measured (100 µl cells + 900 µl SE buffer). When necessary, the OD_{600} had to be adjusted in the range between 0.6 and 0.7. A 2 % (w/v) LGT agarose solution was prepared and cooled down to 45-50 °C. 900 µl of the bacterial suspension was mixed with 900 µl agarose and transferred into appropriate moulds. After solidification at 4 °C, agarose blocks were cut in pieces with an approximately identical size (0.5 × 0.3 × 0.1 cm), and then incubated in 5 ml NDS solution (freshly supplemented with 2 mg/ml proteinase K) overnight at 50 °C on a shaker. For the complete removal of the proteinase K, the agarose plugs were afterwards washed at least four times for 2 h with TE buffer. Plugs were stored at 4 °C in TE buffer until digestion. Before using the agarose plugs, they were incubated in 1 x restriction buffer (1 h, at 50 ºC in 1 ml buffer) to remove traces of TE because of the EDTA included in the TE buffer that could impair proper restriction of the DNA. After 1 hour, the restriction buffer was removed and 150 µl fresh restriction buffer was added that already contained the suitable restriction enzyme. Agarose plugs were incubated for 3 hours with 30 U of the restriction enzyme at 37 ºC.

SE buffer	TE buffer
75 mM NaCl	10 mM Tris-HCl (pH 7.5)
25 mM EDTA (pH 7.4)	5 mM EDTA (pH 7.5)

NDS buffer	LGT buffer
1 % N-laurylsarcosine	10 mM Tris-HCl (pH 7.5)
0.5 M EDTA (pH 9.5)	10 mM $MgCl_2$
Proteinase K (2 mg/ml)	LGT agarose 2 %
	0.1 mM EDTA (pH 7.5)

Separation of the digested high molecular weight DNA fragments was carried out on a 0.8 % (w/v) agarose gel (1 × TBE buffer; see section IV.3.1.1) by horizontal gelelectrophoresis. Gels were run for 21-24 h with pulse periods of 0.5-50 s. After staining in ethidium bromide solution (10 mg/ml), gels were photographed on an UV-transilluminator.

6.15 Southern-blot analysis

DNA fragments were electrophoretically separated according to their size on 0.8-1 % (w/v) agarose gels. After staining in an ethidium bromide solution, and documentation of the fragment pattern by photography, the separated DNA fragments were tansferred to a nylon membrane (Pall Biodyne B, 0,45 μm). Before transfer, the gel was prepared for blotting by rinsing it for nine minutes in the following solutions:

- 1) 0.25 N HCl for fragmentation of the DNA fragments
- 2) Solution 1 for denaturation of the double stranded DNA (0.5 M NaOH; 1.5 M NaCl)
- 3) Solution 2 for neutralisation (0.5 M Tris (pH 7.4); 1.5 M NaCl).

In order to transfer the DNA by *capillary blotting* to the nylon membrane, the gel was placed upside down on top of several layers of Whatmann papers that were sitting in a tray of 20 x SSC buffer. Above the gel, the nylon membrane (pre-soaked in 2 x SSC) was placed avoiding air bubbles between them. Additional layers of Whatman paper (pre-soaked in 20 x SSC) were placed above as well as a thick layer of dry paper towels on top on which a heavy bottle was placed. After overnight blotting, the membrane was treated for 1 min with 0.4 N NaOH and then with 0.2 M Tris-HCl (pH 7.5). After drying, the DNA was fixed on the membrane by UV-light.

Blotting of high molecular weight DNA fragments from PFGE was carried out by *vacuum blotting*. A nylon membrane (Biodyne B) of appropriate size was shortly preincubated in dH_2O and then soaked for 10 min in 20 × SSC. Afterwards, DNA was transferred from an agarose gel to the membrane using a vacuum blotter (Amersham-Pharmacia) by applying a 50 mbar vacuum. DNA transfer from a pulsed-field gel required UV irradiation (for fragmentation of the high molecular weight DNA). The gel was then pre-treated as described for the capillary blot. After this pre-treatment, the gel was covered with 20 x SSC solution and was subjected to a 50 mbar vacuum for 2 h. After DNA transfer, the nylon membrane was incubated for 1 min in 0.4 N NaOH and 1 min in 0.25 M Tris-HCl, pH 7.5 (for neutralisation). The membrane was then shortly dried and the DNA was fixed on the membrane by exposure to UV light.

20x SSC	
NaCl	175.3 g
Na-citrate	88.2 g
H_2O	add to 1000 ml

6.15.1 Hybridisation of a labelled DNA probe to a nylon membrane (”ECL™ kit“, GE Health care/Amersham Biosciences Europe)

To detect a DNA fragment of interest among all the DNA fragments blotted on the nylon membrane, a peroxidase-labelled DNA probe was hybridised to the denatured DNA blotted on the membrane. For this purpose, the membrane was rinsed in 2 x SSC for 10 minutes and then placed into a hybridisation tube that contained 10 ml ECL blocking reagent. For one hour, the membrane was incubated at 42 ºC. For the labelling with horseradish peroxidase, a sample of a suitable DNA fragment was denaturated (5 min at 95 ºC), and then cooled on ice (5 min). Consecutively, corresponding volumes of labelling reagent and glutaraldehyde solution were added and carefully mixed with the DNA. This mixture was incubated for 10 minutes at 37 ºC. The labelled DNA probe was added to the hybridisation tube containing the membrane and the blocking reagent, and was incubated O/N or for at least 4 hours at 42 °C.

After hybridisation, the membrane was washed twice for 20 minutes in wash solution (0.5 x SSC, 0.4 % SDS). After washing the membrane with 2 x SSC solution, the membrane was incubated in a two-component detection solution (5-10 ml) included in the ECL™-kit. One minute later, the

membrane was removed from this solution, shortly dried, packed in saran wrap, and exposed for a suitable time period to the hyperfilm-ECL. The hyperfilm was subsequently developed.

6.16 Colony blot

For screening a cosmid library, the colony blot technique was used where single colonies were transferred from agar plates to a nylon membrane (Pall Biodyne B, 0,45 μm). The bacterial cells were lysed and the DNA was denaturated by incubating the membranes (with the colonies on the upper side) for 5 min on a Whatman filter paper saturated with solution 1 (see section 6.15). After short drying, the membranes were laid on Whatman filter paper saturated with neutralisation solution 2 (see section 6.15) for 5 min. The DNA was fixed at 80 °C for 30 min, and afterwards the membranes were incubated in solution 3 for 6-12 h at 37 °C. After removal of the cell debris for 1 min in 2 × SSC the colony blots were ready for hybridisation as described before.

6.17 Mutagenesis

For the construction of bacterial mutants two different approaches have been used. One of them is based on the the pCVD442 suicide vector and allelic exchange (Donnenberg *et al.*, 1991) while the other took advantage of the use lambda Red integrase system that can mediate site-specific integration of linear DNA fragments into the chromosome (Datsenko and Wanner, 2000).

6.17.1 Allelic exchange using the pCVD442 suicide vector

This method is based on the integration of a DNA construct consisting of the inactivated gene of interest and its flanking regions (~1 kb up- and downstream region) in the suicide vector pCVD442. This plasmid, that contains the π protein-dependent origin of replication (*ori*R6K), can only replicate in *pir*-positive *E. coli* strains such as strains SY327λ*pir* and Sm10λ*pir*. Transfer by conjugation from donor strain Sm10λ*pir* into a non-*pir* recipient (like, e.g. strain 536) results chromosomal integration or loss of the suicide plasmid as the π protein required for plasmid replication is not expressed in this strain. Chromosomal insertion in strain 536 can only occur via homologous recombination between the up- and downstream regions of the inactivated gene construct located on the plasmid and their homologous sequences within the chromosome thereby exchanging the functional chromosomal gene by the inactivated copy introduced on the suicide plasmid. Selection for chromosomal suicide plasmid insertion is based on antibiotic resistances encoded on the suicide plasmid or on the host chromosome. Suitable candidate clones resistant to ampicillin and streptomycin were incubated for two 12 h periods without selective

pressure at 37 ºC in order to allow for allelic exchange and the loss of the suicide plasmid backbone. Selection for mutant candidates was done on agar plates supplemented with suitable antibiotics and 5 % (w/v) sucrose plates at room temperature. If the suicide vector backbone has not been lost, the cells cannot grow on sucrose because the pCVD442-encoded SacB laevan sucrase transforms sucrose to the polymer laevan which is toxic for *E. coli.* Selected mutants were checked by PCR and Southern-blot.

6.17.2 Gene inactivation by lambda Red recombinase-mediated site-specific insertion of linear DNA fragments (Datsenko and Wanner, 2000)

The lambda Red recombinase-expressing plasmid pKD46 was electroporated into UPEC strain 536. The overnight culture (grown at 30 **°C**) of this strain was used as a starter culture and was diluted 1:100 in 50 ml LB^{Amp} medium. For induction of the lambda Red recombinase expression, 20 mmol L-arabinose was added to the medium and the culture was grown at 30 ºC till it reached OD_{600} 0.6. For better induction –because, strain 536 metabolizes L-Arabinose-, at this cell density, additional 10 mmol L-arabinose was added to the culture. It was placed on 30 °C to incubate. After 20 minutes cells were harvested (5,000 rpm, 5 min) and washed twice with 50 ml and with 20 ml ice-cold sterile water, respectively. Then, the cells were washed in 5 ml ice-cold 10 % (v/v) glycerol/water solution. The pellet was afterwards resuspended in 150 µl 10 % (v/v) glycerol/water solution. 50 µl aliquots were directly used for electroporation (10 kVs/cm; 600 Ohm; 0.25 F) with the purified suitable linear DNA fragments usually generated by PCR with specific primers whose 5' ends were homologous to the priming sites 1 and 2 of the plasmids pKD3 or pKD4. The 3' ends of the primers used were homologous to the flanking region of the genes to be inactivated. PCR with these primers using pKD3 or pKD4 as template DNA resulted in amplification of PCR products that consisted of an antibiotic resistance marker flanked by two FRT sites. The extreme 5'- and 3'-ends of the PCR products represented the homologous sequences required for site-specific integration of the DNA fragment into the chromosome. The FRT sites facilitated later the elimination of the resistance marker genes with the help of the pCP20 plasmid. After electroporation cells were incubated in 1 ml LB for 1.5 hours at 30 ºC. For recombination cells were left O/N at 20 ºC temperature and then plated on selective plates. Cm^- and Km-resistant recombinant candidates were selected and tested by PCR and Southern blot analysis.

6.17.3 Integration of genes into the λ attachment site

In order to complement bacterial mutants with a single copy gene, the gene of interest can be inserted into the host chromosome. For this purpose the system that was described by Diederich and coworkers (1992) was used, which allowed the integration of any DNA fragment into the bacteriophage λ attachment site *attB* of the *E. coli* chromosome. The corresponding gene (that must not contain a *NotI* restriction site) was cloned into one of the plasmids pLDR9, 10, or 11. They differ only in their resistance markers (Km, Cm, Tet) and as the result of this in their restriction pattern. After ligation, the plasmid was cut with *NotI* and the two fragments were separated. The fragment containing the gene of interest, the attP site and a *bla* gene, but no the origin of replication was religated. After ligation, the circularised fragment was transformed into the recipient strain that contained the helper plasmid pLDR8 (Km^R) coding for a λ integrase that catalyses the recombinaiton between the *attP* and the *attB* site thereby inserting the gene of interest into the chromosome. The temperature–sensitive replicon of this plasmid allows replication at 30 ºC. Competent cells were prepared from an O/N culture grown at 30 °C that was then diluted 1:20, and incubated for 90 minutes at 37 ºC to partially de-repress the temperature-dependent λ P_R promoter that directs expression of the *int* gene to achieve a basic level of Int protein in the cell. Upon transformation, these competent cells were incubated O/N at 42 ºC on ampicillin plates in order to further induce *int* expression and to inhibit replication of pLDR8. Ampicillin-resistant clones were checked by PCR (primers ATT1 and ATT2) and by Southern blot analysis for correct chromosomal complementation with a single copy gene.

6.18 Sequence analysis

The nucleotide sequence of DNA inserts cloned into BACs, cosmids or plasmids was determined from both ends using dye terminator chemistry. For a final reaction volume of 10 µl 4 µl "Premix" and 10 pmol primers were used. The concentration of the DNA template varied between 0.1-1 µg depending on the quality and the source (cosmid, plasmid, or PCR product). The sequencing reaction includes the following steps: The DNA was initially denaturated at 95 ºC for 2 minutes and then for 30 sec as the first step of every reaction cycle. Annealing (30 sec) of the primer was carried out at a temperature depending on the primer while the elongation step was carried out at 45 to 60 ºC for 4 min. This reaction cycle was repeated 25-35 times. After the PCR, the DNA was precipitated with pure absolute ethanol and was washed with 70 % ethanol. The PCR product was dried in a speed vac, and resuspended in 20 µl Hi-Di formamide.

Large scale sequence determination of the three cosmid clones (pCos6b38, pCos24/23, pCos25/52) was carried out at the Göttingen Genomics Laboratory. For this purpose, small insert libraries (2-2.5 kb) were generated by mechanical shearing of DNA (Oefner *et al.*, 1996). After end repair with T4 polymerase, the fragments were ligated into the prepared pTZ19R vector. Isolated plasmids were sequenced from both ends using the dye terminator chemistry and analyzed on ABI337 sequencers (Applied Biosystems). The Phrap software implemented in the Staden software package was used for assembly and editing of the sequence data (Staden *et al.*, 2000).

Homology searches as well as searches for conserved protein domains were performed with the BLASTN, BLASTX and PSI- and PHI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) programs of the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1997). Putative ORFs were identified using Vector NTI® (InforMax) and the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) softwares. Prediction of membrane spanning regions of proteins was carried out with the TMpred software (http://www.ch.embnet.org/software/TMPRED_form.html). The sequences were submitted to the EMBL nucleotide sequence database.

6.19 RNA methods

6.19.1 Total cell RNA isolation

In order to quantify transcript levels of the genes of interest, total RNA was isolated and analysed by RT-PCR and Northern Blots.

Working with RNA requires a ”nuclease free environment“. All steps were carried out with gloves. All the surfaces were cleaned with absolute ethanol before work and all the solutions used were prepared with 0.1 % (v/v) DEPC (diethyl pyrocarbonate) water. 1 ml DEPC was added to1 liter water, vigorously shaken and then placed O/N at 37 ºC to inacticvate RNases. The next day, the DEPC-treated water was autoclaved and then ready to use. RNases can also be inactivated by the addition of 0.1 M NaOH; 1 M EDTA or chloroform.

6.19.2 Small quantity RNA isolation (Qiagen RNeasy kit)

2 ml of a bacterial culture (OD_{600}=1) were centrifuged (4 min, 5.000 rpm). The pellet was suspended and then vortexed in 100 µl TE buffer containing lysozyme (400 µg/ml). After

incubation for 5 min at room temperature, 350 µl RLT buffer were added. The sample was vortexed and then centrifuged for 2 minutes at 13.000 rpm. The supernatant was transferred to a new tube and suspended with 250 µl absolute ethanol. This mixture was pipetted into a RNeasy mini column and centrifuged for 15 sec, (13,000 rpm). The cloumn was washed with 700 µl RW1 buffer and then with 500 µl RPE buffer (15 sec, 13.000 rpm). The column was washed a third time with RPE buffer (2 min, 13,000 rpm). The RNA was eluted from the column by addition of 30 µl water for two minutes, and centrifuged (1 min, 13,000 rpm).

6.19.3 Large quantity RNA isolation

Large quantities of total RNA were extracted from 10 ml cultures that were immediately poured on equal volume of -80 °C cold crushed ice. The bacterial cells were spun down at 4 °C for 15 min (6,000 rpm). The pellet was resuspended in 0.6 ml of ice-cold buffer (10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris; pH 7.4) and then immediately added to 0.6 ml of hot lysis buffer (0.4 M NaCl, 40 ml EDTA, 1 % sodium dodecyl sulfate) to which 1 % (v/v) β-mercaptoethanol and 100 µl of water-saturated phenol were added immediately before. This mixture was incubated at 100 °C for 40 seconds. Cell debris was removed by centrifugation (10 min, 13,000 rpm) and the supernatant was extracted five times with (1:1, v/v) phenol-chloroform solution. RNA was precipitated with ethanol, washed and dried. The dried pellet was dissolved in sterile DEPC-treated water.

6.20 Reverse Transcription PCR (RT-PCR)

For the Reverse Transcription PCR (RT-PCR) 15 µg of the total RNA preparation was digested with 0.5 units / µl of Rnase-free DNase I (Roche, Germany) for 1 hour at 37 °C to eliminate traces of DNA. The 100 µl DNA-free total RNA solution was mixed with 350 µl RLT buffer (Qiagen, Germany) and 250 µl absolute ethanol and subsequently transferred to a RNeasy spin column. This column was centrifuged (15 sec, 10.000 rpm) and washed with 500 µl RPE buffer. After a second centrifugation –to get rid of the RPE remnants- the purified RNA was eluted wit 30 µl water. For reverse transcription, 2-5 µg RNA was mixed with 3 µg of random primers (Amersham Biosciences, Freiburg) and then denaturated for 10 minutes at 70°C. After cooling down on ice, cDNA synthesis was performed with the Super Script II RNase H^- Reverse Transcriptase (Invitrogen) system at 46°C. After 1 hour, the reaction was stopped by alkaline

hydrolysis of the RNA with 0.25 M NaOH (15 min, 65 °C). The pH was restored by addition of 50 mM Tris-HCl, pH 7.5. The cDNA was purified with QIAquick Gel Extraction Kit PCR purification kit (Hilden, Germany) and amplified by PCR with specific primers. Isolated total RNA (that still contained DNA traces) served as a positive control, whereas the same preparation after DNAse digestion was used as a negative control.

6.21 Gel electrophoresis of RNA

RNA was separated on 1.2 % (w/v) agarose gels to which MOPS buffer and formaldehyde were added after cooling of the agarose solution to 55 °C. After solidification, the gel was placed in an electrophoresis chamber floated with 1x MOPS buffer. Before electrophoresis, the RNA samples (10 µg of the RNA sample was diluted in 40 µl DEPC-treated H_2O to which 10 µl 5x RNA loading buffer was added) were denatured by heating to 65 ºC for 5 minutes. The samples were then cooled on ice. The RNA marker was also treated under identical conditions. Electrophoresis was carried out for 2 hours at 120 V. The gel was washed in water for half an hour in order to remove remaining formaldehde and then stained in ethidium bromide solution.

RNA gel	
Agarose	1.3 g
10 x MOPS buffer	100 ml
cooling down at 60 ºC	
10x MOPS	11.4 ml
37 % Formaldehyde	3.5 ml

10x MOPS	
MOPS	41.8 g
H_2O	800 ml
pH 7 (with 10 N NaOH)	
Na-acetate (3 M)	16.6 ml
EDTA (0.5 M)	20 ml
pH 7 and filtration!	

6.22 Northern blot analysis

After staining, the separated RNA fragments can be transferred to a nylon membrane as described before. The detection of transcripts of interest is carried out as described for Southern blot analysis under 6.15.1.

6.23 Isolation of LPS

The cells from 1 ml overnight culture were collected by centrifugation and treated as described for the isolation of genomic DNA in section 6.2 (Grimberg *et al*., 1989). After incubation with

proteinase K, 600 µl phenol were added to the clear solution, then it was carefully mixed and centrifuged for 10 min (13,000 rpm). The supernatant was transferred to a new tube and 20-50 µl were used for electrophoresis.

6.24 Polyacrylamide gel electrophoresis (PAGE, Laemmli *et al*., 1970)

Protein or LPS samples were separated and analysed on polyacrylamide gels. The size of the gels was 15 × 17.5 × 0.5 cm, and the electrophoresis was performed overnight at RT and 30 V. The samples were loaded on top of the "collecting gel" after heating for 10 min at 90°C in 4 × loading buffer.

Separation gel (13 % (w/v))

30 % acrylamide, 0.8 % bisacrylamide	13 ml
1.5 M Tris-HCl, pH 8.8	7.3 ml
dH_2O	9 ml
10 % SDS	300 µl
APS (100 mg/ml)	150 µl
TEMED	10 µl

Collecting gel (5 % (w/v))

30 % acrylamide, 0.8 % bisacrylamide	2.7 ml
1.5 M Tris-HCl, pH 8.8	4 ml
dH_2O	9 ml
10 % SDS	160 µl
APS (100 mg/ml)	160 µl
TEMED	8 µl

10 × Electrophoresis buffer

Tris	30 g
Glycine	144.4 g
10 % SDS	100 ml
dH_2O to a final volume of	1000 ml

4 × Loading buffer

0.25 M Tris-HCl, pH 6.4	2.5 ml
SDS	0.25 g
EDTA	4 mg
86 % (v/v) Glycerin	4 ml
β-Mercaptoethanol	0.5 ml
Bromphenol blue	0.4 mg
dH_2O to a final volume of	10 ml

6.25 Staining of LPS in polyacrylamide gels with silver nitrate (Tsai *et al.*, 1982)

After electrophoresis, the polyacrylamide gels were stained with $AgNO_3$. All used devices were carefully washed with 70 % (v/v) ethanol. The gels were fixed overnight in 100 ml 1 × fixation solution. Next day, the solution was replaced by 100 ml 1 × periodate solution and the gels were incubated for 5 min. After that, the gels were washed three times for 30 min with dH_2O. Then, the gels were incubated for 10 min in silver nitrate solution and subsequently washed three times for 10 min with dH_2O. The gels were developed in developing solution preheated to 60 °C, and when the colour intensity of the stained bands was appropriate, the reaction was stopped by washing with 50 mM EDTA solution for 10 min.

2 × fixation solution

Isopropanol	250 ml
Acetic acid	70 ml
dH_2O to final volume of	500 ml

Periodate solution

Na-periodate ($NaIO_4$)	0.87 g
1 × fixation solution	100 ml

Silver nitrate solution

1 M NaOH	1.4 ml
NH_3 (33 %)	1 ml
dH_2O	70 ml
20 % (w/v) $AgNO_3$	1.25 ml

Developing solution

2.5 % (w/v) Na_2CO_3	100 ml
40 % (v/v) Formaldehyde	27 µl

Immuno techniques

6.26 Production of a polyclonal antiserum against the K15 capsule antigen

A K15 capsule-specific serum was produced according to the protocol described by Kiesewalter and Seltmann (1968). Briefly, O/N culture of bacteria (the *E. coli* strain 536) was washed and incubated overnight in 50 % (v/v) ethanol at 4 °C to denature protein antigens. After washing, bacteria were diluted to 2 x 10^8 CFU/ml in phosphate-buffered saline (PBS). Rabbits were immunized with 0.2; 0.5; 1.0; and twice 2.0 ml of bacterial suspensions in 4-day intervals. Four days following the last inoculation, test sera was taken and their titres were tested by tube agglutination. Afterwards, the rabbits were sacrificed and their sera were collected and diluted 1:10 in sterile saline containing 0.3 % (v/v) phenol as a preservative. Removal of non-specific antibodies was achieved by absorption with live and boiled cells of uropathogenic *E. coli* strain RZ532 (O6:K^+:H31) which does not express a K15 capsule. For this, a 0.5 g aliquot of wet *E. coli* strain RZ532 cells was suspended in 1 ml of the diluted serum and this suspension was incubated for 1 h at 37 °C and overnight at 4 °C. Next day, the cells were pelleted, resuspended in saline, and boiled for 1 h. Following centrifugation, the pellet was resuspended in the same serum and the suspension was incubated again for 1 h at 37 °C and subsequently overnight at 4 °C. After centrifugation, aliquots of the serum were stored at –20 °C. By this procedure, the serum became free of any immunoreactive antibodies except those against the K15 capsule antigen. This was confirmed by an enzyme-linked immunoabsorbent assay (ELISA).

6.27 Enzyme-linked immunoabsorbent assay (ELISA)

96-well plates (C.E.B., France) were coated overnight with 0.2 ml of bacterial suspension (10^9 CFU/ml in coating buffer) at 4 ºC. The following day, the plates were washed with PBS + 0.5 % Tween20 (washing puffer), and then blocked with PBS containing 2 % (w/v) bovine serum albumin (BSA) for 1h at 37 ºC. The K15-specific serum was diluted in PBS + 0.5 % BSA and pipetted into the antigen-coated 96-well plates. After incubation for 90 min the plates were washed three times with the washing buffer. The bound K15-specific antibodies were detected with a commercial anti-rabbit immunoglobulin conjugated with horseradish peroxidase (HRPO) (Dako A/S, Denmark). The ELISA substrate was *ortho*-phenylenediamin (Sigma) dissolved in citric acid buffer containing 3 % H_2O_2. Optical density was measured at 490 nm in a conventional ELISA plate reader. Duplicates were used for each strain and dilution, and assays were repeated twice.

6.28 Immunofluorescence microscopy

1 ml bacterial O/N culture was washed once and the cells were resuspended in water. 20 μl of this suspension was dropped on a glass slide. After drying (37 ºC for 20 min) a 1:4 dilution of absorbed K15-specific antiserum was added. After 1 hour incubation at 37 ºC, the glass slide was carefully washed twice by dipping it into PBS for ten seconds. Texas red-labelled anti-rabbit IgG (Dianova, Hamburg) was added and the slide was again incubated for 1 hour at 37 ºC. After incubation, the slide was washed again in PBS (two times for ten seconds) and the Texas red-labelled anti-IgG antibody that bound the K15-specifc antibodies was observed by epifluorescence microscopy. To excitate the dye, the wavelength 596 nm was used (emission wavelength is 620 nm).

6.29 Serum resistance assay

O/N culture of bacteria grown in LB medium was washed with saline and diluted to 10^6/ml cells. 100 μl aliquots of the cell suspension were mixed with an equal volume of fresh normal (no bacterial infection, and no antibiotics taken recently) human serum and incubated at 37 ºC for 4 hours in microtiter plates. Samples were taken at 0 h, 0.5 h, 1 h, 2 h, 3 h, and 4 h time points. 10 μl from each sample was taken out and diluted 100x in saline in Eppendorf tubes. The number of viable cells was determined by plating onto LB plates following an overnight incubation at 37 ºC. In addition, serum resistance assays were performed with heat-inactivated (56 ºC for 30 min) serum as a negative control. Three samples of every bacterial strain were analyzed and the assays were performed in triplicates.

In vivo virulence assays

6.30 Ascending urinary tract model

In order to study the virulence of *E. coli* strain 536 and its derivatives, intravesical infection of 2-3-day old suckling CFLP mice (Gödöllö, Hungary) were performed. Bacteria were grown overnight at 37 ºC in LB medium and then harvested by centrifugation, washed once and normalized to the required inoculum density (10^7/ml) in PBS. 0.025 ml of this bacterial suspension containing 0.05 % Pontamin Sky Blue dye (Searle Pharmaceuticas, High Wybcombe) were infected into the bladder directly through the abdominal wall. The stain, which has no toxic or antibacterial effect served as an indicator of successful inoculation. For every strain 6-14 infant

mice were intravesically infected and the assays were repeated four times. Mice that survived infection, were sacrificed 21 days post-infection. The bladder and both kidneys were removed, homogenized in PBS, and aliquots were plated onto agar plates containing a selective antibiotic. Colonies were counted after overnight incubation. Additionally, bacterial counts were determined from the urine and blood samples.

V.
RESULTS

1 Analysis of the fifth PAI of the UPEC strain 536

Genomic differences between the UPEC strain 536 and the non-pathogenic *E. coli* strain MG1655 were previously identified by suppression subtractive hybridisation (SSH) (Janke *et al*., 2001). Altogether, 22 SSH-fragments were found that showed homology to already known strain 536-specific genes or that represented novel genes that were only present in strain 536 but that had not been characterised before. One of these fragments showed similarity to a capsule export protein encoding gene, *kpsC*, of an *E. coli* K10 strain (GenBank accession no. AF127177).

The fact that capsule gene clusters are often mapped near the tRNA genes *serA* (Orskov and Nyman, 1974; Orskov *et al.*, 1976) or *pheV* (Swenson *et al.*, 1996; Welch *et al*., 2002) and that PAIs are also frequently associated with tRNA-encoding genes suggested that a fifth PAI of the UPEC strain 536 (PAI V_{536}) might have been found. A cosmid library was used to verify this hypothesis and to map the genetic structure of the hypothetical PAI and the downstream sequences.

With a $kpsC_{K10}$ specific probe a cosmid clone (pCos6b38) was isolated and sequenced. Sequence analysis of this 41,565 bp- cosmid clone is summarised in the thesis of Janke (2000). It revealed that also in *E. coli* 536, capsule genes are located close to a 5' deleted *pheV*. In this work, junction cosmids of pCos6b38 were identified to further characterise the novel PAI of 536 and to define the borders of the 536 specific DNA region.

1.1 Mapping of PAI V_{536} and its flanking regions by overlapping cosmid clones

1.1.1 Cosmids that overlap the right side of pCos6b38

By using specific DNA probes to both ends of pCos6b38, the genomic cosmid library was screened for overlapping cosmid clones. Primers *Genrev 6buni* and *Genrev 6brev* were used to amplify a 275 bp-product located at the right end of pCos6b38 where *kpsC* is located. Five clones (pCos5/52, pCos8/55, pCos8/75, pCos22/76 and pCos24/23) that contained the right end of pCos6b38 (as was confirmed by the screening PCR and by colony hybridisation) were further analysed for their degree of overlap by determining the sequence of their ends with primers *SuperCos uni* and *SuperCos rev* (Table 2).

Table 2: Sequence analysis of the ends of cosmid clones that overlap with pCos6b38.

	Homology of the sequences obtained	
	Forward primer	Reverse primer
pCos5/52	hypothetical amino transferase	K-12 genome, position 71435-71317, minutes 65-68
pCos8/55	*kpsD*	K-12 genome, position 73270-73150, minutes 65-68
pCos8/75	K-12 genome, position 77250-76447, minutes 65-68	*kpsF*
pCos22/76	K-12 genome, position 81947-81823, minutes 65-68	*kpsC*
pCos24/23	No homology	No homology
pCos6b38	No homology	K-12 genome, position 37653-37748, minutes 84.5-86.5

According to these results and by comparison of the restriction pattern of an *Eco*RI digest, the relative orientation of the five cosmids to pCos6b38 and to each other was determined (Fig. 4). Sequence analysis of four cosmids had revealed homology to the region of 65-68 minutes of the *E. coli* K-12 chromosomal map. The fact that the tRNA gene *pheV* is located at 64 minutes (position 64,760 to 64,835) further supported our hypothesis that a new PAI was found that was associated with *pheV* of the UPEC strain 536.

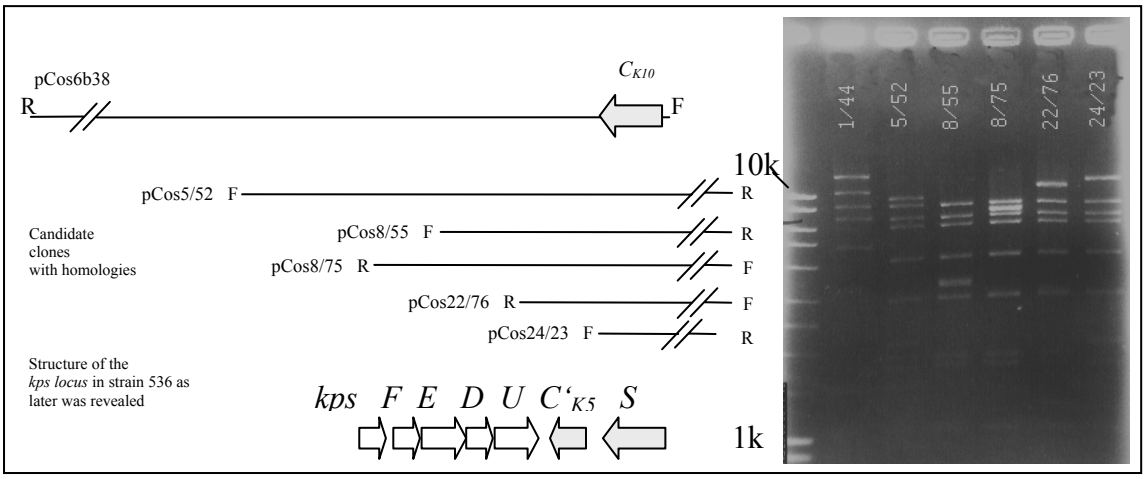


Fig. 4: Relative orientation of the five candidate cosmid clones to pCos6b38. The left side of the figure shows the relative position of the cosmid clones. Below, the orientation and order of *kps* genes as determined from the sequence analysis of pCos6b38 are also depicted. On the right side, the *EcoRI* restriction patterns of the candidate clones (pCos1/44 is included as a negative control that does not contain *kps* genes)) are shown. F: sequenced with primer SuperCos uni; R: sequenced with SuperCos rev.

1.1.2 Conserved components of a capsule locus (*kpsM* and *kpsT*) are present on the cosmids that overlap pCos6b38

The sequence analysis of pCos6b38 had revealed that downstream of a *'pheV* gene, the right end of this cosmid contains the genes of the conserved regions of group 2 and 3 capsules (thesis of Janke, 2000, see also below). Although this capsule locus showed similarity to the capsule determinants of both group 2 and 3, it also showed a novel structure that had not been described before. Nevertheless, for a functional capsule transport machinery two additional conserved genes *kpsM* and *kpsT* were missing. To verify the presence of these two conserved transporter genes on the chromosome of strain 536, primers were designed based on the known sequences of genes *kpsM* and *kpsT* from K5 and K10 capsule types that are the best studied representatives of group 2 and group 3 capsules, respectively (Fig. 1). PCRs with these primers were performed with strain 536 DNA, and with the six cosmid clones containing the capsule gene cluster of strain 536. Additionally, probes for Southern hybridisations were amplified from K5 and K10 strains, respectively. With the exception of pCos6b38, similar results were obtained for *E. coli* 536 and the tested cosmid clones (Table 3).

Table 3: Presence of K5-specific *kpsM* and *kpsT* genes on the chromosome of strain 536

	kpsT	*kpsM*
K5 specific PCR	-	-
K5 specific Southern blot	+	+
K10 specific PCR	-	-
K10 specific Southern blot	-	-

We could gain two pieces of information from these results: (i) The two missing conserved genes that were necessary for the capsule transport through the bacterial inner membrane were present on the overlapping cosmid clones. This suggested that we had identified a functional capsule determinant of strain 536. (ii) Southern blot experiments showed that *kpsT* and *kpsM* of strain 536 were different from that of the K10 capsule type, but both of them were similar to the representatives of K5, which belongs to the group 2 capsules. However, no product was amplified with K5 specific primers suggesting that sequence differences prevented successful binding of the oligonucleotides.

1.1.3 Identification of pCos24/23 as the cosmid covering the most 3' end of PAI V_{536} and its flanking regions

Hybridisation with the K5-specific *kpsM* and *kpsT* probes to the digested 5 candidate cosmid clones revealed that all of them contain these capsule component transport genes. To determine their exact location relative to the conserved genes that were already known from the sequence analysis of pCos6b38, and to find the K15-specific variable region (Region 2) a suitable cosmid clone for further sequence determination had to be identified. The analysis of the cosmid ends revealed that four of them were identical to a region of the core chromosome of *E. coli* K-12 strain MG1655 encoding a putative general secretion pathway (position 71,000 to 82,000) (Table 4). Assuming that the novel PAI was inserted at the 3' end of *pheV* (~ position 65,000) we calculated a nearly 25 kb insertion between *pheV* (*'pheV*) and the secretion pathway genes. On average, cosmid vectors carry inserts of about 40 kb. Only one cosmid (pCos24/23) showed no homology to any known *E. coli* gene at its ends. To unravel whether this region was also present on the other four cosmids (e.g. between capsule genes and the secretion pathway genes) or whether the insert of pCos24/23 was larger thereby covering another possible integration in the

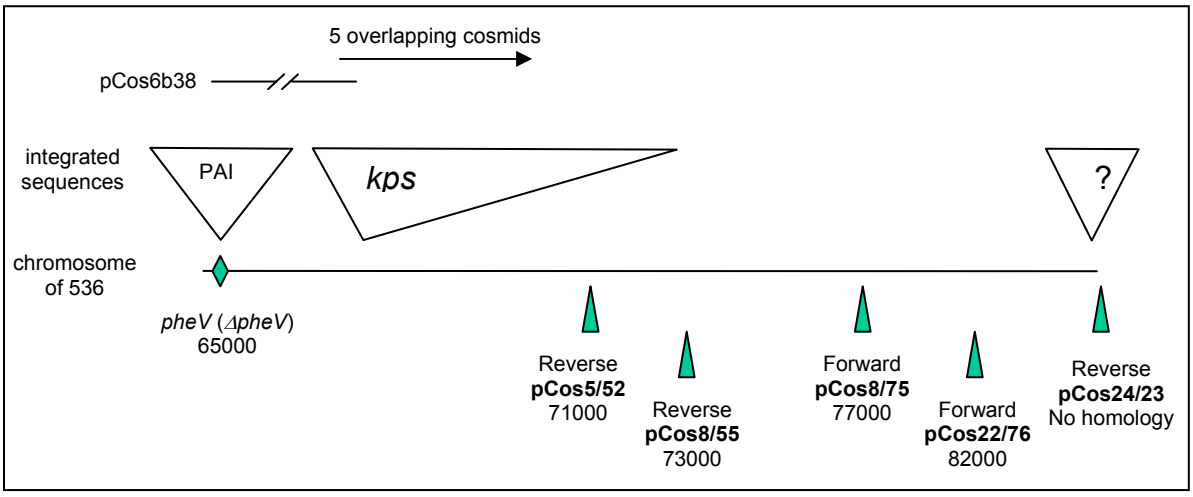


Fig. 5: Sequence analysis of the non-overlapping ends of the five cosmid clones revealed homology to downstream sequences of *pheV* and also suggested integrated 536 specific elements (represented by reversed triangles) into the K-12 backbone. The *kps locus* was suggested to be nearly 25 kb, downstream of it the genes of a general secretion pathway were present and analysis of the reverse end of pCos24/23 revealed an additional integration into the chromosome of strain 536. (Standing green triangles represent the other ends of those cosmids that are overlapping with pCos6b38 on one end. The numbers under the names of each cosmid and *pheV* represent the sequence homology relative to the chromosomal position of MG1655.)

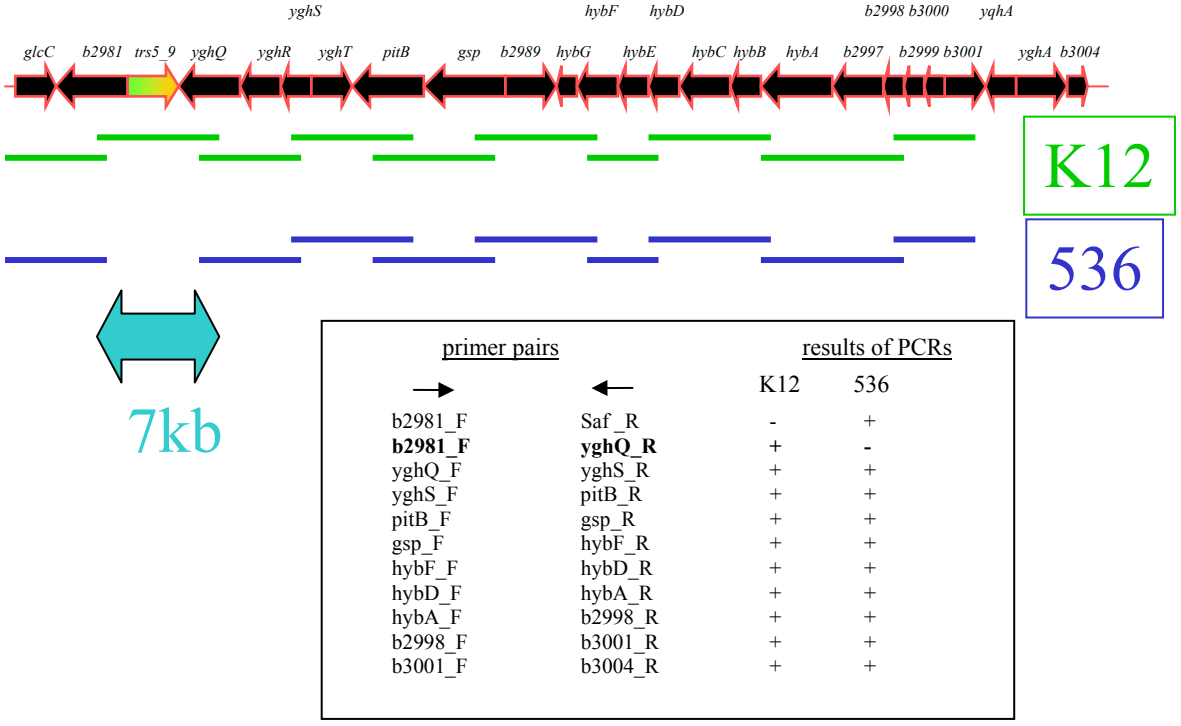
core chromosome, we sequenced all cosmids with two primers. One of them corresponded to the end of pCos5/52 (Fig. 5) and the other to the end of pCos24/23. All five cosmids contained the pCos5/52-specific part, but only pCos24/23 could be sequenced with its specific primer. This result strongly supported that the best candidate for a more thorough sequence analysis to reveal the exact structure of a new PAI-associated capsule locus and the downstream sequences, was the cosmid pCos24/23. Determination of its nucleotide sequence was performed in collaboration with G. Gottschalk's group at the University of Göttingen.

1.1.4 A 7 kb-region is inserted downstream of the general secretion pathway locus in *E. coli* strain 536

As discussed above, the 'right' end of pCos24/23 had no homology to any known sequence at the nucleotide level. Only some weak homology to a saframycin Mx1 synthase B from a *Myxococcus* species (T18551) was detected on the protein level. Continuing to sequence this cosmid with the primer *Safrev*, 1,400 bp upstream of the right end we found similarity to the gene *b2981*. This is one member of the general secretion pathway locus which is present in *E. coli* strain MG1655 and this is the region where the inserts of the other 4 cosmids (that overlapped pCos6b38) ended (Figure 5). During the construction of gene libraries, false assemblies of *Sau*3A I fragments can sometimes occur. Therefore, it had to be verified whether the insert of pCos24/23 corresponds to the real chromosomal structure of strain 536. To rule out the possibility of an insertion of a *Sau*3A I fragment downstream of the general secretion pathway gene cluster, the right end of pCos24/23 was checked for recognition sites of *Sau*3A I, but none was found. Additionally, a PCR was carried out with primers *b2981fw* and *Safrev* that connected *b2981* and the putative saframycin Mx1 synthase B gene. A product of the expected size was amplified with *E. coli* 536 as a template, indicating that downstream of the general secretion pathway genes, an insertion is present in strain 536 that was further investigated.

To determine the size of this integration we screened the 536 genomic library for cosmids overlapping with pCos24/23 by the above described PCR (*b2981* and *Safrev*). Altogether, six cosmid clones (pCos1/33, pCos2/13, pCos9/11, pCos9/47, pCos11/62 and pCos21/63) were found to be positive for this probe. Sequence analysis of the ends of these cosmids revealed that downstream of the general secretion pathway genes, an about 7-kb insertion seemed to be present. Furthermore, it indicated that the major part of the six new cosmids were *E. coli* K-12 sequences that were already known from the database. Overlapping primer pairs were used to

reveal if all of the known genes from this part of the strain MG1655 chromosome were present or some might have been deleted (Figure 6). With these primers, PCRs were carried out with strain 536, pCos2/13 and K-12. Strain 536 and pCos2/13 yielded the same results.



primer pairs		results of PCRs	
→	←	K12	536
b2981_F	Saf _R	-	+
b2981_F	**yghQ_R**	**+**	**-**
yghQ_F	yghS_R	+	+
yghS_F	pitB_R	+	+
pitB_F	gsp_R	+	+
gsp_F	hybF_R	+	+
hybF_F	hybD_R	+	+
hybD_F	hybA_R	+	+
hybA_F	b2998_R	+	+
b2998_F	b3001_R	+	+
b3001_F	b3004_R	+	+

Fig. 6: Overlapping PCRs downsteram of the GSP genes have revealed further differences between the genome of the UPEC strain 536 and the K-12 strain MG1655. Instead of a transposon 5 gene (*trs5_9* - greenish-yellow ORF) a 7 kb integration (*orf99-106*) was localized in the genome of strain 536. Function of the hypothetical proteins encoded by these genes is not known or only hypothetical.

Between *b2981* and *yghQ* in strain K-12, a transposon 5 (*trs5_9*) was present. With a long distance PCR with primers starting from these two genes, a nearly 7-kb product (involving *orf99-106*, see Appendix) was obtained from *E. coli* strain 536. This almost 7-kb fragment was sequenced using pCos2/13 as template. Sequence analysis of these genes revealed a genetic region that's function is not known. Similarites to unknown hypothetical proteins (*orf100, 103, 104*), a hypothetical protein (*orf101*) that shows high similarity to the putative acyl carrier protein AcpP, a protein that shows similarity to a hypothetical transferase (*orf102*), and to a hypothetical protein (*orf106*) with suggested ATP binding motif were revealed. This genetic region is also present in *Shigella flexneri* 2a and in the UPEC strain CFT073.

1.1.5 Identification of cosmids covering the left end of PAI V_{536}

It was reported before, that the first gene of several PAIs integrated into *pheV* or *pheU (Rabbit specific EPEC strain–LEE-PAI* (Tauschek et al. 2002)*, bovine specific E. coli strain - afa-8 gene cluster containing PAI, S. flexneri – she PAI* (Rajakumar et al. 1997)*, EHEC O157:H7, UPEC strain CFT073 – PAI II)* is a P4-like integrase gene and encodes a predicted protein of 421 amino acids that is designated as a site-specific recombinase Int-phe (Figure 7).

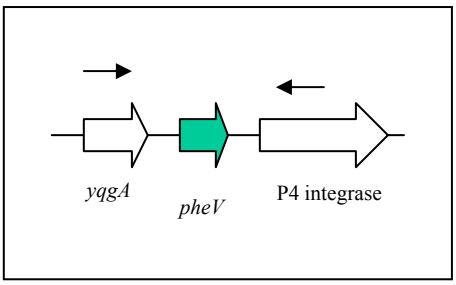


Fig. 7: Screening approach for chromosomal insertions in *pheV*. P4 integrase-specific sequences were used. The primer pair used for screening the genome library for cosmid clones that contain the left end of PAI V_{536} are indicated by arrows.

A primer was designed corresponding to the 5'end of this gene and another corresponding to the 3'end of the gene *yqgA1* located just upstream of *pheV* in the genomes of the EHEC strain and K-12. Screening the 536 genome library, two positive clones were found (pCos1/26 and pCos25/52). Comparison of the ends of these two cosmids revealed that one end of pCos21/26 was homologous to a chromosomal sequence 25 kb upstream of *pheV* while the other end showed no homology. One end of pCos25/52 showed homology to a gene 3 kb upstream of *pheV* in the *E. coli* core chromosome while the other end was overlapping with the left end of pCos6b38. This common part encompassed more than 5 kb. However the two overlapping ends of these cosmids did not fit exactly to each other. Sequence of the last 2 kb of pCos25/52 was uncertain and did not alow the two overlapping cosmids to fit exactly to each other. Analysis of this 2 kb revealed a *Sau3A* cutting site in the beginning of this 2 kb fragment and two additional ones inside it. This made it clear that during the construction of the genomic cosmid library from the chromosome of strain 536 (digested with *Sau3A*) a small piece of DNA was ligated between the cosmid vector and the longer genomic part. Sometimes this happens during the construction of genomic libraries.

In summary, with four cosmids (pCos25/52, pCos6b38, pCos24/23 and pCos2/13) the whole PAI V_{536}, and its downstream sequences were revealed (Figure 8).

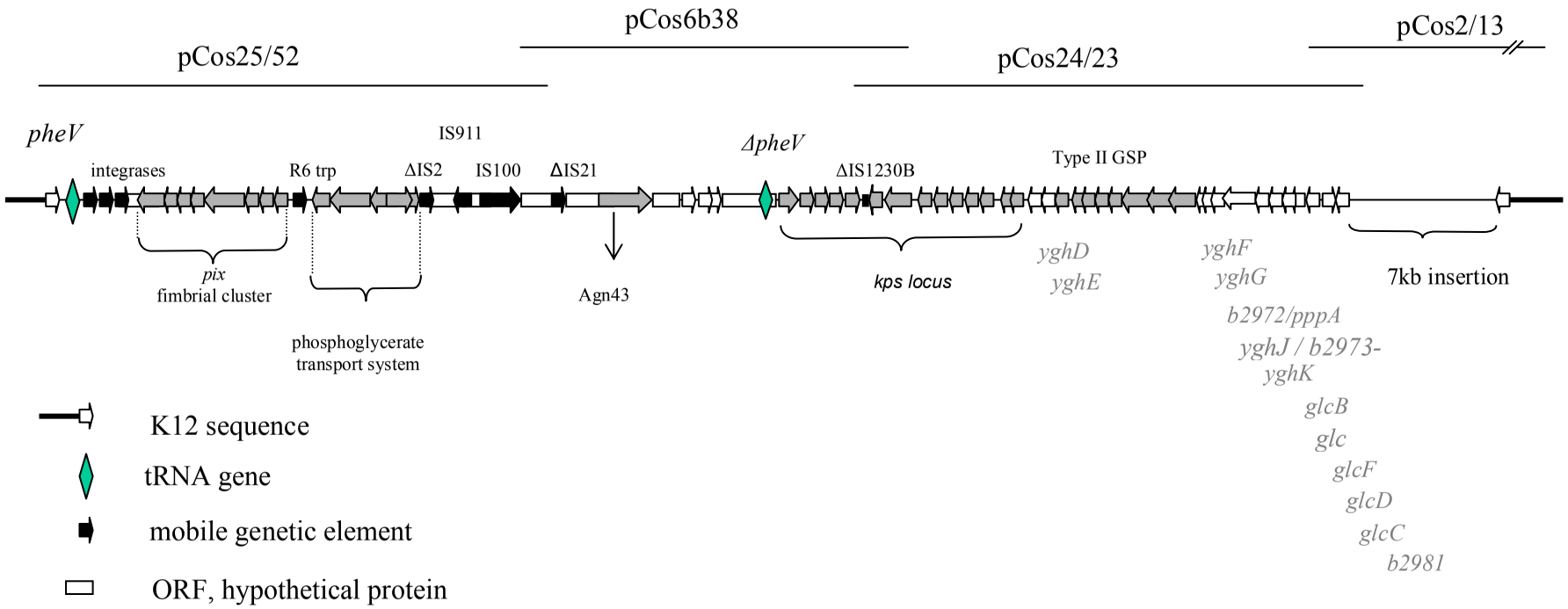


Fig. 8: Genetic structure of PAI V_{536} and its downstream sequences. This chromosomal region of strain 536 was mapped with four overlapping cosmids whose size and localisation to each other is indicated above the map.

1.2 Sequence analysis of PAI V_{536} and its downstream sequences

1.2.1 ORFs of PAI V_{536}

Sequence analysis of the four overlapping cosmids (pCos25/52, pCos6b38, pCos24/23 and pCos2/13) revealed a mosaic-like structure of PAI V_{536}, which is typical for PAIs. PAI V_{536} is inserted at the 3' end of the *pheV* tRNA gene at minute 64 on the chromosome. The upstream region of *pheV* corresponds to the gene order of *E. coli* K-12 strain MG1655. Downstream of *pheV*, a P4-like integrase gene is present which is nearly identical to the integrase genes found in other PAIs integrated at one of the two *phe* tRNA genes (see also above). Beside the complete *pheV*, a 5' truncated copy was identified 49 kb downstream. This organisation strongly resembles to PAI III_{536} of this strain in which downstream of a truncated *thrW* tRNA gene additional integrated sequences are present. In case of PAI V_{536}, the 25-kb K15 *kps* locus , the genes for a general secretion (type II) pathway and a 7-kb insertion were identified downstream of the truncated *pheV*.

The size of the sequence located between the two tRNA genes is 48,708 bp. It contains 58 putative ORFs and has a G+C content of 47.7 %. This part is characterised by the presence of kryptic or active IS elements, transposases, prophage genes, a *pix* fimbrial gene cluster, the genes coding for a phosphoglycerate transport system (*pgt*), an autotransporter (*agn43*), and hypothetical ORFs.

The sequence is available under the **accession number: AJ617685**. The summarised list of the ORFs can be found in the appendix (A/4).

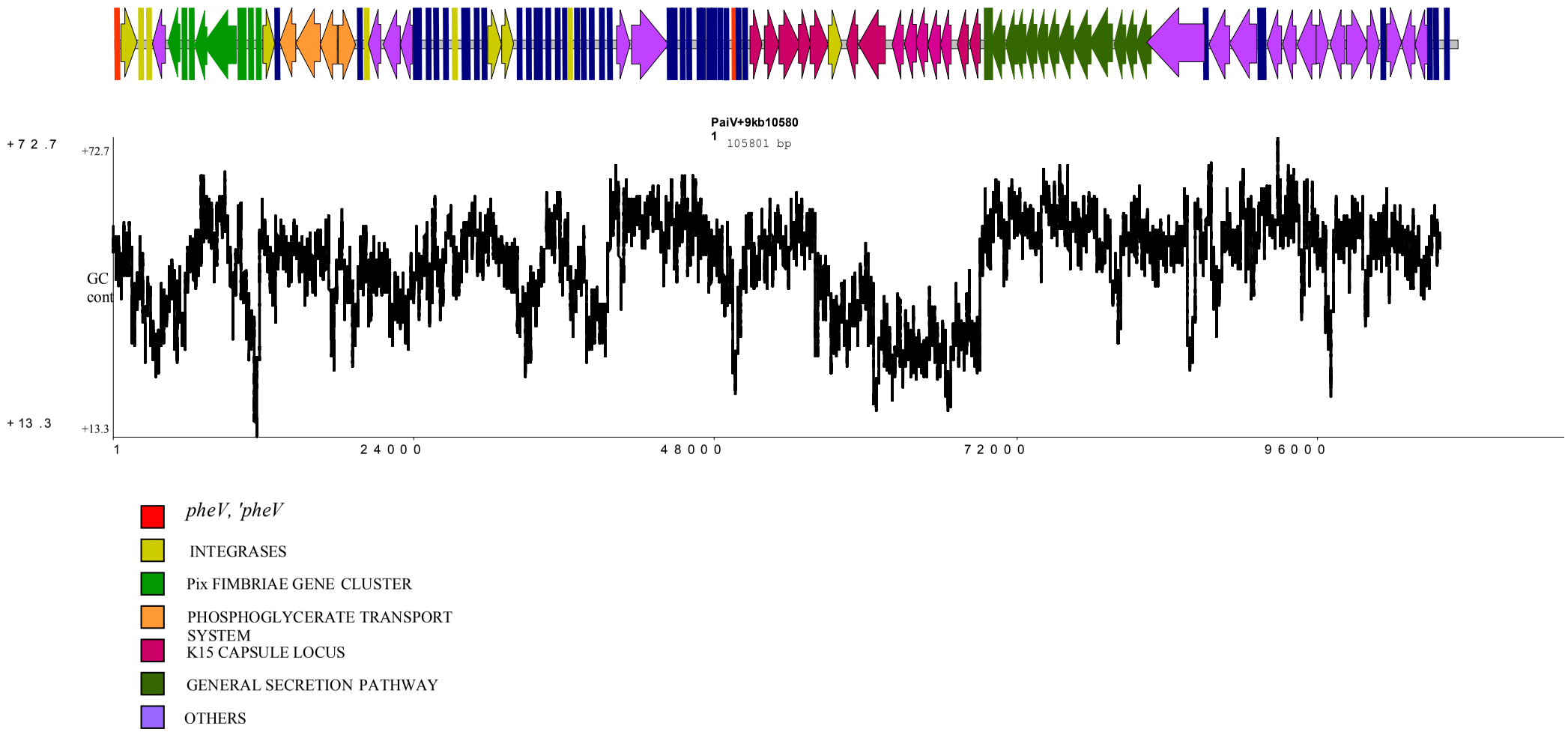


Fig. 9: Genetic structure of PAI V_{536} and its downstream regions. Clusters, mobile genetic elements and DNA fragments of the 105-kb chromosomal region of strain 536 are shown with different colours.

1.2.2 Mobile genetic elements of PAI V_{536}

Mobile genetic elements play an important role in genome flexibility and in gene rearrangements. IS elements, transposase and prophage genes are almost always reported to be present on different PAIs, and are also found on PAI V_{536}.

As already discussed, just downstream of *pheV* is an ORF that shows high homology to a P4-like integrase that is found in many enterobacterial PAIs associated with a Phe-tRNA gene.

Fragments of different IS elements are also indicators for gene rearrangements. Upstream of the *pix* fimbrial gene cluster, two ORFs (*orf2* and *orf3*) are located that code for a putative IS*110* integrase and a transposase like protein. Between the fimbrial gene cluster and the phosphoglycerate transport system, an R6 transposase gene, and just downstream of the phosphoglycerate transport system, an IS*2* transposase gene (*insA*) were found. Furthermore, there are two complete IS elements: IS*911* (*orf29-30*) and IS*100* (*orf33-34*) present. Interestingly, both of them can also be found on PAI I_{536}. An additional remnant of an IS element was detected in the middle of the *kps locus*, between the truncated $kpsC_{K5}$ and the $kpsS_{K10}$ genes. This ORF shows homology at the protein level to IS*1230*B of a *Salmonella enteritidis* strain.

Upstream of the truncated *pheV* gene, high level homology to prophage genes (L0007-L0011) was detected that were first described in the genome of EHEC strain EDL933. These prophage genes are also present in PAI II_{536}.

1.2.3 Gene clusters of PAI V_{536}

Two large gene clusters were localised between *pheV* and *'pheV* (Figure 9, Table 5). From *orf6* to *orf13* the recently described *pix* fimbrial gene cluster is present (Lügering *et al.,* 2003). The operon consists of 8 genes from which the last 5 (*pixC, D, J, G, F*) showed partial homology at the nucleotide level to a novel fimbrial gene cluster that is encoded by the large plasmid (pSFO157 – ECO131667) of the sorbitol-fermenting Enterohaemorrhagic *E. coli* O157:H- (Brunder *et al*., 2001). The first three genes of this cluster are similar to the genes of a pyelonephritis-associated pilus operon (*pap*) of *E. coli* (ECU26449). The organisation of the three fimbrial clusters is compared in Figure 10. The *pap* gene cluster shows a complete structure because it contains the tip fibrillum genes *papK*, *E* and *F* and a complete regulatory system (*papI* and *B*) that is located upstream of the structural genes. It is known that upstream of these latter two genes some small sequences are also important for regulation. $Sfp_{pSFO157}$ completely lacks

the regulatory genes and only part of a fibrillum gene (*sfpF*) is present. The *pix* gene cluster is organised similarly to *sfp* but it has a regulatory gene *pixB* upstream of *pixA*. *pixB* shows only

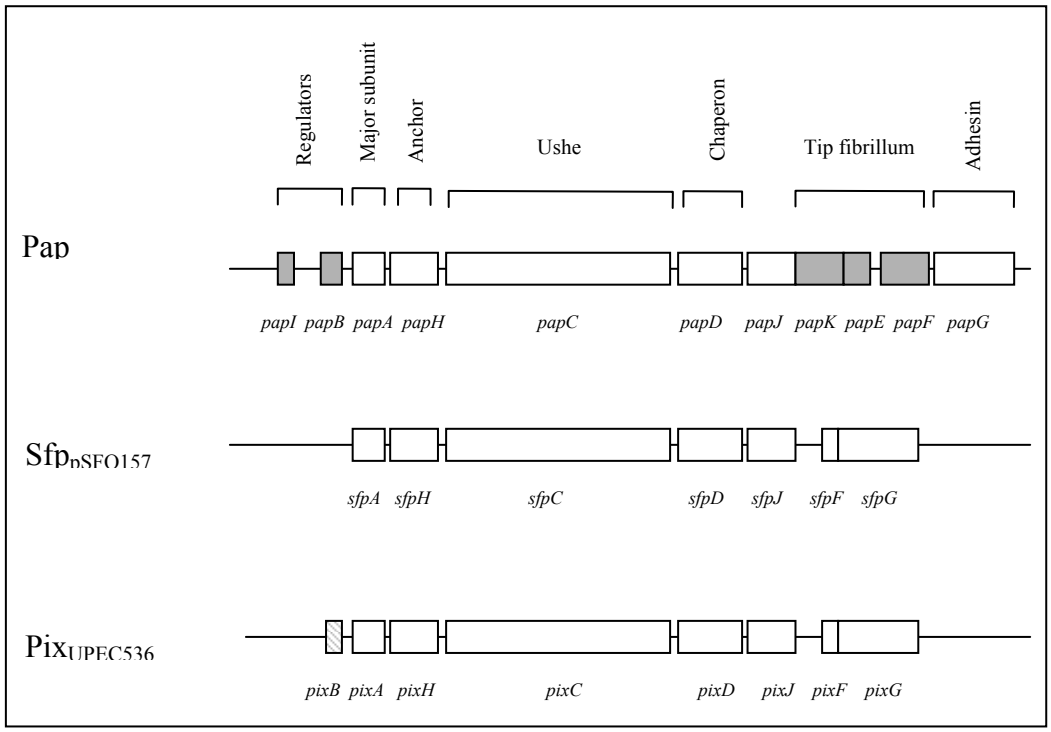
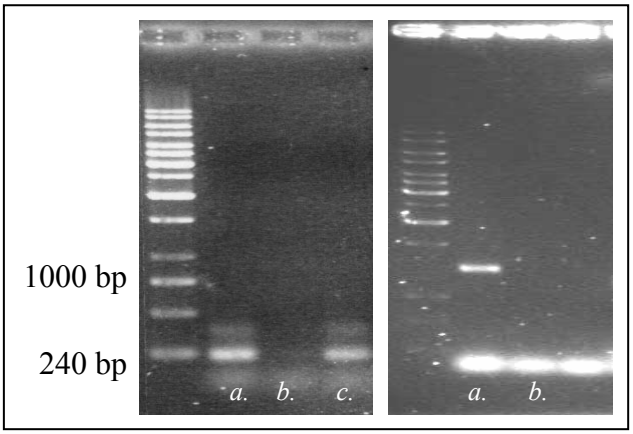


Fig. 10: Comparison of the genetic structures of *pap*, *sfp* and *pix* gene clusters.

partial homology to *papB/prfB*, but seems to be intact. *pixB* encodes a putative negative regulator of the gene cluster, but a hypothetical positive regulator that could have a homologous function to papI is missing. Regulatory sequences upstream of *pixB* are also missing.

Transcriptional analysis with RT-PCR of the *pix* fimbrial gene cluster in strain 536 at 37 ºC revealed that the structural gene *pixC* is not transcribed but that the repressor *pixB* was expressed (Figure 11).

Fig. 11:
Analysis of *pixB* and *pixC* transcription at 37ºC by RT-PCR. Under the conditions used, expression of *pixB* hindered the transcription of *pixC*. (***a.***: PCR from total RNA; ***b.***: PCR from DNAse digested total RNA; ***c.***: PCR after reverse transcription)



The second large gene cluster (*orf16-19*) of PAI V_{536} codes for a complete phosphoglycerate transport system that consists of three regulators and a transporter protein. This system is involved in the uptake of different phosphoglycerates (Goldrick, 1988) and activated sugars, and was originally described in *S. enterica* serovar Typhimurium LT2 (accession no. AE008808). Similar to *S. enterica* and CFT073, the four genes are present but in strain 536 an additional fifth small ORF (*orf20*) was detected that showed weak homology on protein level to a phosphoglycerate mutase of *Bacillus stearothermophilus* (accession no. T46865). Analysis by RT-PCR revealed that the phosphoglycerate transport system is transcribed under laboratory conditions at 37 °C (data not shown).

1.3 Structural analysis of PAI V_{536} by comparing to other bacterial genomes

Many DNA fragments of PAI V_{536} show similarity at the nucleotide level to chromosomal regions of other *E. coli* strains (e.g., strain CFT073 –UPEC, EDL933 –EHEC O157:H7, 239 KH

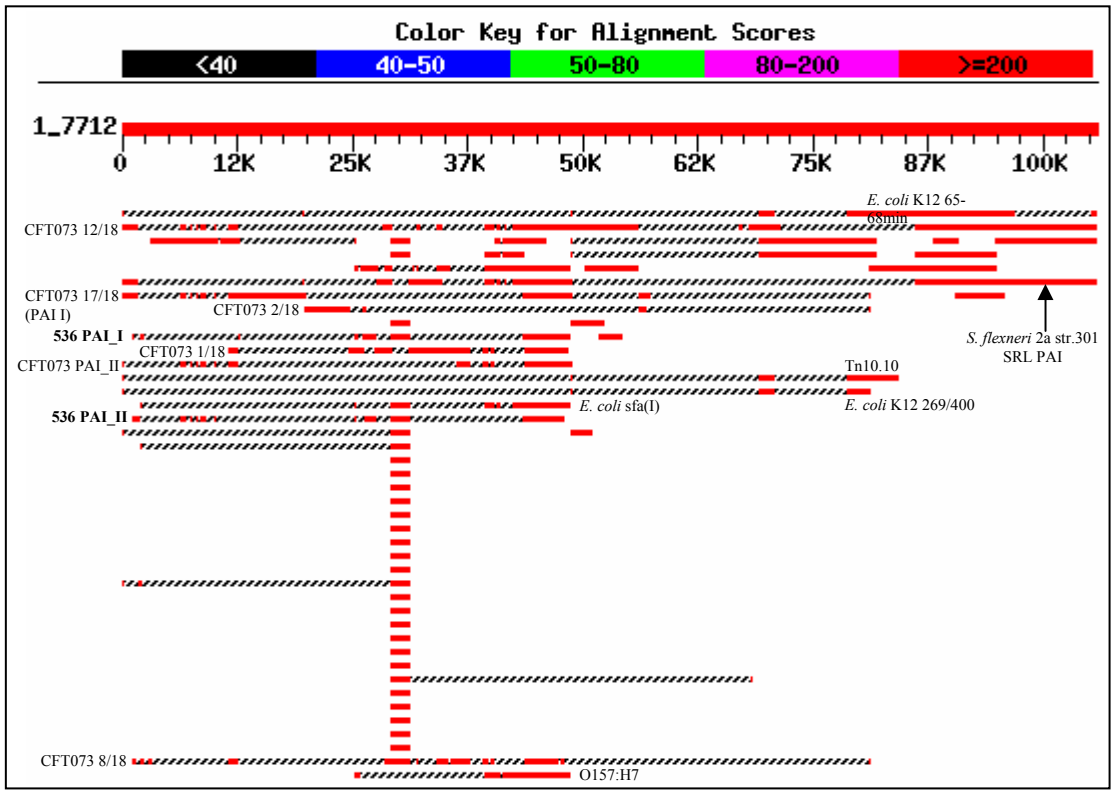


Fig. 12: Sequence comparison of PAI V_{536} to other bacterial sequences using the BLAST software. A mosaic like structure of PAI V_{536} and its downstream sequences was found with high homology to different sections of the genome of UPEC strain CFT073. Additional homology can also be detected with sequences of different other pathogenic strains.

89 -human and bovine pathogenic, two ETEC strains), of *Shigella flexneri* 2a (*she* and SRL PAIs), and of *Salmonella enterica* serovar Typhimurium LT2. Many fragments of PAI V_{536} are also nearly identical to sequences of different virulence plasmids of *E. coli* (pB171, pSFO15), *Shigella* spp. (pWR501, and pWR301) and to the *Y. pestis* plasmids pMT1 and pCD1. The highest homology was shown to the completely sequenced genome of strain CFT073 which is, as strain 536, an uropathogenic *E. coli* isolate. Segments 1, 2, 8, 12 and 17 of the CFT073 genome (18 segments altogether) show homology to PAI V_{536}. These findings indicate a mosaic-like structure between the two UPEC genomes with a high variance between the sequences.

A frame-like homology can be observed among PAI V_{536}, PAI I_{536}, PAI II_{536}, PAI II_{CFT073} and the SRL PAI of *S. flexneri* 2a where the presence of integrase genes and the remnants of prophages are characteristics that have been proposed to have played a role in the acquisition of these PAIs. Inside this frame, homology to additional integrase genes and IS elements were detected that may have taken part in the acquisition and deletion of different genes and gene clusters. Figure 12 demonstrates that the phosphoglycerate transport system shows homology to PAI I_{CFT073} (also designated as *pheU* island). Only partial homology can be seen to the *pix cluster* of PAI V_{536}. Such homology to the conserved part of other fimbrial clusters is evident in the cases of PAI II_{536}, PAI II_{CFT073} and PAI I_{CFT073}.

1.4. Presence of the PAI V_{536} and its genes in a collection of *E. coli* strains

To investigate the distribution of sequences that are present on PAI V_{536} within the species *E. coli*, 140 strains of the institute's strain collection were screened with PCRs. It is noteworthy to mention that the composition of this strain collection is focussed on ExPEC strains. PAI V_{536} screening primers were specific for the *pix*, *pgt*, *kps* and *gsp* gene clusters (the results are shown in the appendix). Because of its novel structure PCRs were performed on all the three regions of the capsule locus.

According to the results, beside strain 536 only one strain was detected to be positive for all the seven PCRs (E642, a newborn meningitis strain). Furthermore, only one additional strain (764- a classic UTI strain) seems to possess the complete K15 specific *kps* sequence. This correlates with the information we have on serotype because this strain also expresses the K15 capsule type. The incidence of the *gspD* gene which is a member of the general secretion pathway is rather high (77%). Interestingly most of the GSP genes are missing from the genome of CFT073. The

phosphoglycerate transport system has a much lower incidence (38%) while the occurence of *kpsC*$_{K5}$ gene is high (63,5%).

2 Structural analysis of the capsule determinant of the UPEC strain 536

2.1 The capsule locus of the UPEC strain 536 shows a "mixed" genetic structure

Sequence analysis of pCos6b38 strongly suggested that PAI V$_{536}$ is inserted at the 3'end of the *pheV* tRNA gene and is associated to the capsule locus. As the capsule locus responsible for the K15 antigenic properties of strain 536 was not yet known it was hypothesised that parts of the functional capsule locus of this strain was found (B. Janke, PhD thesis). In this work, the

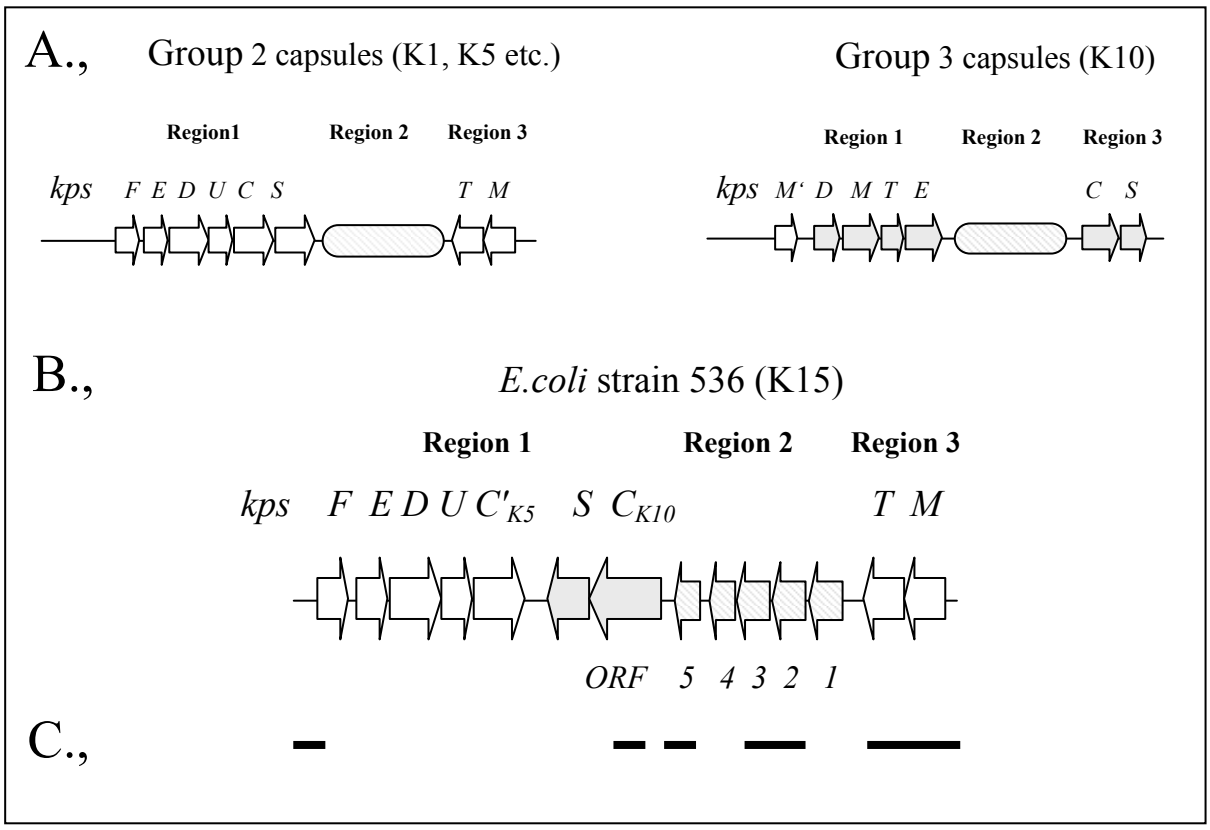


Fig. 13: Genetic organisation of the capsule locus of *E. coli* strain 536. A. Organisation of group 2 (left) and group 3 (right) capsule gene clusters. **B.** A mixed genetic structure of the capsule locus is hypothesised to be responsible for the K15 antigenic properties of *E. coli* 536: Region 1 shows a mixed structure; the first 5 genes (*kpsFEDUC*) show high homology to K5 (one member of the group 2 capsules), the additional *kpsS* and *C* are on the complementary strand and show homology to the capsular determinant of K10 (one member of the group 3 capsules). **C.** Control PCRs were carried out with three ETEC strains that also have K15 capsules. In these cases, the PCRs –similarly to strain 536- were positive and resulted in the appropriate product sizes. Localisation of the regions amplified by the test PCRs are indicated by black lines.

complete gene cluster was identified and its sequence analysed (see chapter 1 of the results). It was verified that the capsule determinant of this strain is associated with the tRNA gene *pheV*. Its organisation showed features that were characteristic for both group 2 and 3 capsule determinants (Figure 13) with two conserved regions flanking a highly variable region. In strain 536, **region 1** that encodes transport functions has 5 ORFs (*kps F, E, D, U, C*) and shows high nucleotid homology to the capsule determinant of K5 and K1, two representatives of group 2 capsules. However, the fifth gene of the transcriptional unit (*kpsC*, later termed $kpsC'_{K5}$) was nearly 150 bases shorter than its homologues in K1 and K5 gene clusters. Downstream of $kpsC'_{K5}$ the sixth gene (*kpsS*) was missing but instead a hypothetical transposase gene was present (*orf66*). This ORF showed homology at protein level to an IS*1230*B transposase from *S. enteritidis* (NP717743).

Complete *kpsC* and *S* genes were identified downstream of the transposase gene but on the complementary strand. *kpsS* showed similarity to *A. hydrophila* (AF375657) and *kpsC* to *E. coli* K10 (termed later $kpsC_{K10}$). The latter is a well studied representative of group 3 capsules (Clarke, 1999).

Upstream of *kpsS* and $kpsC_{K10}$, **region 2** is located that contains 5 ORFs (*orf69-73*) and similarly to the *kpsS* and *C* genes they were transcribed from the complementary strand. The start and stop codons of the first four genes (*ORF73-70*) are overlapping, while the fourth and fifth genes (*ORF69*) are separated by a 290-bp non-coding region. None of these five genes showed significant homology to any known sequence at the nucleotide level, but at the protein level, two of them (*ORFX* and Y) were similar to enzymes of sugar biosynthesis. Homology was found to a putative glycosyltransferase from *Actinobacillus* (AAC26630) and a mannosyltransferase B of an *Aquifex* species (NP213361). Up- and downstream of region 2, small (~50 bp) AT-rich repetitive sequences were identified that could be traces of genetic rearrangements. Nevertheless the G+C content of this region is significantly lower than that of the whole PAI V_{536} and other downstream sequences.

Upstream of the biosynthetic genes, the missing conserved capsular genes were identified. The genes *kpsM* and *T* show homology to the genes for K5, K2 and K4 which belong to group 2 capsules and form **region 3**. They are members of the ABC (ATP binding cassette) transporter family (Silver et al., 2001; Higgins, 2001).

The mixed genetic organisation of the K15 capsule or any other capsule types was not known before. Therefore it was tested whether other isolates with the K15 capsule type have the same

genetic organisation as *E. coli* 536. From the strain collection of the Institute for Molecular Biology of Infectious Diseases, three K15 strains (IMI405, 406, 415) were checked by PCR concentrating on 5 parts of the K15 capsule locus. The following primer pairs were used (Figure 13c): *IHFfw-KpsErev*, *13165fRT-14167rRT*, *14400RT-orf5fw*, *orf2fw-orf3end*, *MT1-MT4*). In all the five cases the size of the products equalled with that of strain 536.

2.2 Regulatory sequences of the *capsule locus*

We have found all the conserved genes that are necessary for capsule transport through the bacterial inner membrane, and our data suggested that the antigen-specific region was also localised. As capsule expression depends also on different environmental signals, the presence of additional regulatory sequences is also expected. Therefore, we compared the regulatory sequences of other capsule determinants with that of K15 sequences.

AGGAAATAATCAATTGATAAATTGTTTTTGGTC**TTAATAGT**CAAGTTGGAAACAT
TT**TAATAAATGAT**AAAAATCCTAAATTCCTTGTTCATAATGTAGGGGTGTGTTAA
ATAGGTGGGCAAAATTTGGTTCCCCTTCTCGCCTGTAATATTGCAGCATCTTCGCA
CCTCCATGAGACATTGCGACTTAATAAGAAGGTGATAAGTCCTGCATATAAGCAT
GGACTGACCATGGTTTTATATTCATTAAAAATTTTGTTACAACCCATTGATTTAGC
ATAAATAAATTATAGTGGGTTCGGGTTTGTTGTGACTGTGGCATTATTTCCGTGCA
AA***GGAG***CTGAT**ATG**TCTGAAAGACATTTACCTGATGACCAGAGCAGTACTATC

Fig. 14: Nucleotide sequence of the 5' end of the *kpsF* gene (region 1) and its upstream region. Bold, start codon of *kpsF*; *italic*, Shine-Dalgarno sequence; underlined, sequences matching the consensus IHF binding site; green bold, conserved AT-rich regions that are also present in the region 3 promoter.

Similar to group 2 capsule gene clusters, two hypothetical integration host factor (IHF) binding sites were found upstream of *kpsF* in *E. coli* 536 (Figure 14). IHF is a histone-like DNA-bending protein and binding of IHF at these sites is predicted to change the DNA conformation thereby altering the efficiency of transcription and/or mediating the action of other regulatory factors. However, IHF is only required for optimal gene expression of group 2 capsules (Rowe *et al.*, 2000).

Region 3 contains the genes *kpsM* and *T* organized in a single transcriptional unit. Similarly to other group 2 capsule representatives, in the case of K15 no consensus sequences for alternative sigma factor or other DNA-binding proteins (like IHF) have been described. However, transcription of region 3 is affected by an antitermination process, conferred by the antiterminator

RfaH (Figure 15) and an *ops* (operon polarity suppressor) element, located within a nucleotide sequence region called JUMPstart (just upstream of many polysaccharide gene start). In this process, RfaH binds to the RNA polymerase holoenzyme and prevents recognition of downstream termination signals. The interaction of RfaH and the RNA polymerase requires the *ops* element that must be located upstream of the genes to be transcribed. At this site, the RNA-polymerase recruits RfaH and possibly other proteins, to promote transcriptional elongation. Mutations in RfaH or deletion of the *ops* sequence abolish detectable group 2 capsule expression at 37 °C.

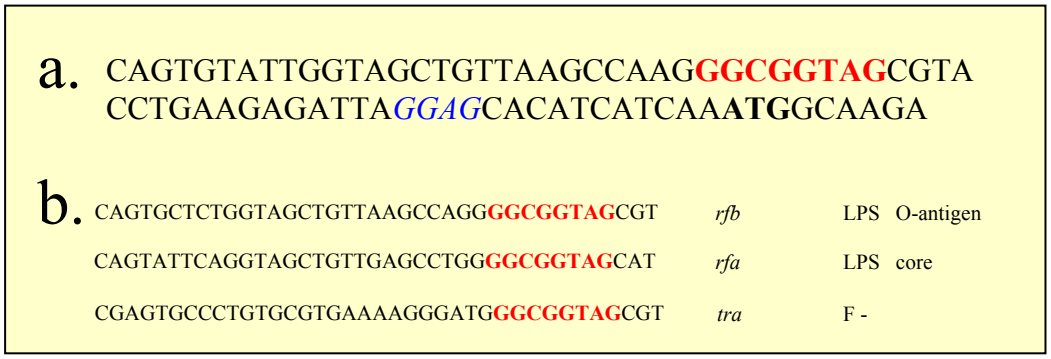


Fig. 15: a., Promoter sequence of the K15-specific Region 3. Bold, start codon for *kpsM*; *italic*, Shine-Dalgarno sequence; red and bold, *ops* element. **b.,** Examples of different JUMPstart sequences of *E. coli* operons that are RfaH dependent (Figure is kindly supplied by G. Nagy).

3 Functional analysis of the K15 capsule in the uropathogenic process

Mapping of the capsule locus has revealed its genetic structure. Similarity to known group 2 and 3 capsule determinants suggested that this was a functional gene cluster, but its structure showed a mixed organisation that was not described before. A transcriptional analysis was carried out to elucidate the role of the two *kpsC* genes and to reveal the transcriptional organisation of this locus. Furthermore, knock-out mutants were constructed to verify that this locus codes for enzymes that are responsible for the K15 antigenic properties of strain 536.

3.1 Transcriptional analysis of the capsule locus

Reverse Transcription PCR (RT-PCR) was carried out with *kpsC* specific primers for the capsule locus of strain 536 to examine their role in capsule transport. Primers *C5in_fw* and *C5in_rev* were used to check the transcriptional activity of the truncated $kpsC_{K5}$ gene, which showed high homology at nucleotide level to K5 while primers *13165fRT* and *14167rRT* were used for the other *kpsC* gene that was encoded on the complementary strand, and showed slight homology at

protein level to the K10-specific allele. The results of the RT-PCR revealed that both *kpsC* genes were transcribed (Figure 16).

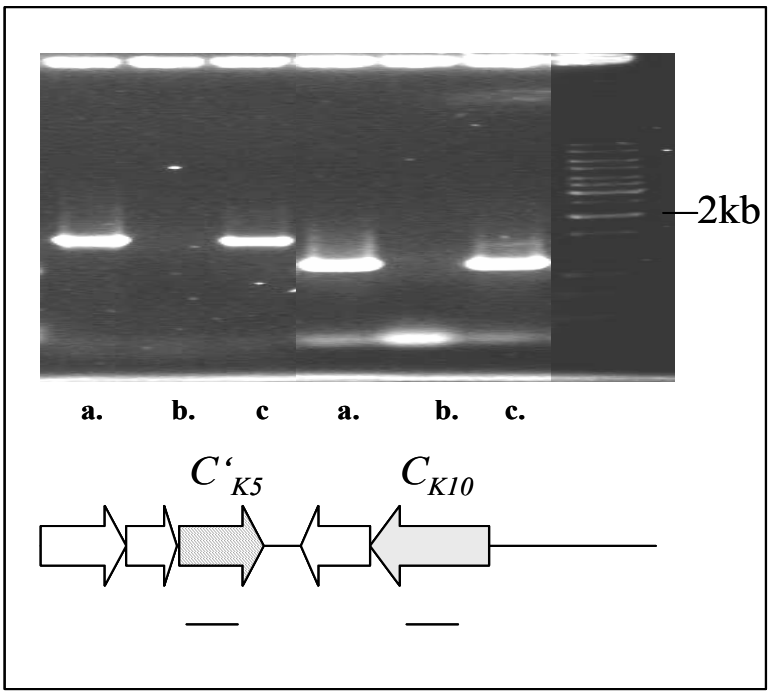


Fig. 16: RT-PCR analysis of *kpsC*'$_{K5}$ and *kpsC*$_{K10}$ has revealed that both of them are transcribed. PCRs were carried out from: **a**, total RNA; **b**, total RNA after treating with DNase; **c**, cDNA. (Left part corresponds to the end of region 1 –see Fig 13; PCR products are symbolised under the arrows)

By producing mutants of both *kpsC* genes, their role was further analysed (see below). To reveal the transcriptional organisation of the *kps locus* additional RT-PCRs were performed. From the isolated total RNA, cDNA was synthesised with random primers. After DNA was totally digested with DNAse the questionable parts of the *kps locus* were amplified with specific primers. From the PCR results, the presence of transcriptional units of the capsule locus could be determined as follows (Figure 17):

Positive products from RNA and cDNA were amplified with primers kpsC$_{K10}$ and orf5, orf5 and orf4, and orf1 and kpsT. These results suggest the presence of a transcript encompassing *kpsT* to *kpsS.* The transposase gene located between *kpsC'$_{K5}$* and *kpsS* could also be amplified with inner primers indicating that it is transcribed. From the literature, it is known that all genes of region 1 in group 2 capsules are organised in a single transcriptional unit (Whitfield, 1999). Therefore, it can be assumed that the K15 capsule locus is organised in two transcriptional units (Figure 17).

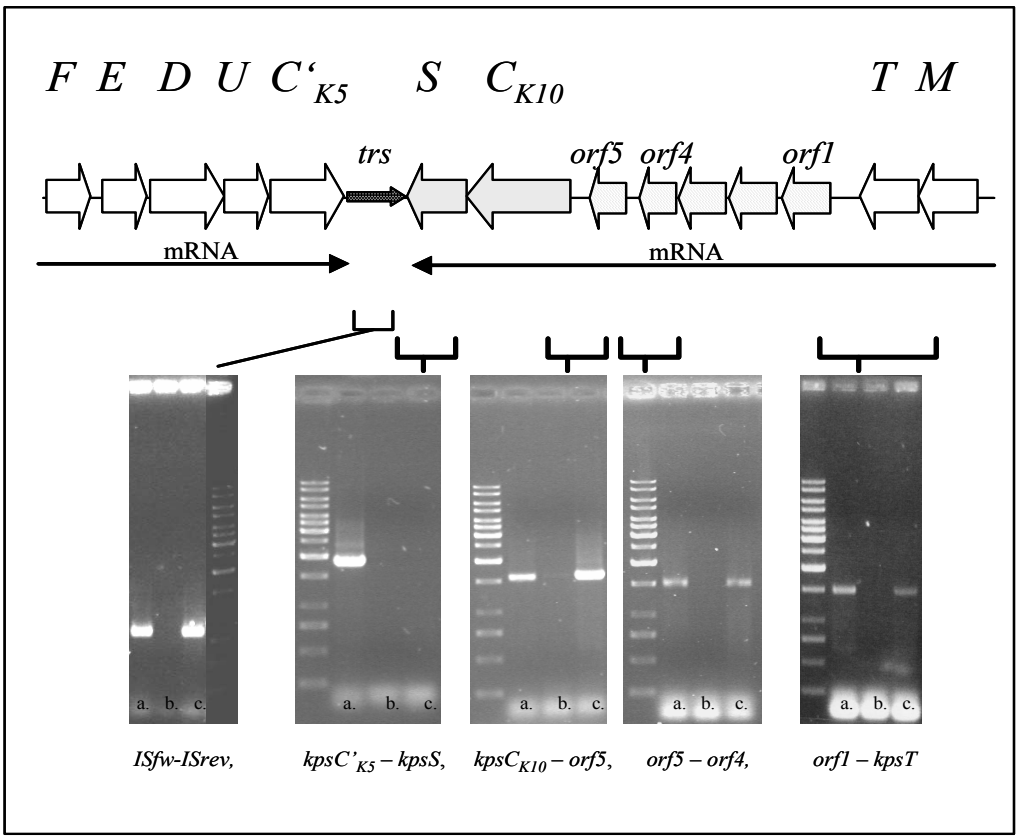


Fig. 17: Analysis of the transcriptional organisation of the K15 capsule locus by RT-PCR. From the results two transcriptional units were identified. The truncated region 1 (*kpsFEDUC'*$_{K5}$) forms one transcriptional unit while the second one involves the genes of region 3 (*kpsMT*), region 2 and the *kpsC*$_{K10}$ and *S* genes. (PCRs were carried out from: **a**, total RNA; **b**, after treating with DNase; **c**, cDNA).

3.2 Mutants of the hypothetical K15 capsule locus

Mutants were constructed for all three regions of the capsule determinant. Region 1 and 2 mutants were prepared with derivatives of the suicide vector pCVD442. In region 1, *kpsS* and *kpsC*$_{K10}$ were replaced by a kanamycin casette while in the case of region 2 the first four genes (*ORF70-73*) were deleted. For deletion of *kpsS* and *kpsC'*$_{K5}$, a 4.6 kb product was amplified with primers *kpsSCfw_XbaI* and *kpsSCrev_SacI* and ligated with pGEM®-T. The resulting plasmid was digested with the restriction enzymes *BstBI* and *BsiWI* that removed an internal 2.8-kb fragment encompassing the two genes, and leaves a ~900-bp flanking region on each side. A kanamycin cassette that had been amplified from pACYC177 with primers *Kan_BstBI* and *Kan_BsiWI,* and subsequently digested with *BstWI* and *BstBI* and then inserted into the linearised vector. Positive clones on kanamycin plates were checked and after plasmid isolation the kanamycin cassette with the two flanking fragments corresponding to the capsule locus was

cut out with restriction enzymes *XbaI* and *SacI*. In a next step, the 2.2-kb fragment was directly ligated with pCVD442 and transformed into SY327 λ*pir*. After selection on sucrose plates as described before clones were checked by PCR and Southern-hybridisation.

For region 2 mutants, the same strategy was used but for the integration of a tetracyclin cassette (also from pACYC177) the restriction enzymes *BglII* and *StyI* were used. Another mutant was also generated for region 2 with linear PCR products according to the method described by Datsenko and Wanner (see materials and methods). Other mutants *(ΔkpsC'$_{K5}$, ΔkpsC$_{K10}$, ΔkpsMT and Δkps$_{K15}$)* of the capsule locus were also constructed with the same method. The exact distances of the mutations relative to *'pheV* are as follows (Table 4):

Table 4: Characteristics of the different deletion mutants within the K15 capsule locus

Region deleted	Position (relative to *'pheV*)	Size of the deletion [bp]
kpsC'$_{K5}$	6,017 – 6,837	820
kpsSC$_{K10}$	8,886 – 11,590	2,704
kpsC$_{K10}$	10,403 – 11,873	1,470
kpsΔR2-1 and *ΔR2-2*	13,548 – 16,308	2,760
kpsMT	18,466 – 19,429	963
kps$_{K15}$	610 - 20,355	19,745

After the mutants have been confirmed with PCR and Southern blot (Figure 18) loss of the capsule was detected with serological methods.

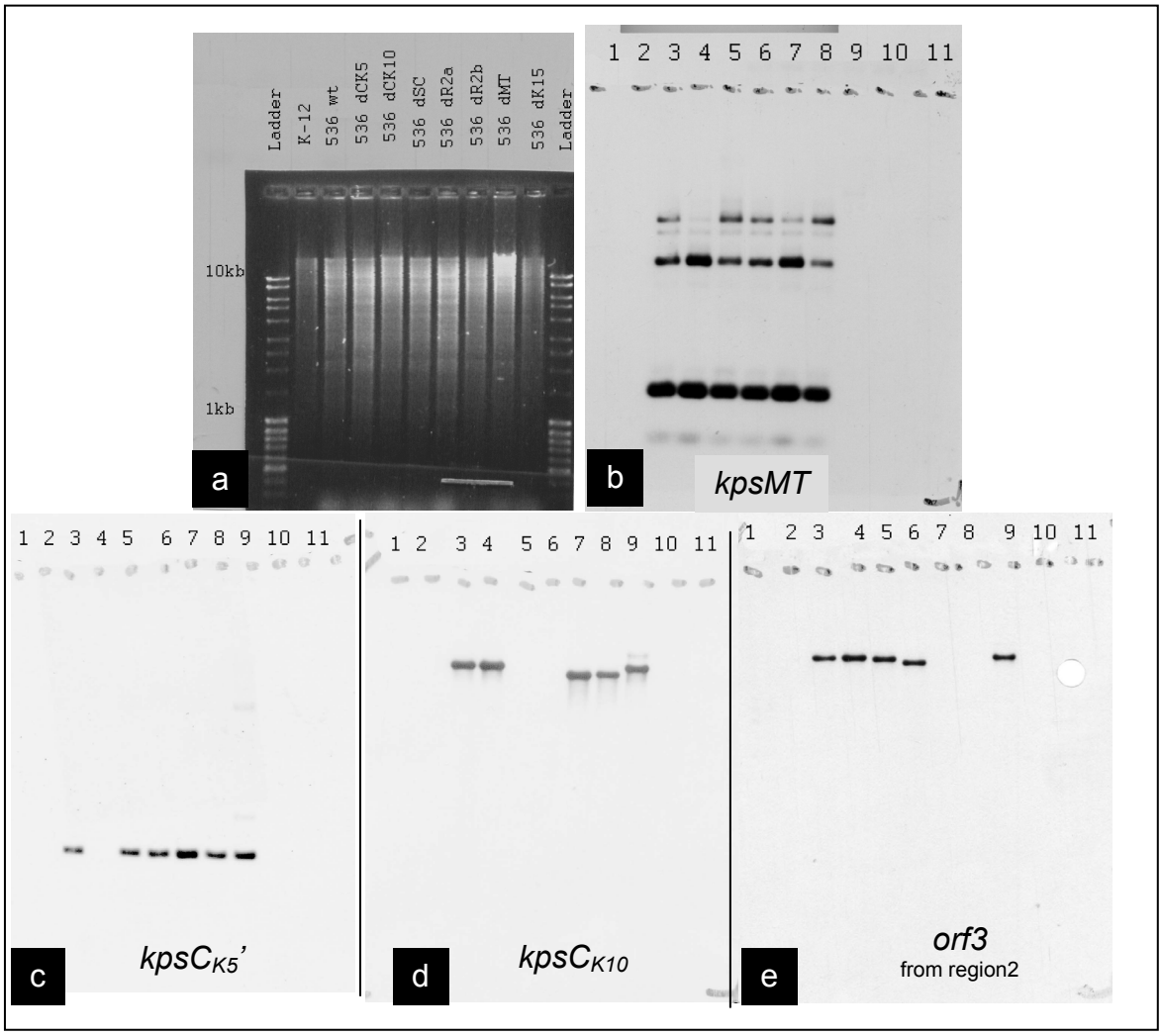


Fig. 18: Mutants of the K15 capsule locus were checked with Southern hybridisation. Chromosomal DNA of each strain was digested with *BglI* (This enzyme cuts only in the hybridised sequences of Region 3 (*kpsMT*).) and was run in 1% agarose gel (**a**). Hybridisation of gene specific probes (written on the bottom of each membrane, **b-e**) were hybridised with the chromosomal DNA of wild type and mutant strains to detect the loss of gene(s) in question (**b-e**). Order of strains is the following: **1**, Marker; **2**, *E. coli* MG1655; **3**, *E. coli* 536 wt; **4**, *E. coli* 536Δ*kpsC'*$_{K5}$; **5**, *E. coli* 536Δ*kpsC*$_{K10}$; **6**, *E. coli* 536Δ*kpsSC*$_{K10}$; **7**, *E. coli* 536ΔR2–1; **8**, *E. coli* 536ΔR2–2; **9**, *E. coli* 536Δ*kpsMT* **10**, *E. coli* 536Δ*kps*$_{K15}$; **11**, Marker.

3.3 The capsule locus downstream of *'pheV* is responsible for K15 antigenic properties in strain 536

Mutants of the K15 capsule locus were first tested with serological methods. All of them were tested for capsule production or loss of capsule with a K15-specific antiserum. With this absorbed polyclonal antiserum, it was revealed in ELISA that with one exception all the mutants had lost their ability to express or synthesise the K15 capsule (Figure 19). In contrast to a $kpsC_{K10}$ deletion, that of $kpsC'_{K5}$ did not have any effect on capsule expression. Therefore it can be assumed that even though both of them are transcribed (Figure 16) only $kpsC_{K10}$ is required for a functional protein.

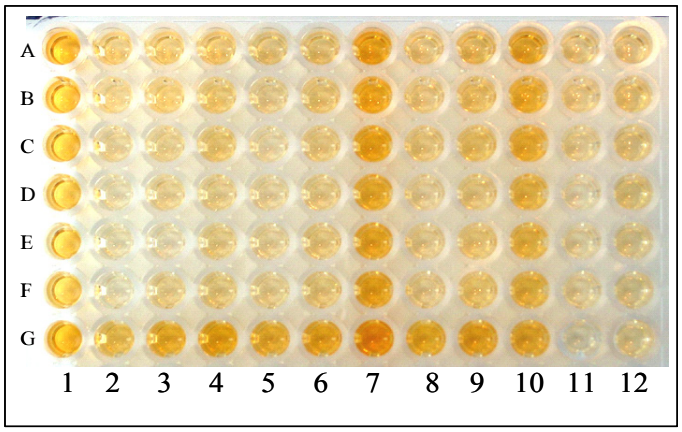


Fig. 19: Detection of K15 capsule production of *E. coli* strain 536 and different derivatives by ELISA. Unabsorbed polyclonal serum (O-K serum) was used in line **G**. In lines **A-C** and **D-F**, two different charges of absorbed serum was used (absorbtion was made with strain RZ532 O6:K+:H31, non-K15). **1**, 536-wt; **2**, RZ532; **3**, *E. coli* 536Δ$kpsSC_{K10}$; **4**, *E. coli* 536ΔR2-2; **5**, *E. coli* 536Δ*kpsMT*; **6**, *E. coli* 536Δ$kpsC_{K10}$; **7**, *E. coli* 536Δ$kpsC'_{K5}$; **8**, *E. coli* 536Δkps_{K15}; **9**, *E. coli* 536Δkps_{K15} (pBeloBAC11); **10**, *E. coli* 536Δkps_{K15} (pBAC); **11**, 536*rfaH*; **12**, *E. coli* 536ΔR2-1.

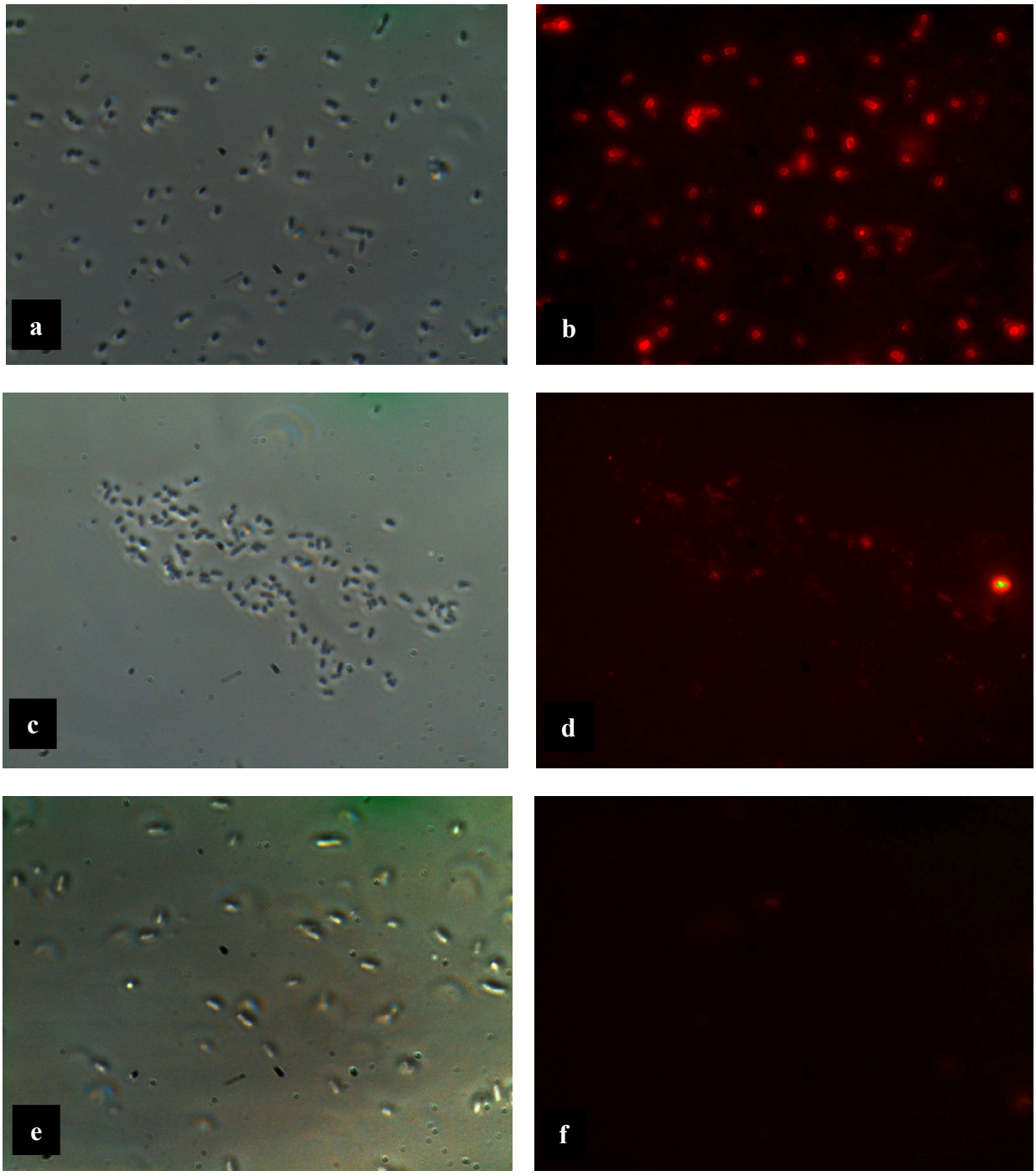


Fig. 20: Detection of K15 capsule expression of *E. coli* strain 536 and derivatives by immunofluorescence microscopy. **a-b**, 536 wt; **c-d,** 536Δkps_{K15}; **e-f**, MG1655. Experiments were made by the T-RED labelled absorbed anti-K15 serum. Phase contrast images are on the left side, while immunfluorescence images of the same preparation are on the right side.

3.4 Deletion and complementation of the whole capsule locus of strain 536

For complementation experiments the capsule mutant of *E. coli* strain 536Δ*kps*$_{K15}$ was used which lacks the complete *kps* gene cluster. This ~20-kb deletion was complemented with a BAC clone (pBAC29B-41) that contained the complete *kps*$_{K15}$ locus of strain 536 (personal communication M. Emmerth). Since in this BAC clone additional upstream and downstream sequences were present, the risk for homologous recombination was relatively high. Therefore we constructed a double mutant of strain 536 in which beside the K15 capsule-encoding locus the gene *recA* was also deleted. In the absence of functional RecA, the BAC clone was no longer able to integrate into the chromosome by homologous recombination. For further experiments, a derivative of the BAC clone 29B-41 was constructed which carried a deletion of a 30-kb *Pml*I fragment (designated later pBAC29B-41/30S).

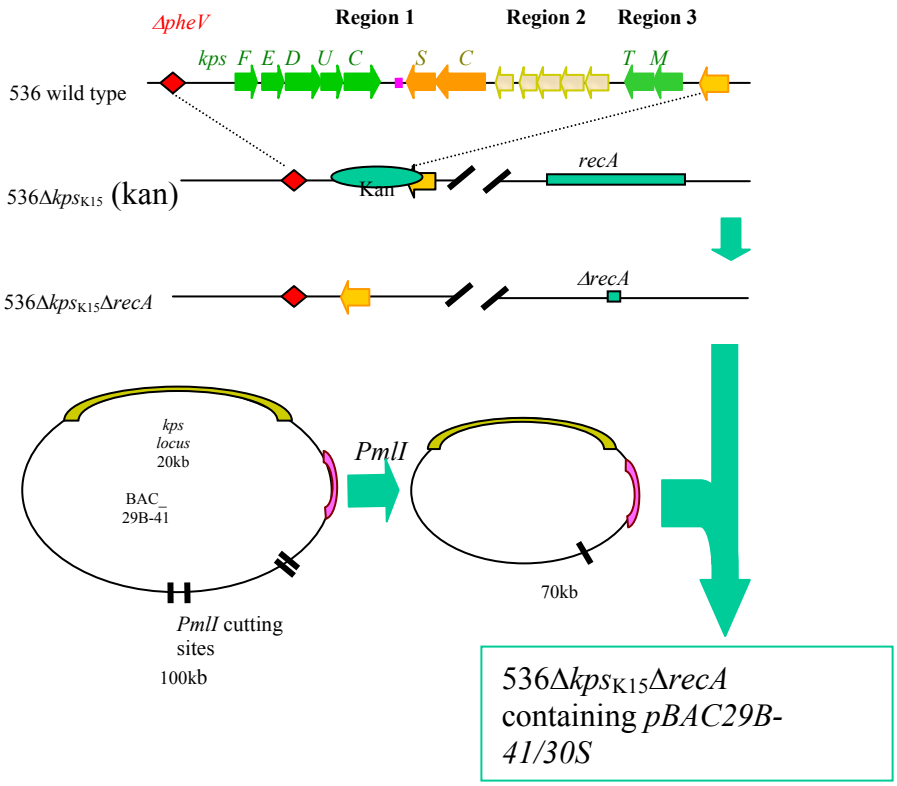


Fig. 21: Complementation strategy for strain 536Δ*kps*$_{K15}$. The K15 capsule mutant of strain 536 was complemented with pBAC29B-41. For size reduction, a 30-kb *PmlI* fragment was cut out from pBAC29B-41. The vector was then religated (pBAC29B-41/30S) and electroporated into the double mutant 536ΔK15Δ*recA*.

The resulting 70-kb recombinant BAC vector pBAC29B-41/30S contained the complete K15 capsule locus but its size was reduced compared to the original clone (pBAC29B-41). The BAC clone pBAC29B-41/30S was electroporated into strain 536Δ*kps*$_{K15}$Δ*recA*. After re-isolation of the plasmid, it was verified that integration into the chromosome had not occurred and complementation was detected by ELISA (Figure 19).

3.5 The K15 capsule contributes to pathogenicity of *E. coli* strain 536

After verification that the capsule locus that was found downstream of PAI V_{536} was really functional, encoding the enzymes of K15 capsule biosynthesis and export, we tested all the mutants in an ascending urinary tract infection model (Table 5). In this model, bacteria were inoculated into the bladder of newborn mice. Non-pathogenic strains cause no death while the lethality of the wild type UPEC strain 536 is nearly 100%.

Table 5: Virulence of *E. coli* strain 536 and different K15 capsule mutants in an ascending urinary tract infection model.

Strain	No. of infected mice	Lethality [%]
MG1655	19	0
536 wt	21	100
536Δ*kpsSC*$_{K10}$	27	14.8
536ΔR2-2	20	10
536Δ*kpsMT*	24	8.3
536Δ*kps*$_{K15}$	21	19
536Δ*kpsC'*$_{K5}$	12	91.7
536Δ*kpsC*$_{K10}$	13	15.4

With the exception of strain 536Δ*kpsC'*$_{K5}$, all tested mutants proved to be avirulent. This correlates with the results of the ELISA assay where strain 536Δ*kpsC*$_{K5}$ was shown to express the K15 capsule. Loss of the K15-specific extracellular polysaccharide had attenuated the strain 536

indicating that the K15 capsule plays an important role in the pathogenic process of an ascending urinary tract infection.

Serum resistance experiments with 50 % human serum were carried out with selected mutants to examine further the role of the K15 capsule in systemic infection. As a negative control, the *rfaH* mutant of strain 536 was used, which expresses neither LPS nor capsule, and is also known to be serum sensitive.

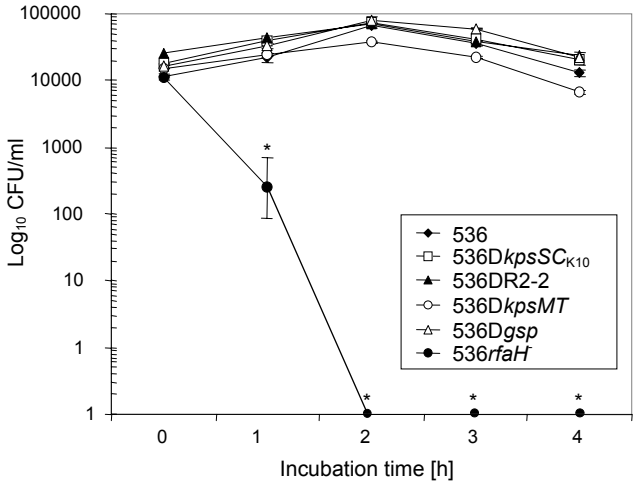


Fig. 21: Role of the K15 capsule for serum resistance of *E. coli* strain 536 and different mutants was investigated in 50 % human serum. Strain 536 has the ability to avoid the bactericidal activity of human serum. Loss of the K15 capsule showed no effect on this ability because in those mutants where only capsule was lost (536Δ*kpsSCK10*, 536ΔR2-2, 536Δ*kspMT*) bacteria stayed resistant against serum. Because of the impaired LPS synthesis the RfaH mutant of strain 536 was known to lost its serum resistance and it was used as a negative control. Serum resistance experiment was also made with the GSP mutant 536Δgsp (resistant) and the 536ΔR2-1 (sensitive – data not shown, details see in text).

Almost all tested K15 capsule mutants were serum resistant and a significant decrease in serum resistance was not detected. Only small reductions were observed indicating that the K15 capsule type does not play a role for serum resistance. Only one mutant was serum sensitive (536ΔR2-1). This mutant was constructed with the help of the suicide plasmid vector pCVD442. However, the other region 2 mutant, (536ΔR2-2) that was constructed using linear DNA fragments and practically deleted exactly the same sequence did not show a reduced serum resistance. It can be assumed that the first mutant may carry a secondary mutation somewhere in the chromosome which is responsible for causing the serum sensitivity. Clearing up this question we prepared LPS from strain 536 and its different mutants and detected the O6 LPS side chain expression by SDS-

PAGE (Fig. 22). It became evident that the "first" region 2 mutant (536ΔR2-1) that was made with the suicide vector pCVD442 had a more fainter LPS pattern and the core region seemed also damaged that had correlated with the serum sensitivity of this mutant and not loss of capsule.

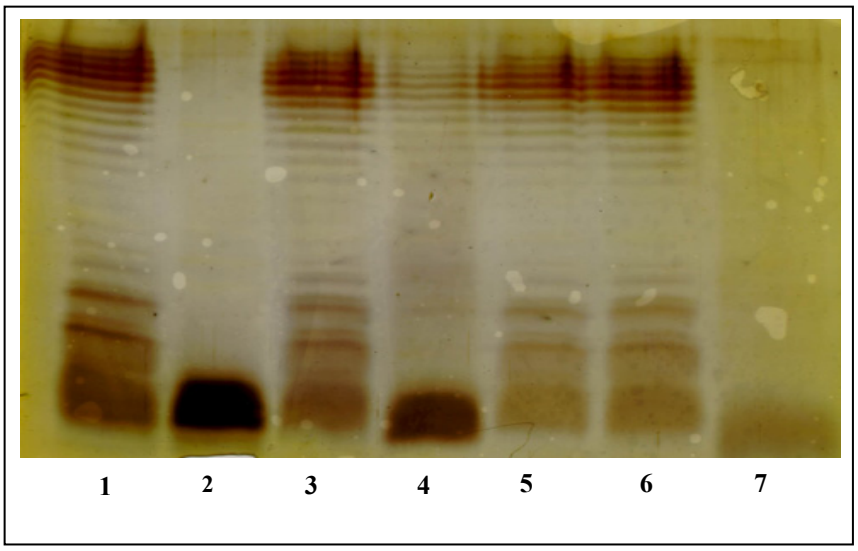


Fig. 22: Detection of O6 LPS side chain expression of strain 536 and different capsule mutants by SDS-PAGE: lane **1**, *E. coli* strain 536; lane **2**, *E. coli* K-12 strain MG1655; lane **3**, *E. coli* strain 536Δ*kpsSC*$_{K10}$ (region 1 mutant); lane **4**, *E. coli* strain 536ΔR2-1 ("first region 2 mutant"); lane **5**, *E. coli* strain 536Δ*kpsMT* (region 3 mutant); lane **6**, *E. coli* strain 536 ΔR2-2 ("new region 2 mutant"); lane **7**, *E. coli* strain 536*rfaH* (negative control).

4 Analysis of downstream sequences of the K15 capsule determinant

Structural analysis of the pCos24/23 revealed that downstream of the K15 capsule determinant a genetic region was localised that showed no significant homology at the nucleotide level to known genes. Nevertheless, considerable homology was detectable at the deduced amino acid level. The proteins encoded by eight putative ORFs localised in this region of PAI V_{536} exhibited homology to components of a type II secretion system (general secretion pathway, *gsp*). Type II secretion systems play a role in the assembly and secretion of the cholera toxin in *Vibrio cholerae* (*eps*) (Overbye *et al.*, 1993), the secretion of pullulanase in *Klebsiella oxytoca* (*pul*) (Pugsley and Reyss, 1990), the secretion of extracellular enzymes of *Erwinia carotovora* (*out*) (Reeves *et al.*, 1993, and are also present in *Pseudomonas aeruginosa* (*xcp*) (Filloux *et al.*, 1990) and *Xanthomonas campestris* (*xps*) (Thomas and Trust, 1995).

A DNA region with 99 % homology at the nucleotide level was also described in an enterotoxigenic *E. coli* strain (Tauschek, 2002; AF426313). In this ETEC strain, the type II secretion system is involved in secretion of a heat-labile enterotoxin.

Comparison of this chromosomal region of strain 536 to that of *E. coli* strains MG1655 and CFT073 (Fig. 23) revealed that *yghD* (*orf76*) is the last gene, which is commonly present in all strains analysed. According to a new nomenclature, this gene is the M-type component (*gspM*) of the general secretion pathway. A major part of the next gene (*gspL*) that is partially absent in strain MG1655 is completely present in strains CFT073 and 536. The next gene *gspK* (*orf78*) is already completely missing from the chromosome of MG1655, and is only partially present in the genome of the UPEC strain CFT073. From this gene additional seven genes (*gspJ, I, H, G, F, E, D*) are present in this genomic region of strain 536 that are completely missing from the genome of MG1655 and CFT073. This part shows high homology at the nucleotide level to the recently described type II secretion system-encoding gene cluster of an O78:H11 ETEC strain, and plays a role in the secretion of a heat-labile enterotoxin of the ETEC strain H10407 (Tauschek, 2002; AF426313). The next gene that was common again in MG1655 and 536 was *gspC* (*orf86*) or *yghF*. 81,194 bases downstream of *pheV* in strain 536 an ORF is located (*orf89, yghJ*) that is also present in MG1655 and was found to be the largest orf in PAI V_{536} and downstream sequences. *yghJ* can also be found in MG1655 and earlier was thought to be two genes (*b2973* and *b2974*). Sequence homology to the genome of CFT073 comes back at *yghK*. This is the gene, which is

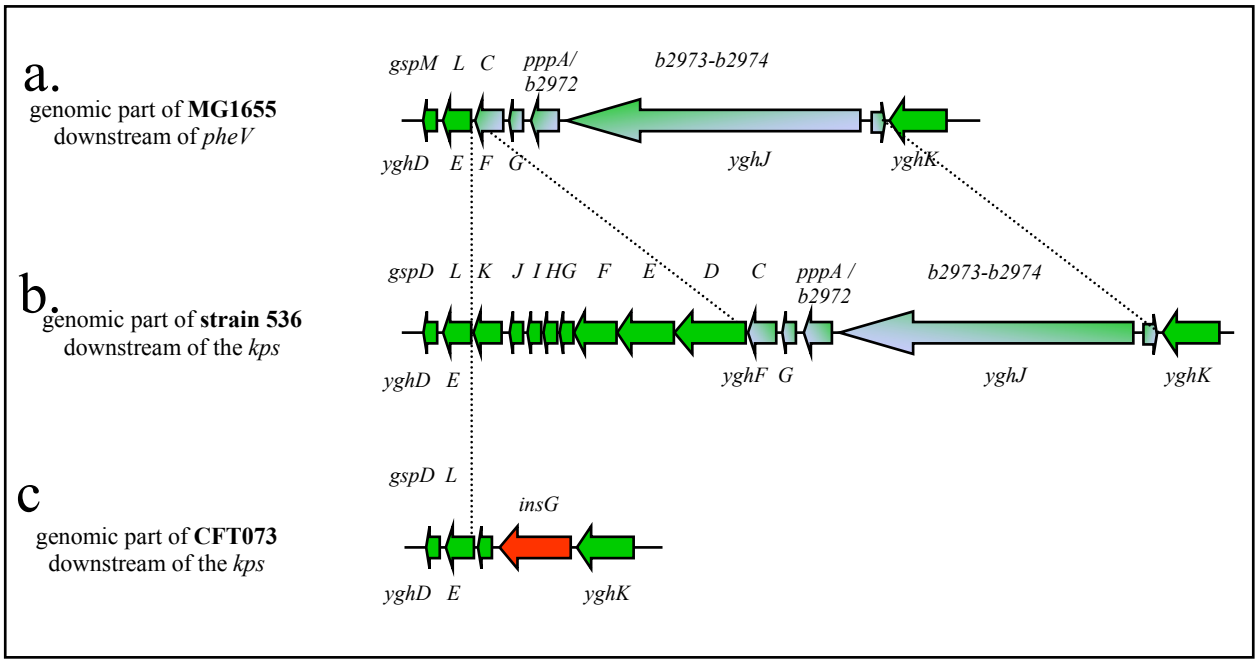


Fig. 23: Comparison the common parts of the general secretion pathway genes among three different *E. coli* strains. A nearly 8 kb insertion can be found in strain 536 (**b**) relative to the equivalent genomic part of the K-12 strain MG1655 (**a**), while the genome of CFT073 (**c**) shows a dramatic reduction of this part, hypothetically because of an integration of an IS element.

common again in all the three strains. In CFT073, members of the type II secretion system are missing. Between the truncated *gspK* and the *yghK* gene an *insG* (c3703, accession number AE016766) can be found, which is a transposase gene of the insertion element IS4.
Transcription of the gsp gene cluster was studied by RT-PCR. With primers *gspDfwRT* and *gspDrevRT*, *gspD* transcripts could not be detected from cDNA of exponentially growing cells (not shown). The *gsp* genes that were absent in strain MG1655 but were present in strain 536 have been deleted: altogether 6,870 bases were deleted involving the genes *gspD, E, F, G, H, I, J* and *K*. This deletion started 70,970 bases downstream of *pheV* and ended at position 77,840. The resulting deletion mutant was tested for capsule production and was also tested in the ascending urinary tract infection model. Neither loss of capsule expression nor virulence attenuation could be detected.

As described in section 1.1.4, a 7 kb insertion was localised on the cosmid pCos2/13 relative to the *E. coli* K-12 situation. This DNA region exhibited homology to the genomic regions of *S. flexneri* 2a (accession number: AE015315) and the UPEC strain CFT073 (accession number: AE016766) genomes. According to the sequence analysis, some of the putative ORFs localised in this region encode membrane bound proteins, e.g. permeases (*orf102-orf103*) or a hypothetical protein with similarity to ATP-binding proteins (*orf105*).

5 Mobilisation of PAI II_{536} into recipient strain SY327

5.1 A modified PAI II_{536} derivative is transferred by the conjugative plasmid RP4

Studies of the instability of PAIs from UPEC strain 536 have revealed that four of them (PAI I_{536}, PAI II_{536}, PAI III_{536} and PAI V_{536}) are able to delete from the chromosome with different frequencies. Additionally, extrachromosomal forms (circular intermediate, CI) of PAI II_{536} and PAI III_{536} were detected by a specific PCR assay (Middendorf *et al*., 2004). PAIs are thought to exist at least transiently as CIs after excision from the chromosome by integrase-mediated site-specific recombination between the flanking direct repeats (Blum *et al.,* 1994, Middendorf *et al.*, 2004, Hochhut unpublished). Since the islands of strain 536 contain no origin of replication, it has been also hypothesised that CIs are lost upon cell division if they fail to reintegrate into the chromosome. The processes involved in deletion and probably also in integration of PAIs are relatively well studied, but little is yet known about the mechanisms that have been involved in acquisition of PAIs by horizontal gene transfer. To evaluate the potential of PAIs to be transferred by conjugation, PAI II_{536} was labelled with a resistance gene (*cat*) for selection, an origin of replication ($oriV_{R6K}$) and an origin of transfer ($oriT_{RP4}$).

An overview of the main steps of the mobilisation experiment is shown in Figure 24. First, the chloramphenicol resistant derivative pGS704 of the suicide vector pGP704 was integrated into a non-coding part of PAI II_{536} by homologous recombination. This plasmid carries a mobilisation region containing *oriT*, that served later as the origin of transfer and originates from the conjugative plasmid RP4. Furthermore, the plasmid contains an *oriV* derived from plasmid R6K that functions only in the presence of the *trans* encoded π-protein (*pir*). Second, after labelling PAI II_{536}, the *pir* gene was integrated into the chromosome of strain 536 by inserting it into the λ attachment site (*λatt*) to ensure that PAI II_{536} can replicate and be stably maintained in the extrachromosomal form after excision from the chromosome. Finally, the plasmid RP4 was introduced to deliver the genes for mating pair formation and conjugative DNA metabolism (*tra* genes) that are required for transfer of PAI II_{536} by conjugation. As a recipient strain, the K-12 derivative SY327 was used, that contains a λ phage carrying the *pir* gene and that is resistant to nalidixic acid due to a mutation in *gyrA*. Transconjugants were selected on agar plates containing Nal (for SY327), Cm (for labelled PAI II_{536}) and 5% blood because PAI II_{536} codes for an α-haemolysin determinant.

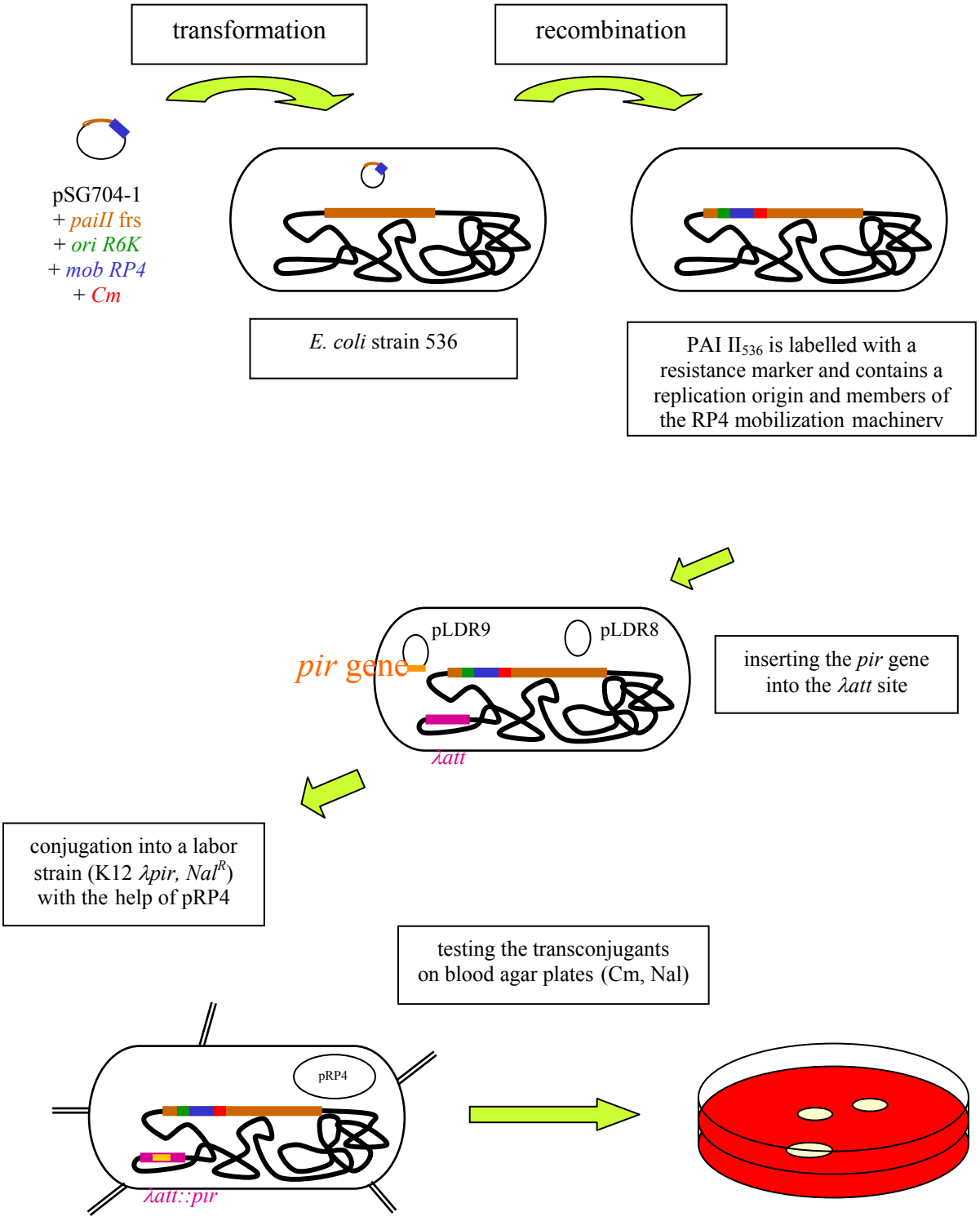


Fig. 24: A general overview of the main steps of the mobilisation experiment. PAI II_{536} was labelled and then transferred by conjugation. For more details, see text.

5.2 Labelling of PAI II_{536} with a resistance marker, an origin of replication and an origin of transfer

For inserting a *cat* cassette into pGP704, the vector was cut with the restriction endonuclease *Pst*I. Thereby, a 700-bp fragment that encompasses two thirds of the ampicillin resistance gene *bla* was deleted and replaced by the *cat* cassette that was amplified from pACYC184 with flanking *PstI* sites. This Cm-resistant pGP704 derivative was named pGS704. Into this plasmid, two PCR products that correspond to non-coding sequences of PAI II_{536} 2500 bp downstream of *leuX* (using primer pairs *paiII_1XhoI*, *paiII_1Sac* and *paiII_2Sac*, *paiII_2XhoI*) were ligated into

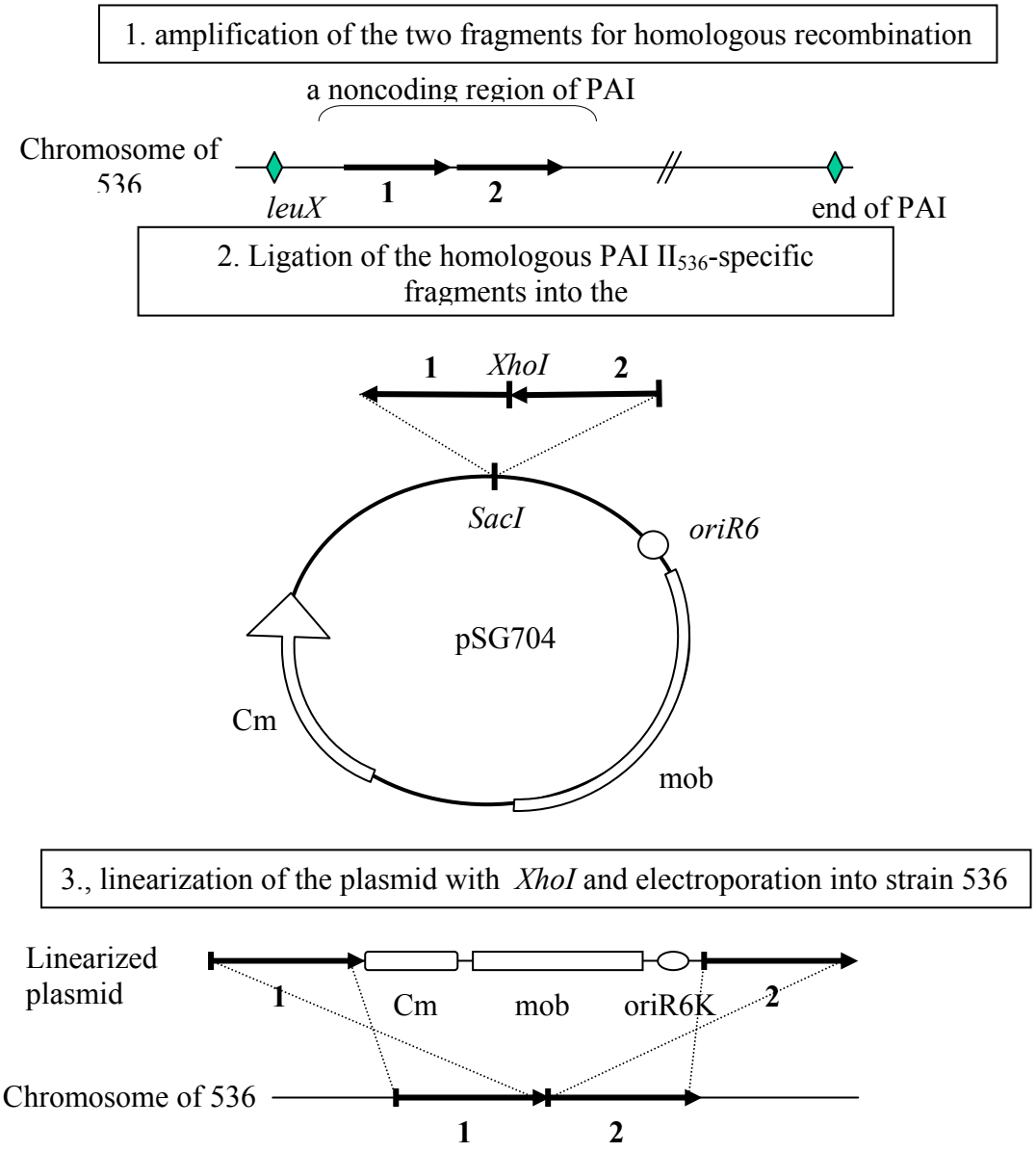


Fig. 25: PAI II_{536} was labelled by homologous recombination with a replication origin (*oriV* from plasmid R6K), a Cm resistance marker (*cat*), and a *mob*-region that contains *oriT*.

a *SacI* restriction site. The orientation of the two fragments was inverted in the circular form, and only corresponds to the original order in PAI II_{536} after linearization with *Xho*I (Figure 25.).

The purified linearised vector with the flanking regions homologous to PAI II_{536} was electroporated into strain 536 containing pKD46. Recombinant colonies with the integrated pGS704 suicide vector were selected on antibiotics containing LB plates. Cm- and Sm-resistant clones were tested by PCR and then analysed by Southern hybridisation whether the insertion of the pGS704 was really correct (Figure 26). For further experiments, clone 10 (536mob_10) was chosen.

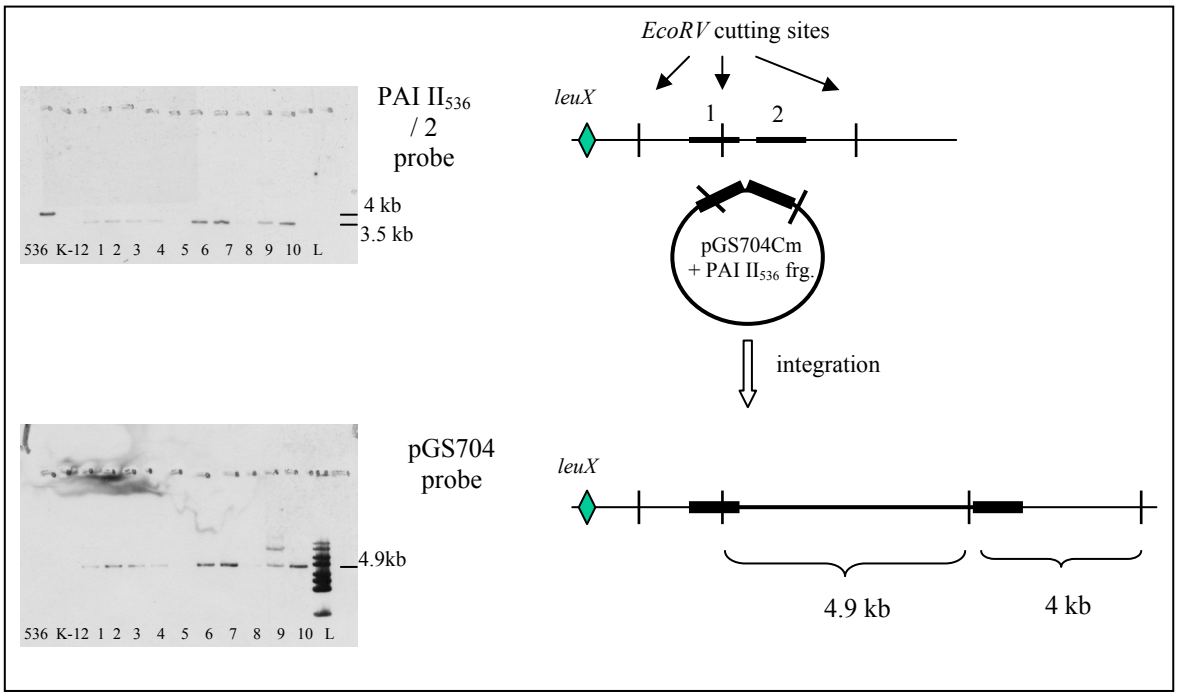


Fig. 26: Verification of pGS704 integration into the PAI II_{536} by Southern hybridisation. On the left side, the digested (*EcoRV*) chromosomal DNA of 10 candidate clones are shown that were hybridised with a specific probe to fragment 2 of PAI II_{536} (top) and with labelled pGS704 (bottom). Clones 1, 2, 3, 4, 6, 7, 8, and 10 appeared to be integrated as expected; (a 4-kb fragment hybridised with the PAI II_{536}/2-specific probe and a 5-kb fragment hybridised with pGS704), while clones 5 and 9 were not correctly integrated. (thick black line: PAI II_{536} sequences for homologous recombination; middle thick black line: pGS704Cm, vertical lines: *EcoRV* sites).

5.3 Integration of the *pir* gene into the λ attachment site of the chromosome

With the help of the pLDR plasmid family, the *pir* gene was integrated into the chromosome of strain 536mob_10. The *pir* gene was amplified from strain Sm10λpir with the primers *pir_fw_SacI* and *pir_revStop_EcoRI*. Primer *pir_revStop_EcoRI* was designed cautiously because

when *pir* of plasmid R6K was cloned by partial digestion with *Hin*dIII, only a fragment containing the first two third of this gene was integrated into the chromosome of strain Sm10λ*pir* (personal communication, J. Reidl and S. Schild). Downstream of the gene an alternative STOP codon is present. Transcription of the resulting 3' truncated *pir* allele is sufficient for activity of the π-protein. Based on the sequence of the *pir* gene, the reverse primer was designed in a way that a complementary STOP codon (TGA) was introduced and overlapped one bp with the *HindIII* recognition site (Figure 27).

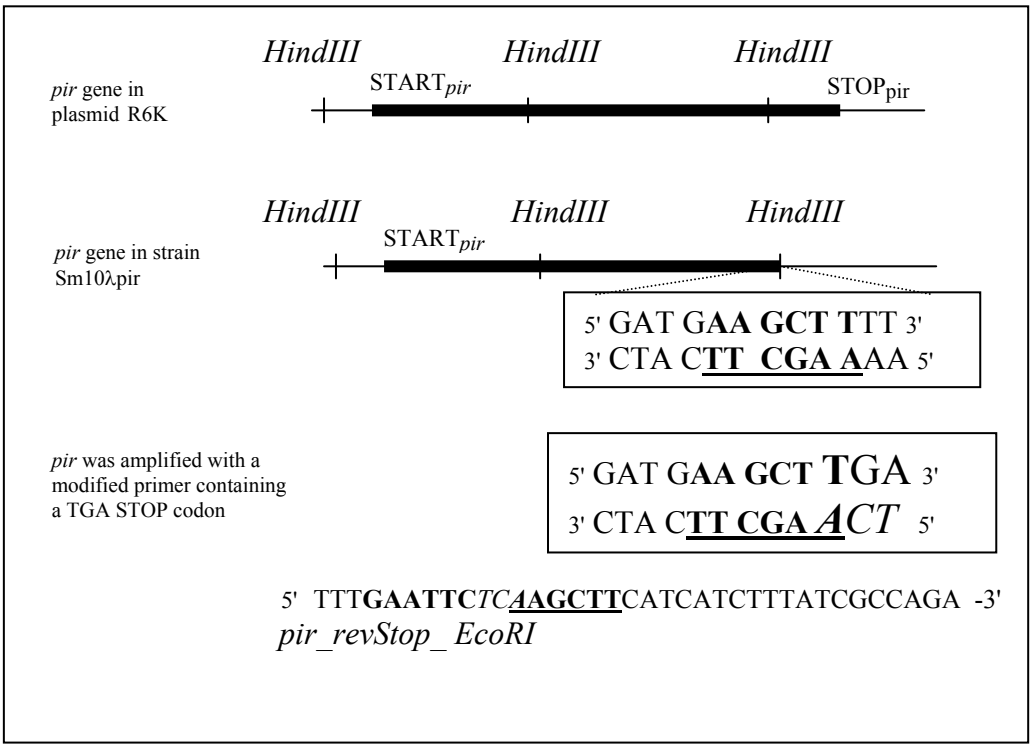


Fig. 27: Variations of the *pir* gene present in pR6K and *E. coli* strain Sm10λpir. For the integration into the λ-*attB* site of the *E. coli* 536*mob*_10, *pir* was amplified from the genome of *E. coli* strain Sm10λpir. Constructing this strain, the plasmid R6K was partially digested (*HindIII*) and *pir* was indirectly integrated into the chromosome (*via λ* phage). The last third of the original gene is missing and ends with an alternative STOP codon in Sm10λpir. Amplification of the incomplete *pir* gene (the first two third) was performed from the chromosome of Sm10λpir by using a primer that contained a complementary TGA STOP codon that overlaps with the *HindIII* recognition site (AAGCTT).

The 950-bp PCR product (*pir* gene) was ligated with *EcoRI* and *SacI* restriction sites into pLDR9. After restriction with *NotI* the bigger fragment was religated and electroporated into the pLDR8 containing strain 536mob_10. Pools of clones were screened with PCRs (primers ATT1 and ATT2) for the integration of *pir* into the λ attachment site (Figure 28). From positive clones,

a 3-kb fragment was amplified. The 290-bp fragment corresponding to the size of the PCR product without integration remained there but became fainter. One clone was chosen and tested for the expression of π-protein. For this, a pCVD442 derivative with a tetracycline resistance cassette was used. pCVD442 carries an *ori*R6K and a functional π-protein supports the replication of pCVD442-Tc under selective conditions after electroporation. The clone 536-19mob was chosen for further experiments. For the mobilisation experiments this clone was used as a negative control.

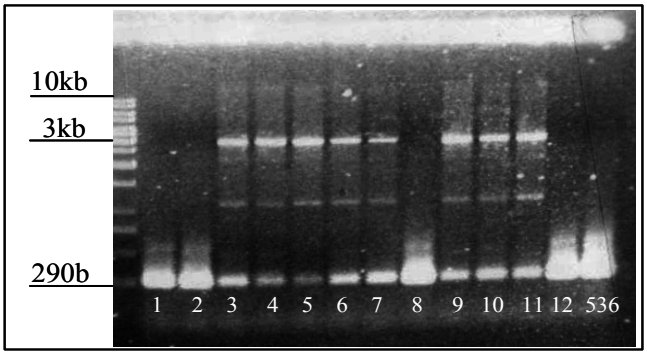


Fig. 28: Detection of *pir* insertion into the λ-*attB* site of strain 536mob_10. Candidate clones (lanes 1-12) were checked by PCR with the primer pair ATT1 and ATT2. Successful *pir* insertion into the chromosome resulted a 3 kb PCR product (lanes 3-7, 9-11). Without integration, a 290 bp fragment was only amplified (lanes 1, 2, 8, 12) similarly to the wild type strain 536 (last column).

5.4 The labelled PAI II_{536} is mobilisable by RP4

After labelling of PAI II_{536} and integrating the *pir* gene into the chromosome, the plasmid RP4 was conjugated into the strain 536-19mob. Conjugation with RP4 was very effective and one clone (536-19/1mob) was chosen for further experiments. Later, in the conjugation experiments, this strain was used as the donor strain. The recipient strain was the nalidixic acid resistant SY327 laboratory strain. For the first conjugation experiments O/N cultures were used with a donor to recipient ratio of 1:1. The total volume (200 µl) was spread on LB plates and incubated for 24 hours at 37 ºC, for 48 hours at 30 ºC, for 72 hours at 20 ºC and for 168 hours at 4 ºC. Cells were resuspended and for selection of transconjugants, 100-µl aliquots of the undiluted suspension, a 10^{-1}-, and 10^{-2}-dilution were plated on Cm-Nal-blood agar. We have found 90 haemolytic clones that were further analysed by PCR. First we looked for those haemolytic clones that were not strain 536 but were the laboratory strain SY327, that is why the primers specific for *E. coli* strain 536 (*orf4bico* and *orf5bico*, bind in region 2 in the K15 gene cluster) and strain K-12 (*K12R* and *K12L* or *K12R* and *K12ISL*, Kuhnert *et al*., 1995), respectively, were used. Those 16 clones that were negative in the 536-specific PCR assay and positive in the K-12-specific PCR assay were then tested with primer pairs specific to PAI II_{536}. In this latter PCR the

following primer pairs were used: *17kDup* and *17kDin*, *hlyDup* and *hlyDin*, *hec_down1* and *hec_down2*, *dsdXin* and *dsdAup*, *ORFAin* and *Na-Anti_pdo* (Fig. 28). From those six clones that were positive with all the five screening primer pairs, overlapping PCRs were carried out that covered the complete PAI II_{536} (102 kb). 53 primer pairs were designed that amplify overlapping products each with a size of 2 kb. With these PCRs, it was verified that the clones in question contained the complete PAI II_{536} without internal deletions. Subsequent PCRs were performed to check whether the mobilised PAI II_{536} was present in the recipient strain in the circular form or integrated into the chromosome. If there was no integration at *leuX,* the primer pair *M803b* and *M805c* allowed amplifying of an 800-bp product and if the mobilised PAI was present in the cell as a stable circular intermediate, the PCR with primers (*leu2* and C*oncat1* or alternatively with *PaiII_1rev* and *PaiII_53fw*) was positive. These primers were designed in a way that they are oriented towards the ends of chromosomally integrated PAI II_{536} and only amplify a specific product when PAI II_{536} is present in the circular form. Finally, PAI II_{536} carrying transconjugants were analysed by pulse field gelelectrophoresis to reveal differences in the restriction pattern of the transconjugants carrying integrated or extrachromosomal PAI II_{536}.

5.4.1 Mobilisation of the whole pathogenicity island was detected

Results of the first conjugation have successfully shown that intercellular transfer of a whole pathogenicity island could occur with our system, although with very low rates. These results are summarised in Figure 29.

Altogether, 90 haemolytic that were also Cm/Nal resistant were isolated. However, checking these clones with 536- and K-12 specific PCRs revealed that most of them were only spontaneous nalidixic acid resistant mutants of strain 536. The remaining 16 clones were analysed with PAI II_{536}-specific PCRs to determine whether they had obtained the complete PAI II_{536}. Six transconjugants of SY327 seemed to contain the whole PAI II_{536} while the other 10 contained only parts of it (Figure 28). With the six clones, additional PCRs with primers amplifying overlapping products were carried out to verify that no internal deletion had occurred within PAI II_{536} (Figure 30).

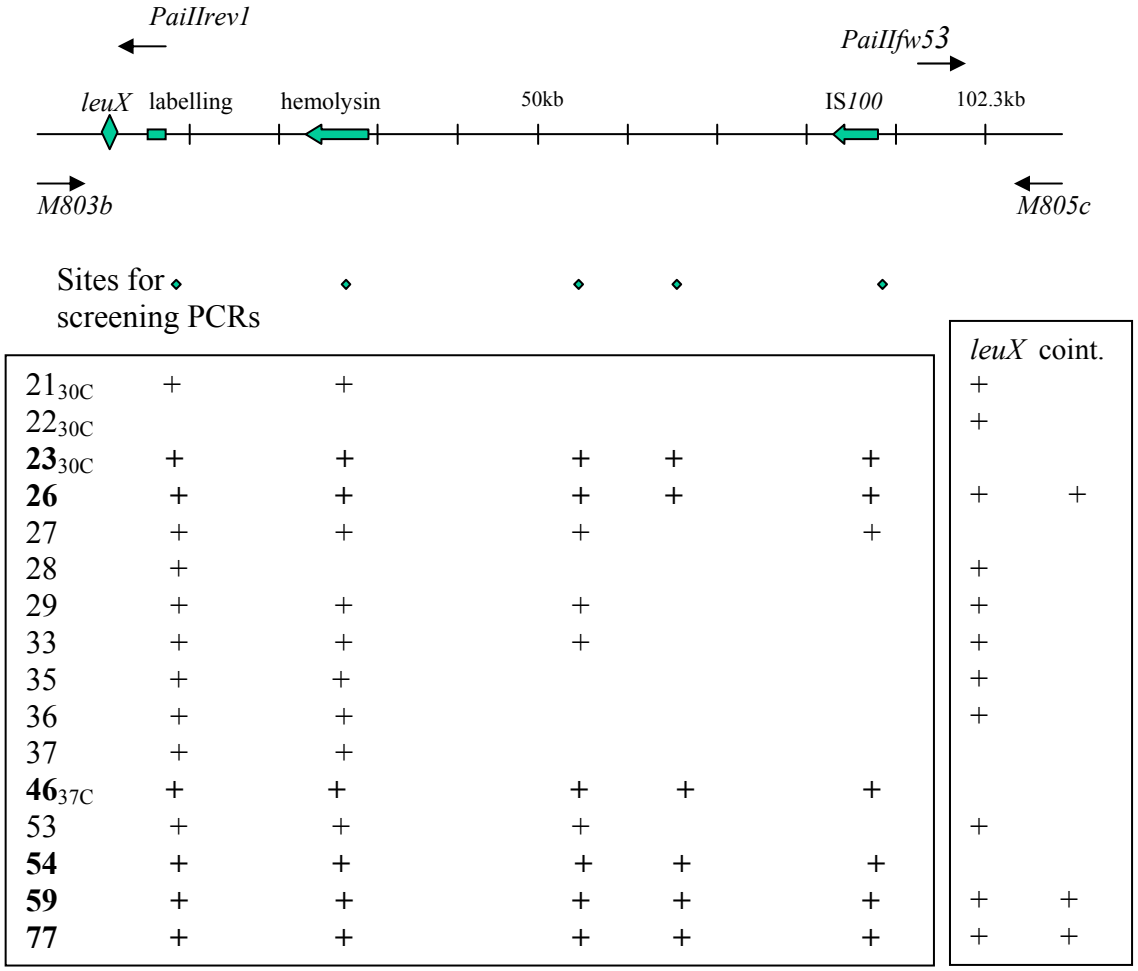


Fig. 29: Detection of partial as well as complete transfer of PAI II_{536} into strain SY327 by PAI II_{536} specific PCRs. Primer binding places of the screening PCRs are shown with small dots under the line representing of PAI II_{536} in the upper part of the figure. 6 out of 16 clones (shown in bold letters) were positive with all six primer pairs. PCRs with the *leuX* and cointegrate primers (right) suggested that in clones 23, 46 and 54 the mobilised PAI II_{536} has been integrated into the chromosome of strain SY327 at *leuX*, whereas clones 26, 59 and 77 seem to carry the extrachromosomal form. Except clones 21-23 and 46, transconjugants result from matings at 20 °C. Orientation of "integration primers" and M803b and M805c are labelled above (see also Fig. 30)

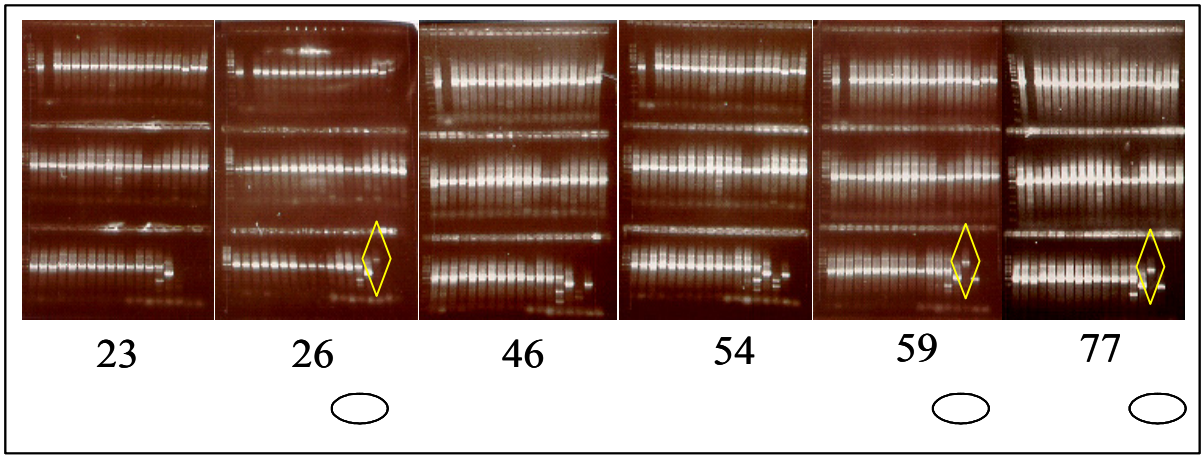


Fig. 30: Transfer of the complete PAI II_{536} into strain SY327 as verified with overlapping PCRs. All the six candidate clones were positive for every PAI II_{536}-specific PCR (53x2kb). In clones 26, 59 and 77 the positive signal (◊) of the primers *PaiII_1rev* and *PaiII_53fw* (see Fig. 31/c) suggests that PAI II_{536} is present in the circular from as also indicated by circles beneath the gel photos.

5.4.2 PAI II_{536} can integrate into *leuX* of the recipient's chromosome

Further analysis by PCR revealed that only three (no. 26, 59 and 77) out of the six transconjugants containing a complete PAI II_{536} carried the island in the extrachromosomal form (Figure 29). From the other three transconjugants (no. 23, 46 and 54) no product was obtained with primers specific for the circular form. Furthermore, *leuX*-specific primers failed to amplify a product suggesting that, as in *E. coli* strain 536, PAI II_{536} had integrated into *leuX*. Therefore, a PCR assay specific for the junctions between chromosome and island was designed to prove this assumption (Figure 31). From the results it became evident that after mobilisation and transfer of PAI II_{536}, the island can either be maintained as a stable circular element or integrates into the recipient's chromosome thereby using the same insertion site as in the donor.

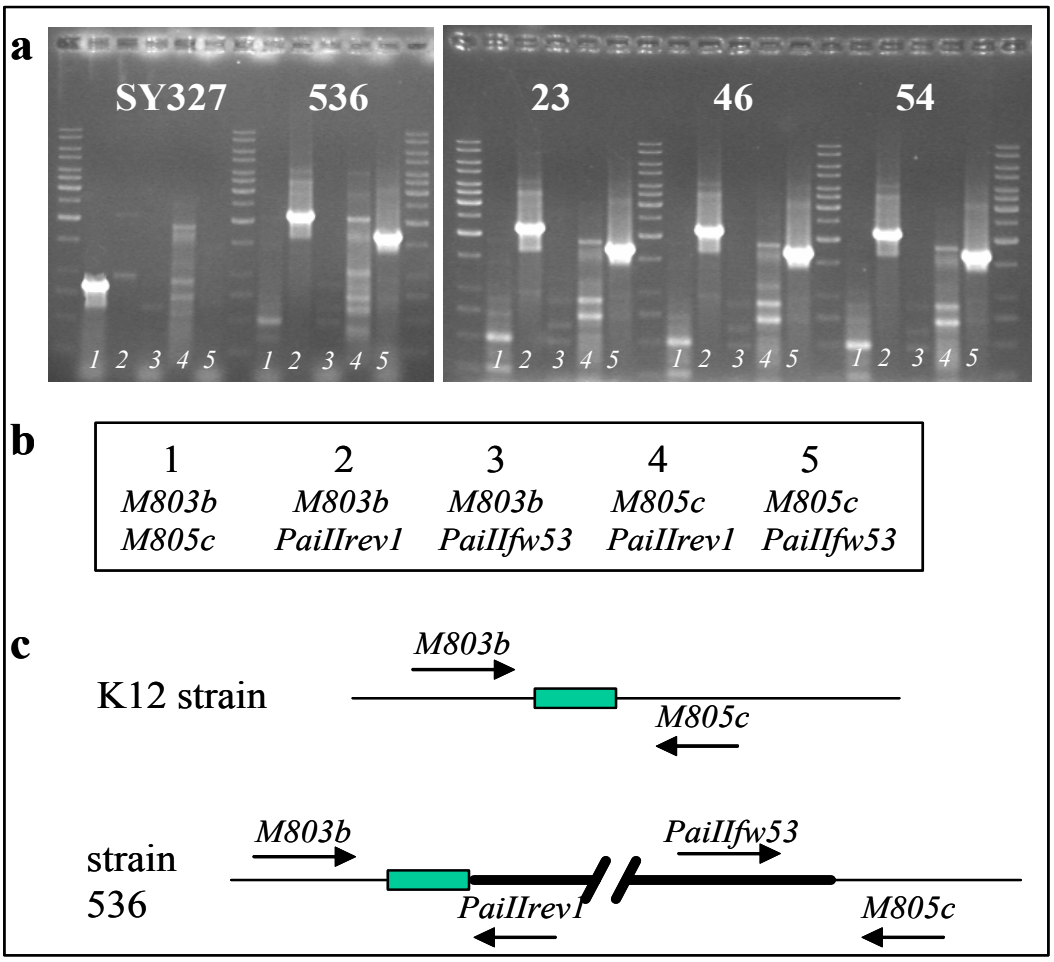


Fig. 31: Integration of the mobilized PAI II_{536} into the chromosome of SY327 at *leuX* was detected by PCR. *leuX* and PAI II_{536} specific PCRs were carried out (**a**) with the laboratory K-12 strain SY327, the wild type strain 536, and the SY327 clones 23, 46, 54. For this purpose four testing primers (*M803b, M805b, PaiIIrev1, PaiIIfw53*) were used in different combinations (**b**) that is also detected on the bottom of the gelphotos (**a**). Orientation of the primers relative to *leuX* (green box) in a K-12 strain and in the wild type strain 536 is depicted in the lower part of the figure (**c**).

5.4.3 Clones with the mobilised PAI II_{536} were analysed by PFGE

PAI II_{536} has a size of 102.3 kb. Deletion of the island from the chromosome (strain 536-225) could clearly be visualised by PFGE (dissertation of G. Blum). We also checked the six transconjugants with this method to see whether differences could be detected between isolates carrying the PAI integrated into the chromosome or in the circular form, respectively (Figure 32).

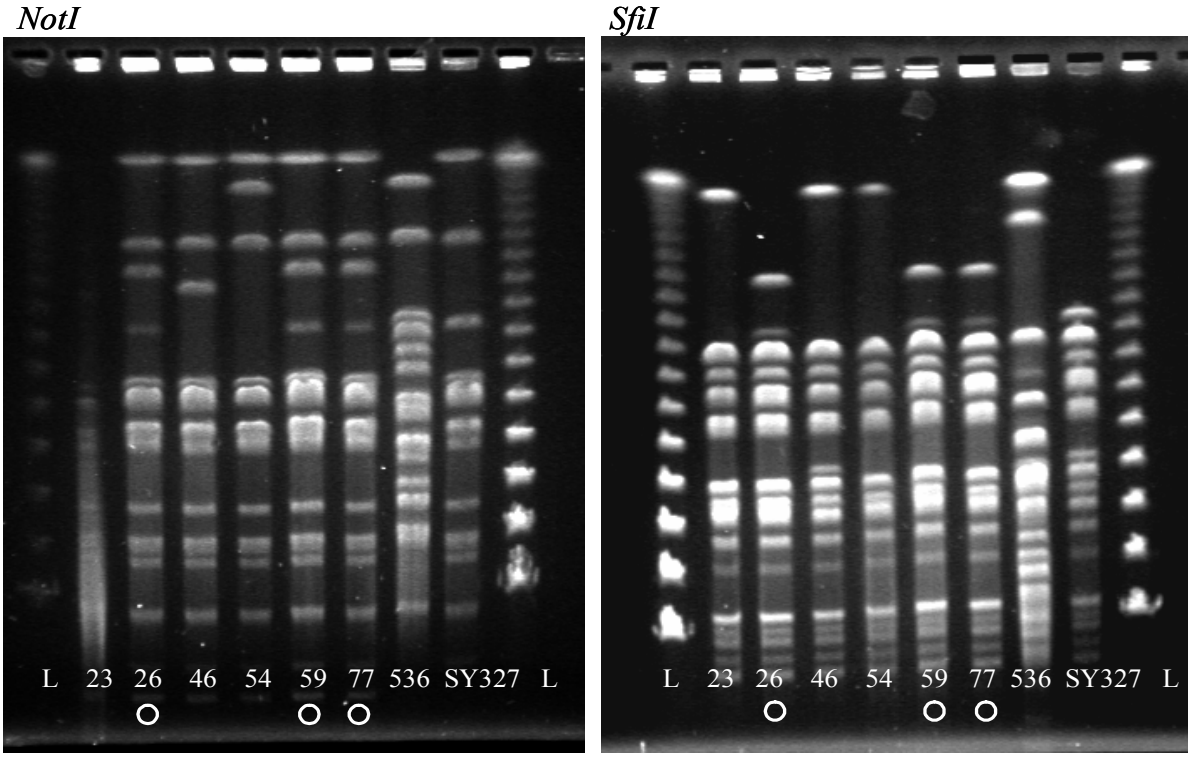


Fig. 32: Similarities and differences among the clones where PAI transfer has occured were confirmed by PFGE. DNA was digested with *Not*I (left) or *Sfi*I (right). All the six clones where PAI II_{536} was present show the typical pattern of strain SY327, but because of the presence of PAI II_{536} additional bands are also present. (L –ladder, 23, 26, 46, 54, 59, 77 are the six transconjugants; 536, SY327 are controls; mark '**o**': in these clones the mobilised PAI II_{536} is present as a circular form, while in clones 23, 46 and 54 integration of the mobilised PAI into the chromosome of strain SY327 has occurred.)

First, the analysis by PFGE confirmed that the transconjugants are derivatives of SY327 as they show a similar restriction pattern to the K-12 strain, but not to 536. Furthermore, the transconjugants with the circular form of PAI II_{536} (26, 59 and 77) have an identical restriction pattern. Similarly, clones 23 and 46 (PAI II_{536} integrated into the chromosome) showed the same pattern with both restriction enzymes, but clone 54 showed a little difference compared to 23 and 46. It can be speculated that other DNA rearrangements may have occurred in this strain, but this was not further investigated.

6 Remobilisation of PAI II_{536} from SY327 into 536-21

We could successfully demonstrate that mobilisation of a whole pathogenicity island from one strain to another is possible. Following deletion from the chromosome, the modified PAI II_{536} was transferred by conjugation mediated by plasmid RP4. In the recipient strain SY327, (i) the transferred PAI could either integrate into the chromosome or (ii) be stably maintained as a circular form that replicates via *ori*R6K in the *pir* containing recipient.

In the next part of the mobilization experiments we wanted to know whether remobilisation of PAI II_{536} could occur or not. Experiments were divided into two groups according to the state of the mobilised PAI. In the first group, clone 77 was used as a donor strain with PAI II_{536} present in the circular form. In the second group, clones 23 and 54 were used as donor strains. As shown before, they both contain PAI II_{536} integrated into *leuX* in their chromosome, but could be distinguished by small differences in their restriction patterns.

In all cases strain 536-21, a nonhaemolytic derivative of strain 536 lacking both islands encoding a haemolysin determinant (PAI I_{536} and PAI II_{536}), was used as a recipient strain. Remobilisation experiments were carried out in a way that the π-protein was only expressed in the donor strains (SY327 – 23, 54 and 77) but not in the recipient strain 536-21.

For selection M9 minimal agar was used with chloramphenicol because strain SY327 cannot use lactose as the only C-source due to a chromosomal deletion encompassing the *lac* operon. After mating at 37 ºC, 30 ºC and 20 ºC, respectively, transconjugants were checked by PCR. With clone 77 (with extrachromosomal PAI II_{536}) as a donor strain, PAI II_{536} was transferred with relatively high frequencies (Table 6). 90 transconjugants were tested with strain 536 and K-12-specific primers. For all the clones, the 536-specific PCRs were positive while the K-12-specific PCRs were negative. Similar results were obtained with clones 23 and 54 as donor strains although the transfer rate was less sufficient because in these strains, PAI II_{536} has to excise from the chromosome prior to conjugative transfer (Table 8). From four independent experiments, 127 transconjugants were tested, and they all proved to be transconjugants of strain 536-21. Interestingly, remobilisation of PAI II_{536} from clones 23 and 54 was significantly higher at 20 ºC. At this temperature, a tenfold higher deletion rate of PAI II_{536} had been observed in the stationary growth phase (Middendorf *et al.*, 2004).

In strain 536-21, the remobilised PAI II_{536} cannot be maintained as a circular intermediate because this strain does not contain *pir*. Integration of PAI II_{536} into the chromosome of strain 536-21 was investigated using the primer combinations described before (Figure 31). With the SY327 transconjugant no. 77 as a donor strain, 9 clones of strain 536-21 were tested and all of them showed a PCR fragment pattern corresponding to integration of PAI II_{536} into *leuX*. With SY327 transconjugants no. 23 and 54 as donor strains, in eight out of nine transconjugants of strain 536-21 tested, integration had occurred into *leuX*, whereas in one 536-21 transconjugant integration may have occurred in another site, because it showed another PCR pattern. However, this was not further investigated.

From these results, it can be concluded that PAI II_{536} was transferred with higher frequencies from those strains that carried the island in an extrachromosomal form. However, in the *pir* negative recipient 536-21, PAI II_{536} integrated specifically into the tRNA gene *leuX*, independently whether it had been present in the circular or integrated form in the donor.

Table 6: Remobilisation of PAI II_{536} from strain SY327 into the strain 536-21.

a

Starting conditions of conj.		Results of the conjugation	
	Donor:Recipent ratio		
Temperature	23:536/21	Efficiency of	conjugation
A37	1,44:1	**1x10-9**	
B30	1,44:1	**7,3x10-8**	
C20	1,44:1	**0**	
D37	7,6:1	**0**	
E30	7,6:1	**1x10-9**	
F20	7,6:1	**3,1x10-6**	
G37	40,0:1	**0**	
H30	40,0:1	**6,6x10-8**	
I20	40,0:1	**6,6x10-7**	

Starting conditions of conj.		Results of the conjugation	
	Donor:Recipent ratio		
Temperature	23:536/21	Efficiency of	conjugation
A37	2,2:1	**0**	
B30	2,2:1	**3x10-7**	
C20	2,2:1	**1,8x10-7**	
D37	6,9:1	**0**	
E30	6,9:1	**2,5x10-7**	
F20	6,9:1	**5,75x10-7**	
G37	24,2:1	**0**	
H30	24,2:1	**5x10-7**	
I20	24,2:1	**1,6x10-7**	

b

Starting conditions of conj.		Results of the conjugation	
	Donor:Recipent ratio		
Temperature	54:536/21	Efficiency of	conjugation
A37	1,1:1	**1x10-8**	
B30	1,1:1	**2,14x10-7**	
C20	1,1:1	**9x10-7**	
D37	5,8:1	**0**	
E30	5,8:1	**2,7x10-7**	
F20	5,8:1	**3,15x10-7**	
G37	30,9:1	**4x10-8**	
H30	30,9:1	**2,3x10-8**	
I20	30,9:1	**1x10-8**	

Starting conditions of conj.		Results of the conjugation	
	Donor:Recipent ratio		
Temperature	54:536/21	Efficiency of	conjugation
A37	2,2:1	**0**	
B30	2,2:1	**6,25x10-7**	
C20	2,2:1	**2x10-7**	
D37	6,9:1	**0**	
E30	6,9:1	**6,6x10-7**	
F20	6,9:1	**4,5x10-7**	
G37	24,2:1	**0**	
H30	24,2:1	**0**	
I20	24,2:1	**3,63x10-7**	

c

Starting conditions of conj.		Results of the conjugation	
	Donor:Recipent ratio		
Temperature	77:536/21	Efficiency of	conjugation
A37	2,2:1	**5,4x10-5**	
B30	2,2:1	**1,2x10-4**	
C20	2,2:1	**1,2x10-5**	
D37	6,9:1	**4,2x10-5**	
E30	6,9:1	**8,4x10-5**	
F20	6,9:1	**1x10-5**	
G37	24,2:1	**4,3x10-5**	
H30	24,2:1	**6x10-5**	
I20	24,2:1	**1,9x10-5**	

Frequencies of the remobilisation are shown where PAI II_{536} was integrated into the chromosome of SY327 (**a** and **b**) and where PAI II_{536} was remobilised from the circular intermediate form (**c**). More details can be found in Appendix.

6.2 Localisation of a possible growth factor on PAI II_{536}

Sequence and phenotypical analyses of PAI II_{536} have revealed that it carries a haemolysin determinant, a fimbrial- and a putative adhesin gene cluster. Additionally, several ORFs encoding hypothetical proteins with unknown functions are also present.

During the remobilisation experiments, CFUs of the starting mixture and of the cell resuspension after coincubation of donor and recipient strains (PAI II_{536} transcjonjugants of SY327 and 536-21) were determined. SY327 alone grows relatively slowly, due to several mutations (especially *recA*). Interestingly, when coincubated, PAI II_{536}-containing SY327 was able to overgrow 536-21. This phenomenon was only observed at 37 ºC and was not striking at 20 °C and 30 °C.

Table 7: Analysis of the PAI II_{536}-dependent growth advantage by competitive growth experiments between strain 536-21 and different PAI II_{536} fragment-containing SY327 derivatives.

	Start **SY327 transconjugant : 536-21**			Stop **SY327 transconjugant : 536-21**
SY327 transconjugant no.	Experiment no.	Ratio of strains		Ratio of strains
21	A	0.18:1		0.022:1
	B	0.95:1		0.3:1
	C	5:1		0.66:1
22	A	2.7:1		0.47:1
	B	14.2:1		6:1
	C	75:1		62:1
27	A	0.87:1		3:1
	B	4.6:1		13.8:1
	C	24:1		3.,5:1
28	A	0.24:1		0.038:1
	B	1.2:1		0.2:1
	C	6.6:1		0.83:1
77	A	0.83:1		3.3:1
	B	4.37:1		24.5:1
	C	23:1		86:1
SY327	A	0.036:1		0.026:1
	B	0.19:1		0.067:1
	C	1:1		0.16:1

In the left column transconjugants of the the laboratory strain SY327 are listed that contain the whole (77) and parts (21, 22, 27, 28) of the mobilized PAI II_{536} (see Fig. 28). Each strain was mixed in three different ratios (A, B, C) with strain 536-21 (middle main column) and was incubated at 37 °C. After 24 hours CFUs and ratios were determined (last column).

A competitive growth experiment was performed by using those clones that were received in the mobilisation experiments. Some of these SY327 derivatives did not contain the whole PAI II_{536} but only a part of it. SY327 transconjugants 22, 28, 21, 27 (Figure 29) were chosen for a competitive growth experiment with wild type strain SY327 as a negative control and SY327 transconjugant 77 as a positive control because this is one clone that contained the whole mobilized PAI II_{536}.

Comparing the cell ratios at the starting time point and after 24 h incubation, not only SY327 transconjugant 77 was able to overgrow strain 536-21 but also transconjugant 27. In contrast, with transconjugant 21 that contains a smaller fragment of PAI II_{536} than transconjugant 27, no overgrowth could be detected. The comparison of the fragment length of PAI II_{536} in the different SY327 transconjugants suggests that genes located in the region between 32 kb and 58 kb account for the growth advantage of SY327 at 37 °C.

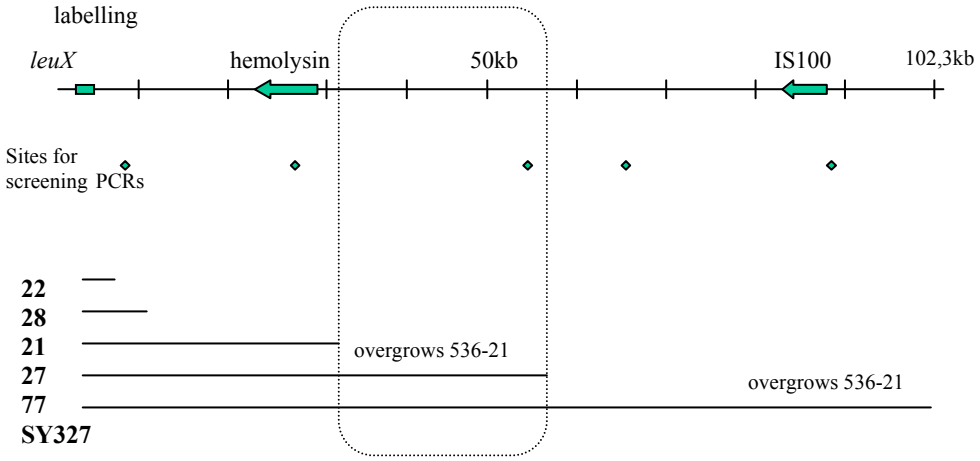


Fig. 33: Localisation of the region within PAI II_{536} identified by competition experiments responsible for the growth advantage relative to PAI II_{536}-negative strains.

Precise localisation of the gene that can be responsible for this phenomenon has not been performed yet.

VI.
DISCUSSION

1. Genome plasticity and evolution of enterobacterial variants

The sequence analysis of complete bacterial genomes made it possible to get detailed insight into the genetic structure of different bacterial genomes. The first bacterial genome sequence that was completely determined was that of *Haemophilus influenzae* (Fleischman et al., 1995) and since then complete genome sequences of more than 200 bacterial genomes have been finished (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html) or are in progress. Comparison of complete genome sequence information obtained from different pathogenic, symbiotic or non-pathogenic strains reveals characteristic differences between certain pathotypes or non-pathogenic variants. For a long time, phenotypic differences have been considered to be frequently associated with the presence of extrachromosomal elements such as plasmids, e.g. encoding toxins or invasion factors. The bacterial chromosome was generally regarded as a well conserved replicon that is relatively stable and static (Brunder and Karch, 2000). However, the increasing number of comparative studies of bacterial genomes demonstrates that they may be relatively variable structures constantly undergoing changes. This so-called genome plasticity depends on the bacterial organisms and is not only manifested by the acquisition or loss of genetic information, but also by large-scale rearrangements affecting genome organisation (Brunder and Karch, 2000). Resulting from genome plasticity, an enterobacterial bacterial genome is composed of a conserved ”core“ genome, which contains the genetic information that is required for essential cellular functions and a ”flexible“ gene pool, which encodes additional traits that can be beneficial under certain growth conditions (Dobrindt *et al.*, 2004). This latter one also includes genetic information that codes for disease-related factors in pathogenic bacteria that are usually absent from non-pathogenic species. This flexible gene pool consists of, e.g. genomic islands (GEIs) and pathogenicity islands (PAIs) as well as of plasmids, bacteriophages and accessorial DNA elements such as IS elements and transposons (Dobrindt and Hacker, 2001).

1.1 Pathogenicity islands and their contribution to genome plasticity of UPEC strain 536

Spontaneous loss of the haemolytic activity of UPEC strain 536 due to deletion of two large alpha-haemolysin encoding genomic regions was the first observation that led to the concept of pathogenicity islands. It was the first clear indication that virulence-associated genes can be deleted from the core chromosome, resulting in attenuation of a bacterial pathogen (Blum *et al*., 1994). Since these first experiments, four PAIs of strain 536 have been identified and characterised in detail. The four PAIs of strain 536 exhibit typical features of PAIs (Kaper and

Hacker, 1999). They carry least one virulence-associated gene, they have a different G+C content relative to that of the core chromosome, they are associated with a tRNA gene, they carry kryptic or functional "mobility" genes and phage sequences, and they have a typical mosaic-like structure indicative of multiple genetic rearrangements.

With the help of suppressive subtractive hybridisation (SSH) analysis of strain 536 and the K-12 strain MG1655, the genome of this UPEC isolate was screened for additional DNA fragments absent from that of *E. coli* K-12 strain MG1655 representing so far uncharacterised PAIs that could be important during pathogenesis This analysis resulted in the identification of a DNA fragment that exhibited homology to the gene coding for the capsule transport protein KpsC of the K10 capsule (Janke *et al*., 2001). The *E. coli* strain 536-specific K15 capsule-encoding gene cluster was unknown at that time, but it was clear from the literature that *kps* loci were usually chromosomally integrated downstream of the tRNA genes *pheV* and *serA* (Orskov and Nyman, 1974; Orskov *et al.*, 1976; Swenson *et al.*, 1996). Furthermore, more and more data accumulated suggesting that *pheV* and the highly homologous *pheU* gene are frequently associated with PAIs. Examples for *pheV*-associated PAIs are the *she* PAI of *S. flexneri*, the LEE PAI of the REPEC strain 84/110-1, PAI I of UPEC strain J96 and a putative PAI of an EHEC O157:H7 strain (Tauschek, 2002; Swenson *et al.*, 1996). *pheU* has been shown to be the chromosomal integration site of the LEE islands of two REPEC strains (RDEC-1 and 83/39) and PAI II of UPEC strain J96 (Al Hasani, 2001; Tauschek, 2002; Swenson *et al.*, 1996). Another PAI which carries the *afa-8* gene cluster in *E. coli* strain XXX was associated both with *pheV* and *pheU* (Lalioui and Le Bouguenec, 2001). These findings led to the conclusion that analysis of the chromosomal localisation and the flanking sequence context of the $kpsC_{K10}$–specific SSH fragment will result in the characterisation of a so far uncharacterized PAI (PAI V) of strain 536.

Analysis of the sequence data obtained revealed a mosaic-like structure of the composite *pheV*-associated PAI V_{536}. The genetic structure of PAI V_{536} is typical for many PAIs and results from the insertion and deletion of DNA fragments as well as from other genetic rearrangements driven by homologous and site-specific recombinations. These mechanisms are involved in the evolution of genomic regions of the flexible gene pool: genetic information that may be beneficial under certain growth conditions can be acquired and stabilised within the chromosome or others which are not important can be deleted. Comparison of PAI V_{536} with the *pheV*- and

pheU-associated islands of *E. coli* strain CFT073 revealed that these islands are partially similar. The acquisition of a functional phosphoglycerate transport system (*pgt*) on PAI V_{536} appears to have occurred via horizontal gene transfer. Its broad distribution among the different extraintestinal *E. coli* isolates (Appendix 5) suggests that the phosphoglycerate transport sytem may play role in the pathogenic process. We do not know much about the chromosomal localisation of the *pgt* clusters of the other strains of the IMIB strain collection, but the same *pgt* gene cluster as in strain 536 is also present in strain CFT073. In this strain, the *pgt* gene cluster is not located on the *pheV*- but on the *pheU*–associated island and is also flanked by mobile genetic elements, an R6 transposase gene and an IS2 element. Thus, it could be speculated that the phosphoglycerate transport system gene cluster could be deleted or integrated into the chromosome with the help of IS elements. This is supported by the fact that on PAI V_{536} this cluster is flanked by an R6 transposase gene and a truncated IS*2* element that may have been involved in its transfer.

It seems that IS*2* elements served as a target site for integration of the *pgt* gene cluster into PAI V of strain 536. This hypothesis is based on the fact that this cluster is not flanked by IS*2* remnants in the *pheU* island of strain CFT073, whereas this IS element is localised in the corresponding region of the *pheV*–associated islands of UPEC strain CFT073 and probiotic *E. coli* strain Nissle 1917 (GEI $II_{Nissle\ 191}$). These latter two islands do not carry remnants of a *pgt* gene cluster. Interestingly, in case of GEI $II_{Nissle1917}$, IS*2* sequences are involved in deletion of a chromosomal region (IS*2*-*iuc*-*sat*-ORFs-*iha*-IS*2*) flanked by them (L. Grozdanov, PhD thesis, Würzburg 2004).

Downstream of the phosphoglycerate transport system-encoding gene cluster, a putative autotransporter gene (*sap*) was localised. Similar, but not identical genes are also located at the same chromosomal site on GEI $II_{Nissle\ 1917}$ and on the *pheV* island of strain CFT073. These genes code for an antigen 43-homologue. Another variant of this autotransporter gene was localised on the chromosome of strain 536 which is encoded on PAI III_{536}. It is well known that Ag43 expression is important for biofilm formation of *E. coli* (Danese *et al*., 2000; Kjaergaard *et al*., 2000; K.Michaelis and U. Dobrindt, personal communication). The two Agn43 variants of strain 536 are similar and probably closely related. Their role and function requires further analysis but they represent a good example of genome plasticity: The fact that these autotransporter genes are always located on GEIs/PAIs or plasmids rather implies that these genes have been acquired by horizontal gene transfer. Even in K-12, the *agn*43 gene is part of a prophage. Multiple copies of

this gene can be the result of spontaneous chromosomal duplications that are common in populations of *E. coli* and *Salmonella typhimurium*, and under the selective pressure of laboratory conditions their frequency is in the range 10^{-2} to 10^{-4} (Haack and Roth, 1995).

These three *pheV*-associated islands show another striking similarity: Downstream of the truncated copy of *pheV* (*'pheV*) the capsule determinant is localised. From this and from the fact that these islands carry the same integrase gene it can be concluded that PAI V_{536} and the *pheV*-associated islands of strains CFT073 and Nissle 1917 might be considered as members of one and the same family of *pheV*-associated capsule-encoding islands of *E. coli*. The common origin could further be supported by the fact that in PAI V_{536} the *pix* fimbrial gene cluster (Lügering *et al*., 2003) is localised nearly 4 kb downstream of the *pheV* tRNA gene. Similarly, a complete *pap* fimbrial gene cluster is present in the same sequence context of the *pheV* island of strain CFT073. Interestingly, only remnants of the *pap* operon are present in the corresponding DNA context in GEI $II_{Nissle\ 1917}$ (Grozdanov *et al.*, 2004). Instead of the 30-kb *hly/pap* region present in strain CFT073, only a fragment of *papA* (*papA'*) and the intact *papI* gene exist in the corresponding region of GEI $II_{Nissle1917}$. The presence of the P-fimbriae family-encoding gene clusters implies that these three *pheV*-associated islands have a common ancestor. It could be speculated that the fimbrial cluster remained intact and functional in strain CFT073 but was almost completely deleted from GEI $II_{Nissle1917}$. Nevertheless, in strain 536 the situation is not so simple because the *pix* fimbriae-encoding gene cluster does not show significant homology to the *pap* fimbrial cluster of the *pheV* island of CFT073. Despite similarity of the genetic organisation between *pix* and *pap* (Figure 10), they show no significant homology to each other at the nucleotide level. Similarly, on the protein level, only the chaperone and usher proteins exhibit some similarity to the corresponding PapC and PapD proteins. We can hypothezise that the *pix* fimbrial cluster has been acquired and inserted later into the *pheV* island of strain 536 thereby replacing a pre-existing *pap* fimbrial gene cluster. Another option could be that *pap* and *pix* gene clusters are located on different mobile genetic elements and have been independently inserted into a pre-existing *pheV* PAI ancestor. Comparison of this region on the different *pheV*-associated PAIs revealed that in case of strain CFT073 upstream of the *pap* fimbrial cluster an alpha-hemolysin determinant is present, which is completely missing from the corresponding region of GEI $II_{Nissle\ 1917}$ and PAI V_{536}. On PAI V_{536} only remnants of the upstream region of the alpha-hemolysin operon can be detected.

These data demonstrate that horisontal gene transfer and genetic rearrangements catalysed by homologous and site-specific recombination can result in acquisition and/or deletion of genetic information thus contributing to the generation of different variants of *pheV* and *pheU*-associated islands in pathogenic and non-pathogenic *E. coli*.

Horisontal gene transfer includes a variety of processes by which bacterial genomes can be changed rapidly. These dramatic changes are those key factors that keep bacterial evolution in motion and that lead to 'evolution in quantum leaps' (Hacker and Carniel, 2001). By incorporating of transferred genetic elements directly into the genome of another organism, a 'genomic island' can be formed whose functions can increase bacterial fitness either directly or undirectly. The so called 'fitness islands' can be divided into several subtypes: 'ecological islands' in environmental bacteria and 'saprophytic islands', 'symbiosis islands' or 'pathogenicity islands' (PAIs) in microorganisms that interact with living hosts. Differences in the structure among the similar islands (islands with common origin) can also be observed that represents quite well that those genes that are not necessary for a certain pathotype are deleted while in the meantime new genes or entire locuses can be aquired by horisontal transfer. This leads to phenotypic (virulence) variations.

1.2 Evolution of capsule gene clusters

For the species *E. coli* alone, more than 80 chemically different capsular polysaccharides or K antigens have already been described (Ørskov, 1992). On the basis of chemical structure and gene organisation bacterial capsules can be divided into four groups (Whitfield, 1999).

Gene duplication is considered to be the first step during evolution of group 1 and group 4 capsules. Their genetic organisation and chromosome localisation suggests that these capsule gene clusters originated from the duplication and modification of the *rfb* gene cluster, which is responsible for the synthesis of the lipopolysaccharide (LPS). This hypothesis is supported by the analysis of the K30 capsule locus, one of the most studied archetypal group 1 gene clusters. This capsule locus is 16 kb in size and comprises 12 open reading frames. The first four genes (*orfX, wza, wzb, wzc*) of this polycistronic operon are highly conserved among different group 1 capsule gene clusters (Rahn, 1999) and have been designated as ”translocation-surface assembly“ region. Although the precise role of *orfX* remains unclear but the other components are thought to encode an outer membrane lipoprotein, a cytoplasmic phosphatase and an ATP binding protein,

respectively (Drummelsmith and Whitfield; 1999). Similar genes have been described in diverse systems including *E. coli* strain K-12 (*wza, wzb, wzc*) (Stevenson, 1996), *Klebsiella* K2 (*orf4, orf5, orf6*) (Arakawa, 1995), and *Erwinia amylowora* (*amsH, amsI, amsA*) (Bugert, 1995). According to the available data, biosynthesis and transport of group 1 capsule components to the cell surface is summarised in the current model: Individual repeat units are assembled on a carrier lipid (undecaprenyl phospate) by the sequential activities of glycosyltransferases. The initial step requires the enzyme WbaP (Drummelsmith, 2000) which is a member of an UDP-hexose family. Then, these repeat units are transported by the Wzx protein to the periplasmic face of the cytoplasma membrane. They are assembled by the Wzy protein, which catalyses the polymerisation step at the periplasmic site of the cytoplasma membrane. Translocation and surface assembly is carried out by the outer membrane proteins Wza, Wzb and Wzc (Whitfield, 1999). In case of the group 4 capsule prototypes K40 and O111 (Amor, 1997; Wang, 1998), capsule synthesis also involves the Wzy-dependent pathways. Group 1 and 4 capsule polymerisation differs only in the initiating glycosyltransferase which is WecA in case of group 4 capsules. This enzyme is able to transfer either GlcNAc-1-P or GalNAc-1-P to undecaprenyl phosphate. This step is common for group 4 capsules and all studied *E. coli* LPS O antigens (Valvano, 1994). Beside the genetic similarity, this functional similarity among group 1 and 4 capsule determinants and the LPS gene clusters underlines their common origin. Beside the similarities, functional differences can be observed between regulation of the polysaccharide chain length of LPS and group 1 and 4 capsules. LPS side chain length is controlled by the chain-length determining protein Wzz. This enzyme controls the Wzy-dependent chain elongation step with the ligase WaaL (Whitfield, 1997) which transfers the synthetised polymer (or oligosaccharide) from the lipid intermediate onto the lipid A-core acceptor (Heinrichs, 1998). It has been confirmed that this system does not function in case of group 1 and 4 capsules. In group 1 gene clusters, the *wzz* gene is completely deleted whereas *wzz* exists in group 4 determinants but has evidently no effect on chain elongation (Dodgson, 1996; Amor, 1997; Drummelsmith, 1999). Accordingly, it seems to be clear that there is an alternative polymerisation control for group 1 and 4 capsular antigens. This is presumably due to the fact that ligation to lipid A-core is not required for capsule expression on the cell surface (MacLachlan, 1993; Krallmann-Wenzel, 1994). This probably was another important evolutionary step for capsule biosynthesis gene clusters: after gene duplication capsule biosynthesis gains independence from LPS synthesis and turns in a new direction that is different from that of the ancestral mechanisms.

Cloning and analysis of a large number of *E. coli* group 2 and 3 capsule gene clusters has revealed that they represent another alternative route for extracellular polysaccharide synthesis and transport. Their genetic organisation and localisation in the core chromosome is completely different from those of group 1 and 4 capsules. Most of the known group 2 and 3 capsule gene clusters are inserted at 64 minutes on the *E. coli* K-12 chromosome and are associated with the tRNA genes *serA* (K10, K54) and *pheV* (Ørskov, 1974; Ørskov, 1976; Vimr, 1991) (K5, K1, K15). This is far away from other sugar biosynthesis gene clusters (at 45 min on the *E. coli* chromosome (Berlyn, 1998)) like *rfb* (for LPS) and *cps* (for colanic acid capsule). The genetic structure of group 2 and 3 capsule determinants shows no similarity to that of the LPS gene cluster. Sequence analysis of different group 2 and 3 capsule clusters revealed their typical organisation: Two conserved regions (region 1 and region 3) flank a highly variable region (region 2). The genes of the conserved regions code for enzymes required for the transport of the capsule components through the cytoplasma membrane while the genes of the highly variable region 2 encode enzymes involved in the polymerisation of the polysaccharide and, where necessary, for the biosynthesis of the specific monosaccharide components of the polysaccharide (Roberts, 1996). The size variation of region 2 among different group 2 capsule types is indicated in Figure 33.

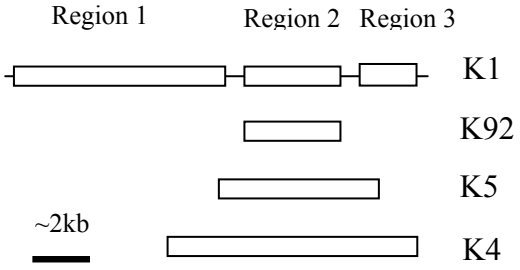


Fig. 33: Schematic representation of the size variation of region 2 of different group 2 capsule types (Whitfield and Roberts, 1999).

Whereas some ORFs of region 2 of different capsule types show homology on the nucleotide level to each other, others are completely different. In region 2 of the K15 capsule determinant of strain 536, we identified five ORFs that exhibited no homology to any known gene on the nucleotide level. The deduced amino acid sequences showed weak homology to sugar transferases like mannosyl- and glycosyltransferase. Analysis of the different capsule loci aims at

the answers to two main questions: (i) where does this high variability of the region 2 genes come from, and (ii) what is the mechanism by which a new region 2 variant can be acquired.
The analysis of the different region 2 sequences of representatives of different group 2 and 3 capsule gene clusters revealed, that the G+C content of this region is always lower than that of the two conserved flanking regions 1 and 3. This indicates that these genes have been acquired by horizontal gene transfer (Roberts, 1995).
IS element-dependent deletion or integration is one way by which genes can be lost or acquired. If so, IS elements or remnants thereof should be present in the different capsule loci in the neighbourhood of region 2 genes. Studies to find evidence for the presence of such accessory genetic elements in different capsule loci were performed but were not successful in Gram-negative bacteria (Roberts, 1996). However, acquisition of new capsule gene clusters by site-specific transposition was more thoroughly investigated in *Streptococcus pneumoniae*. Analysis of the capsule gene clusters among *S. pneumoniae* strains supports an IS element-mediated recombination event that may play a role in the transmission of capsule genes between *S. pneumoniae* and other encapsulated pathogens (Caimano *et al.*, 1998).

In case of UPEC strain 536, analysis of the flanking sequences of region 2 has revealed four A+T rich repetitive sequences (between *orf68-69* and *orf73-74*) that were 40-55 bases long and were very similar to the sequences also identified up- and downstream of region 2 within the K5 capsule determinant (Simpson *et al.*, 1996; Rowe *et al.*, 2000). It is known that after their deletion from the chromosome certain transposons leave their “footprint” at the chromosomal site from which it was deleted. These sequences are usually repeat sequences (Roeder and Fink, 1980). The identified four A+T rich sequences upstream and downstream of region 2 of the K15 capsule gene cluster could be due to such genetic rearrangements in the capsule locus of the UPEC strain 536. Homologous recombination could have been another mechanism involved in the acquisition of the K15-specific region 2. In general, group 2 and 3 capsule determinants contain conserved DNA stretches, i.e. region 1 and region 3 that flank the serotype-specific region 2. Accordingly, because of their conservative sequence, region 1 and 3 are good target sites for homologous recombination between different group 2 and 3 capsule determinants. With their help acquisition of new sugarbiosynthetic genes (region 2) can happen relatively easily. This hypothesis is further supported by the analysis of the C-termini of different KpsT and KpsS proteins of group 2 capsules. They show a marked divergence among different capsule types (Drake, 1991; Pavelka,

1994; Petit, 1995) that means that closer to the region 2 genes there is a higher variability of the C termini of region 1 and 3 has.

The mixed genetic structure of the K15 capsule locus of strain 536 is reminiscent of genetic rearrangements and lateral gene transfer. The G+C content of region 2 is markedly lower than that of the conserved flanking regions, and the presence of repetitive up- and downstream

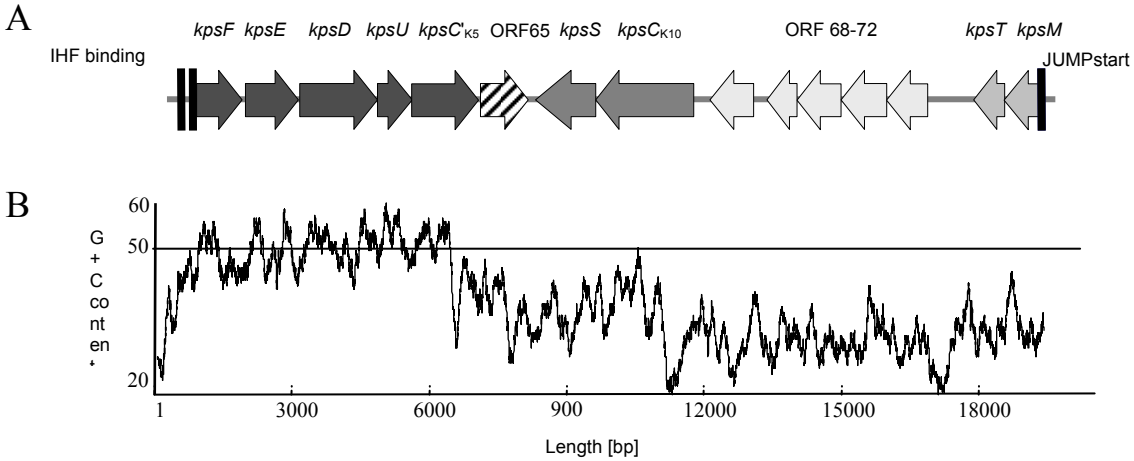


Fig. 34: Genetic organization of the K15 capsule determinant shows the traces of genetic rearrangements. (A) Regions 1 to 3 of the *kps*K15 gene cluster are indicated by different shades of gray. The IS1230B transposase gene is indicated by a hatched arrow. (B) Percent G+C plot of the *kps*K15 gene cluster indicative of the segmental structure of this capsule locus representing DNA regions from different sources. The average percent G+C content of the *E. coli* chromosomal backbone (50.4%) is indicated by a horizontal line.

sequences also suggests that genetic rearrangements occurred during evolution. In the case of the K15 capsule locus an IS element-mediated genetic rearangement occured resulting in the fusion of a group 2 capsule determinant with that of a group 3 capsule type. According to the DNA sequence, it appears that IS*1230B* played a role in this process. As a consequence of the IS*1230B* integration into region 1 of a group 2 capsule gene cluster, the last gene (*kpsS*) of region 1 was deleted and the upstream located *kpsC* gene was truncated. Consequently, the IS*1230B* insertion would have destroyed the ability to express extracellular K15 capsule as KpsC and KpsS are necessary for the translocation of capsule components through the cytoplasmic membrane. Lack of these proteins results in the accumulation of capsule components in the cytoplasm. The KpsC and KpsS proteins seem to be involved in the attachment of KDO (2 keto-3-deoxy-octulosanic acid) to the phospholipid and the subsequent ligation of the polysaccharide to the phosphatidyl-KDO residue prior to its export across the cytoplasmic membrane catalyzed by KpsM and KpsT

(Bronner, 1993; Roberts, 1995). It appears that without these proteins there is no connection between capsule biosynthesis and the ABC transporter (KpsM and T)-mediated translocation through the inner membrane. However, in the K15 capsule-expressing strain 536, a complete *kpsS* and *kpsC* gene which exhibit homology to those of the K10 capsule type (a member of group 3 capsules) are located downstream of the *IS1230B* remnant. We demonstrated that these two genes compensate for the truncated *kpsC* and the deleted *kpsS* gene of the group 2 capsule-specific region 1. From unpublished experimental results of Clarke and Roberts it was already clear that KpsC and KpsS are homologous among group 2 and 3 capsules and that they are functionally interchangeable (Whitfield and Roberts, 1999).

IS element-mediated rearrangements resulting in inactivation of a functional *kps* gene have been described for the K10 capsule determinant of strain D1114 (O25:K10:H16): upstream of the functional *kpsD* gene a truncated *kpsM* was identified (Clarke *et al.*, 1999) (Fig. 35). This

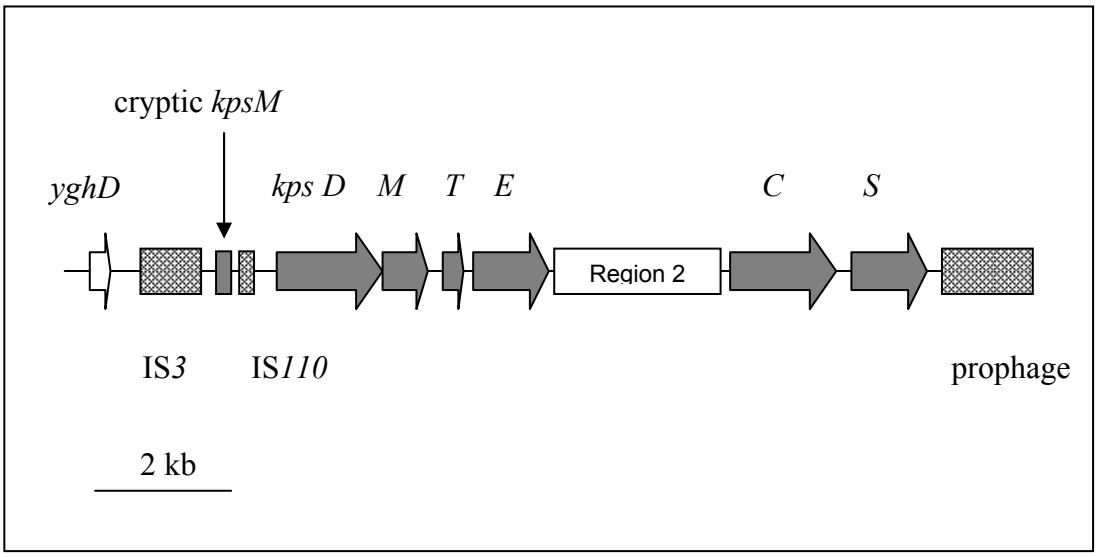


Fig. 35: Traces of IS element-mediated rearrangements in the capsule locus of the *E. coli* strain D1114 was revealed. In this strain, that is one representative of the capsule type K10, upstream of the functional *kps* locus two IS elements and between them a truncated *kpsM* gene were localised.

truncated *kpsM* gene represents a group 2 variant of the functional *kpsM* gene of the K10 capsule determinant that is localted downstream of *kpsD*. Between the truncated *kpsM* gene and the functional *kpsD* gene the 5'-end of a truncated IS*3* element was identified. In the upstream region of the K10 capsule locus remnants another IS element (IS*110*) were found. IS*110*-specific sequences have also been identified next to the K54 capsule determinant, which belongs to group

3 capsules. The presence of the IS*110* element in both, group 2 and 3 capsule gene clusters suggests that this IS element may have played a role in the acquisition of an ancestral group 3 capsule gene cluster. The detection of IS*3* remnants in region 1 of the K10 capsule gene cluster, but not in that of the K54-specific genes, indicate that the latter IS element has been involved in the events specific for evolution of the K10 capsule gene cluster (Clarke *et al.,* 1999). The examples of K10 and K15 capsule loci where truncated IS elements and truncated homologous genes are both present mirror steps involved in evolution of bacterial capsule gene clusters in which both site-specific as well as homologous recombination events occurred.

1.3 Distribution of DNA sequences homologous to PAI V_{536} among isolates from different origin

In order to study the distribution of PAI V_{536}-specific sequences among 137 non-pathogenic and pathogenic *E. coli* isolates as well as to find out whether an identical repertoir of genetic information can be detected in other *E. coli* strains, PCR reactions were designed for the amplification of selected regions of PAI V_{536} including the *pixC*, *pgtC* and *gspD* genes as well as parts of the region 1 to 3 of the kps_{K15} determinant (see Figure 8 and 9). The results obtained (see Appendix A/5) demonstrate that generally PAI V_{536}-specific sequences were more frequently distributed among ExPEC and, to a lesser extent, in commensal isolates than in diarrheagenic isolates. More than 50 % of the ExPEC strains tested carried $kpsC_{K5}$ indicative of group 2 capsule determinants whereas $kpsC_{K10}$ indicative of DNA regions of group 3 capsule-encoding gene clusters has been rarely detected among the strains included into this study. The general secretion pathway determinant, although more frequently distributed among ExPEC strains, was also detectable in at least 50 % of the diarrheagenic and commensal isolates. These findings underline that PAI V_{536}-associated DNA regions are also detectable in commensal isolates indicating that the commensal flora represents the natural reservoir for extraintestinal pathogenic *E. coli* strains and that horizontal gene transfer plays an important role for the spread of pathogenicity islands.

2. Characterization of virulence associated genes located on PAI V_{536}

For a successful urinary tract infection, bacteria should express virulence factors like adhesins, toxins, iron uptake sytems and LPS. These are the most important and best studied virulence factors that were also described for UPEC strain 536. The majority of these virulence factors are encoded on PAIs.

A major feature of uropathogenic *E. coli* is the expression of capsules (Johnson, 1991; Jann, 1992). More than 80 capsule types have been described so far for *E. coli*, but only few of them are typically expressed by uropathogenic strains. The realisation that (i) certain capsule types (K1, K2, K5, K6, K12, K13, K29 and K51) are typical for uropathogenic strains, (ii) most of these capsule types are the members of group 2 capsules and (iii) in fecal isolates the presence of group 2 capsules is not as prevalent as in uropathogenic strains (Kaiser, 1977; Sandberg, 1988) led to the speculation that group 2 capsules contribute to urovirulence by means of their ability to impede phagocytosis (Timmis, 1985; Cross, 1986) and confer resistance to the bactericidal activity of serum (Cross, 1986; Allen, 1987; Leying, 1990; Russo, 1993). Furthermore, a signature-tagged mutagenesis screen has previously suggested the role of the K2 capsule of UPEC strain CFT073 in UTI (Bahrani-Mougeot *et al.*, 2002). However, other findings do not support a role of group 2 capsules as a virulence factor of uropathogenic *E. coli* and favour a role rather in systemic infections than in an ascending UTI (Russo *et al.*, 1993, 1994, 1996). These data from *in vivo* experiments suggest that LPS directly contributes to the development of UTI (Taylor, 1976). Several *E. coli* O serogroups are associated with UTIs (O1, O2, O4, O6, O7, O8, O16, O18, O25, O50 and O75) (Johnson, 1991). Nevertheless, the monocyte phagocytosis killing assays with capsule and LPS mutants of an O75:K5 UPEC strain suggested a more differentiated phenomenon: they demonstrated that the K5 capsule prevents bacterial attachment to phagocytic cells and that both, the O75 and K5, antigens confer protection from internalization (Moxon, 1990).

As not all K or O antigens may have identical biological functions, we have investigated whether the K15 capsule plays a role for uropathogensis. *In vivo* virulence assays with the capsule deficient mutants of UPEC strain 536 in an ascending urinary tract infection model revealed that loss of capsule drastically attenuates the virulent strain. As already suggested by others, a capsule may protect the bacteria by masking the underlying opsonized surface and serving as a physical barrier to the phagocytic cells like polymorphonuclear leukocytes and monocytes (Cross, 1990; Timmis, 1985; Horwitz, 1982; Moxon, 1990). Furthermore, we found out that capsule-deficient mutants were still serum resistant. It is worth to mention that one region 2 mutant of strain 536, that was constructed with the help of suicide vector pCVD442, was serum sensitive and showed a reduced expression of long chain LPS as well as a striking difference in the structure of the LPS core region (Figure 22). This mutant's LPS core exhibited similarity to those of the K-12 strain MG1655 and the rough *rfaH* mutant of strain 536. From these results we concluded that the

expression of the sugar biosynthetic genes may interfere with that of the O6 LPS expression. This idea was also supported by the fact that ORF71 of PAI V_{536} exhibits homology at the protein level to a putative lipopolysaccharide core biosynthesis protein of *Pseudomonas putida* strain (AE016492 / NP_745150). However, this mutant could not successfully be complemented. Possible other integration sites of suicide plasmid-derived sequences, e.g. in the LPS gene cluster, could not be detected by Southern blot analysis and overlapping PCRs. We can only conclude that during mutagenesis probably other mutations occured in this mutant that interfered with other chromosomal regions that are important for regulation of LPS- or sugar biosynthesis. This hypothesis can be supported by the fact that a point mutation within *wzy* gene is responsible for the semi-rough phenotype of strain Nissle 1917 (Grozdanov, 2003). This point mutation results in a defective O6 LPS side chain expression and in serum sensitivity. Consequently, we constructed another kps_{K15} region 2 mutant using the method described by Datsenko and Wanner (2002). This mutant, *E. coli* strain 536ΔR2-2, exhibited serum resistance and the same LPS side chain pattern as the wild type strain 536 (Figure 22). Also to clear up this question a complete kps_{K15} deletion mutant (*E. coli* strain 536ΔK15) was also constructed. This strain was serum resistant as well. From the above mentioned results we can conclude that the K15 capsule is essential for uropathogenesis, but does not play any role in resistance to human serum. This is in accordance with results published before (Burns, 1998).

The *pix* gene cluster on PAI V_{536} was found to be incomplete. Compared to the structurally related *pap* operon, the negative regulatory gene *pixB* of the *pix* gene cluster is completely present and apparently transcribed at 37 ºC, whereas a corresponding positive regulatory gene *pixI* and upstream sequences that also play a role in regulation are absent. Transcriptional analysis of the *pixC* gene showed that the structural genes of this cluster are not transcribed under the conditions tested (Figure 11). We think that this cluster is permanently downregulated due to *pixB* expression as the positive regulator gene *pixI* is missing.
Nevertheless, cloning of the truncated *pix* cluster of UPEC strain X2194 resulted in fimbriae expression in K-12 strain DH5α (Lügering *et al*., 2003). This could be an artifact resulting from the use of *Sau3A* digested DNA that the authors have used to clone the *pix* determinant. The *Sau3A*-DNA fragment contains only a truncated *pixB* gene that should be inactive. Accordingly, the inactivated negative regulator PixB could not repress tanscription of the *pix* structural genes and eventually Pix fimbriae expression was detected by electron microscopy. Because of the

truncated regulatory sequences upstream of the *pix* structural genes, the authors hypothesized that a fragmented R6 transposase gene somehow regulates this cluster as was demonstrated with *lacZ* fusions. Supporting this, they reported that deletion of the upstream region including the truncated R6 transposase gene drastically lowered transcription of the cloned *pix* operon. They showed that transcription of the cloned *pix* determinant seemed to be upregulated at 37 ºC and was downregulated already at 26 ºC (Lügering *et al*., 2003). This correlates with the observation that transcriptional regulation of pili expression can be modulated by environmental factors such as temperature (Uhlin, 1994). Further indication that pix_{536} is really inactive resulted from the characterisation of double mutants of strain 536 that lack the *prf* and *sfa* operons and in which no fimbriae expression under laboratory conditions was observed by electron microsocopy (Dobrindt, personal communication).

On the other hand, the presence of a non-functional *pix* operon of strain 536 and a functional one in UPEC strain X2194 (O2:K-:H6) could be explained by the fact that some slight differences between the two *pix* cluster sequences can be observed. These slight differences may cause different transcriptional activities and eventually fimbrial expression. It is important to mention, that Schmidt and co-workers demonstrated that the cloned, truncated fimbrial determinant was expressed in strain DH5α (Lügering *et al*., 2003). It was not reported whether the UPEC strain X2194 itself expressed this fimbrial gene cluster. It was also not mentioned whether Pix fimbriae were involved in uropathogenesis or whether this strain also expresses other fimbrial gene clusters. Furthermore, the authors mentioned that binding and inhibition studies indicated that Pix pili apparently recognize surface antigens which are distinct from the known UTI *E. coli* adhesin receptors, which may contradict the role of *pix* in the uropathogenic process. The very low distribution of the *pix* fimbrial cluster among UTI isolates (see Appendix A/5) also supports this hypothesis. On the other hand we can not rule out that Pix could recognize so far unknown receptors on uroepithelial cells and may represent another alternative adhesion that contributes to uroepithelial colonisation. Interestingly, the presence of this fimbrial determinant was not only rare among UTI isolates but also among other non-fecal *E. coli* isolates.

In strain 536, three other fimbrial gene clusters are known. In addition to the *fim* gene cluster located on the core chromosome, one pyelonephritis-associated fimbrial cluster (*prf, papG* allele III) is located on PAI II_{536}. The third one is located on PAI III_{536} and codes for S-fimbriae (*sfa*). Additionally, two putative fimbrial gene clusters are located on PAI I_{536} (Dobrindt *et al.*, 2002).

The *mat* fimbrial gene cluster described to be expressed by *E. coli* O18:K1 isolates (Pouttu *et al.*, 2001) is present, but silent in strain 536. It could be speculated that because of the presence of several functional fimbrial gene clusters, strain 536 can afford to possess a non-functional *pix* fimbrial gene cluster. This is an important aspect of genome plasticity implying that strain 536 originally possessed several fimbrial gene clusters and that the most important one were selected while others, including the *pix* operon, became inactivated.

3. Mobilisation of PAI II_{536}

An increasing number of studies of genes and genomes indicate that a considerable amount of horizontal gene transfer (HGT) has occurred between prokaryotes. The comparison of complete prokaryotic genomes revealed that HGT has significantly contributed to bacterial evolution.

By accumulating a variety of fitness or virulence factors over long time periods, different pathotypes and pathogens have evolved from non-pathogenic strains. Virulence associated genes are mostly carried on plasmids, pathogenicity islands (PAIs) and bacteriophages (Hacker and Kaper, 2000).

Discovery of transferable drug resistance R factor plasmids and their role in the spread of the antibiotic resistance genes have underlined the importance of horizontal gene transfer. Since then a number of conjugative plasmids have been isolated and characterised that beside resistance marker(s) encode proteins that mediate transfer from a donor cell to a recipient cell. Furthermore, novel genetic elements were found that also carried resistance genes but were integrated into the chromosome. These elements excise from and integrate into the chromosome similarly to bacteriophages, are transferred by conjugation similarly to plasmids, and have been described in various bacteria (overview in: Burrus *et al.*, 2002). Traces of such elements have also been found in large unstable chromosomal regions that have been called pathogenicity islands. Beside virulence associated genes, PAIs typically carry mobility genes such as phage related integrases, transposases or part of insertion sequence (IS) elements. It has been hypothesised that these sequences have played a role in the acquisition of PAIs. Based on the current knowledge, two main theories have been proposed how PAIs have evolved. The role of bacteriophages in the evolution and transfer of bacterial virulence determinants was discussed by Cheetham and Katz (1995). A close functional association between PAIs and bacteriophages were reported for Gram-negative *Vibrio cholerae* as well as for Gram-positive *Staphylococcus aureus* where bacteriophage mediated transfer of PAIs between bacterial isolates were shown. In *V. cholerae*

transfer of the entire 39.5 kb Vibrio Pathogenicity Island (VPI) by the general transducing phage CP-T1 was reported (O'Shea *et al*., 2002). In *S. aureus* the 15.2 kb SapI1 element, which encodes the toxic shock syndrome toxin requires the helper bacteriophage 80α to excise, replicate and to be transduced to recipient strains at very high frequencies (Lindsay *et al*., 1998; Boyd *et al.*, 2001). However, it has been argued that the 15.2 kb SaPl1 element does not entirely conform to the definition of a PAI and may rather represent a defective phage that requires a helper phage similar to the P2/P4 phage interaction (Boyd *et al*., 2001). Bacteriophage-mediated transfer is limited by the amount of DNA that can be packed into the phage capsid, but in some cases it can expand beyond 100 kb (Ochman, 2000).

Beside transduction the other possible mechanism of acquisition of PAIs is by bacterial conjugation. This process requires a close cell-to-cell contact. Mating pair formation (Mpf) (Willetts, 1981) is essential for the transmission of genetic material and it has been shown that part of the chromosome (Hfr strains), conjugative plasmids (e.g. RP4) or in the presence of a helper plasmid other mobilisable plasmids (e.g. RSF1010) can be transmitted to recipient strains by this mechanism.

Deletion of PAIs from the core chromosome was already described in the early eighties when the formation of spontanous non-haemolytic uropathogenic *E. coli* 536 mutants was observed (Hacker *et al.*, 1983). This led to the discovery of PAIs and since then similar elements have been described in several bacterial species (see also introduction).

The presence of direct repeat sequences (DRs) at the border of PAIs was found to be important for the instability of PAIs. Additionally, the presence and activity of integrase genes are necessary for the deletion from the core chromosome. The UPEC strain 536 carries at least five PAIs in its chromosome. Their stability was characterised in detail and showed differences (Middendorf *et al.*, 2004). Furthermore, the deletion rates of PAI II_{536} and PAI III_{536} are influenced by certain environmental conditions such as temperature and salt concentration.

The deletion rate of the newly characterised PAI V_{536} was also determined and was found to be relatively low ($1x10^{-6}$) (Middendorf *et al*., 2004).

Comparable to the excision mechanism of bacteriophages, PAIs are thought to exist at least transiently as circular intermediates after excision from the chromosome. Such extrachromosomal forms have been detected for PAI II_{536} and PAI III_{536} (Middendorf *et al.*, 2004). It can be speculated that in the presence of a helper mechanism (for example a conjugative plasmid) the

deletion process could proceed and the circular intermediate could be transferred into a recipient strain. In our work, we have verified this hypothesis by constructing a derivative of PAI II_{536} that fulfils the criteria of a possible conjugative element. For this purpose PAI II_{536} was chosen for several reasons: (i) it shows a high deletion rate at 20ºC (~10^{-5}), (ii) its deletion by site-specific recombination always shows the same pattern, (iii) a circular intermediate could be detected and (iv) it has a well detectable marker.

It was also evident from the sequence of PAI II_{536} that similarly to other known PAIs and in contrast to conjugative transposons, a mobilisation machinery is not encoded on the island. Therefore, we introduced into PAI II_{536} a plasmid that helps the deleted and circularised PAI to replicate and to be mobilised by the self-transmissible IncPα plasmid RP4 (Pansegrau, 1994). This 60-kb plasmid has been known for its extremely broad host range and is stably maintained in all of the tested Gram-negative bacteria. Furthermore, RP4 has served as a widely used model system for the study of plasmid biology in general and in conjugation. It was shown that the mobilisable IncQ plasmid RSF1010 is efficiently transmitted in the presence of IncP plasmids. RSF1010 only encodes relaxosomal components and its transfer relies on an Mpf system from conjugative plasmids. A relaxosome is formed by the gene products TraH, TraI, TraJ and TraK at the transfer origin (*oriT*) (Fürste, 1998; Pansegrau, 1996). These four proteins are the products of the genes of the Tra1 region which beside Tra2 encodes the essential transfer functions of RP4. Tra1 region mainly codes DNA processing functions for generation of the single stranded DNA, which is transferred to the recipient cell (Pansegrau, 1996). DNA processing functions are also present on the suicide plasmid pGP704 that has been used for the mobilisation experiments. At the *oriT* site that was introduced into PAI II, a nick could be formed as the starting step of the conjugative process. Single stranded DNA was then transferred into the recipient strain through the membrane-spanning transfer apparatus that was encoded on the RP4 plasmid.

With this construct we were able to detect the transfer of a complete pathogenicity island. However, the frequency of gene transfer with this construct was very low (10^{-8} and 10^{-9}), even though we introduced the *pir* gene into the donor strain (*E. coli* 536). The *pir* encoded π-protein is necessary for the function of the introduced $oriV_{R6K}$ that should enable the deleted PAI II_{536} to replicate as a plasmid in the cell and thereby increasing the chance of a possible PAI transfer.

When we started the mobilisation experiments with this relatively artificial construct, there were no data available about a possible PAI transfer by conjugation. Therefore it is quite interesting that recent data of Schubert *et al.* (2004) support the hypothesis that at least some PAIs may have

encoded a conjugation machinery in their early evolutionary steps that has played a role in their spread by horizontal transfer. In his work, Schubert concentrated on the evolution of the high-pathogenicity island (HPI) by analysing and comparing the sequences of this island from different bacteria. HPI was previously identified in *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica,* and a siderophore-mediated iron acquisition system has been characterized on it. Strikingly, an orthologous HPI is widely distributed among *E. coli* and other *Enterobacteriaceae* that cause extraintestinal infections (Schubert *et al*., 1998, 1999). Therefore, HPI is a good model to analyse the evolution of PAIs. Schubert and coworkers identified a novel variant of HPI in *E. coli* strain ECOR31 isolated from leopard stool. The right border of HPI_{ECOR31} (RB-HPI_{ECOR31}) contained an additional 35 kb sequence that was not described before from any PAI. Analysis of this part revealed striking homology to conjugative plasmids. RB-HPI_{ECOR31} consists of three regions that provide a mating pair formation system and a DNA-processing region for conjugative transfer.

The newly found variant of the HPI also contains the P4-like integrase gene on the 5'-part of the island and is flanked by 17 bp DR sequences. Interestingly these flanking DRs are partially missing from other *E. coli* isolates but they are characteristic for the HPIs in most *Yersinia* spp. HPI_{ECOR31} with the DRs, the integrase gene and the presence of RB-HPI_{ECOR31} fulfils all structural criteria of the recently defined family of integrative and conjugative elements, ICE (Hochhut *et al.,* 1999; Burrus *et al.,* 2002).

It is noteworthy, that no conserved *repABC* genes or any other indication of a plasmid replicon have been found on the RB-HPI_{ECOR31}. Till now, mobilisation of this island has not been shown but integrase specific excision and circularization have been demonstrated (Schubert *et al*., 2004). A functional replication system may significantly increase the chance of a possible PAI transfer as we have shown in the case of PAI II_{536}. It can be hypothesised that HPI_{ECOR31} had a progenitor encoding a plasmid replicon. For this, one argument could be that between region two and three in the RB-HPI_{ECOR31} an IS element IS630 homologue was detected and nearby sequences can be found that show homology to the sequence of the conjugative plasmid R6K. We can only hypothetise that integration of the IS630 element might have eliminated the plasmid replicon and with this moment a stabilisation process of this PAI has started. The other side of the coin is that ICEs can be successfully transferred even though they are not able to replicate themselves (Hocchut, personal communication).

We could successfully demonstrate that in the presence of all the necessary components of a conjugative machinery and in the presence of a plasmid replicon a whole PAI can be mobilised after excision from the core chromosome. Even though several clones were isolated where only parts of PAI II were transferred, we focussed only on those clones where the whole 102.3 kb PAI with its nearly 6 kb (derivative of pGP704) insertion was transferred into the recipient strain. Two different classes of transconjugants were detected. In one class, PAI II_{536} was present as a circular intermediate and replicated further as a plasmid due to the prescence of the *pir* gene in the recipient. For the second class, integration of PAI II_{536} occurred at *leuX,* which is also the integration site of PAI II in the donor strain. This underlines the site-specificity of the integration mechanism of this island. Site-specific integration of PAIs has already been described previously; however, if more than one allele of the target tRNA gene is present on the chromosome, all sites may be used. HPI of *Y. pestis* is for example associated only with the *asnT* tRNA locus, but the HPI of *Y. pseudotuberculosis* shows no preference and can insert into any of the three *asn* tRNA loci in the chromosome (Buchrieser *et al.,* 1998). The site-specific recombination events are determined by the recombinases that are encoded on the 5' end of PAIs.

These PAI-specific recombinases control the island's excision and integration from and into the chromosome. Site-specific recombinases are grouped into two unrelated families, the serine recombinase family and the tyrosine recombinase family. Related serine recombinases were found to be involved in insertion or excision of some prophages and some other integrative elements (Smith and Thorpe, 2002), whereas the tyrosine recombinases are involved in integration and excision of a very large array of integrative elements including most of the prophages such as λ, ICEs and PAIs (Argos, 1986, Williams, 2002). Most of the integrases belonging to the tyrosine recombinase family catalyse site-specific integration into the 3' end of a gene encoding a tRNA. Integrases are important components of the conjugative transposons or integrative and conjugative elements (ICEs) which show differences in their site-specificity. The conjugative transposon Tn5276 from *Lactococcus lactis* integrates into at least five chromosomal sites in *L. lactis* MG1614 (Rauch *et al*., 1992), and a large array of conjugative transposons was found in *Bacterioides* (Salyers, 1995; Smith, 1998) whose integration specificity is relatively narrow as they can be integrated only at three to seven chromosomal sites depending on the element. Elements with a high site-specificity were detected in *Salmonella enterica* (CTn*scr94*), and *Streptococcus pneumoniae* (Tn5252). Other conjugative elements, such as *bph-sal* from *Pseudomonas putida*, *clc* from *Pseudomonas sp*., symbiosis islands from *Mezorhizobium loti* and

SXT from *Vibrio cholerae* are also site-specific and integrative (O'Shea *et al*., 2002; overview in: Burrus *et al.*, 2002). Certainly, in the case of PAI II_{536} we can not speak of an ICE although it is true that it excises and also integrates, but the proteins that were necessary for the conjugal transfer were encoded on a helper plasmid (RP4).

Site-specific recombination was also observed in the case of PAI II_{536} when it was remobilised from the laboratory strain SY327 into *E. coli* 536-21. 536-21 is a spontaneous deletion mutant of strain 536 that has lost PAI I and PAI II. In this strain, *leuX* lacks the last 6 bp due to the deletion process, but still contains one copy of the 18-bp DR. As in 536, *asnT, asnW, pheV, thrW* and *selC* are occupied by other PAIs whereas other tRNA genes seem to be 'empty'. All positive transconjugants must carry PAI II_{536} integrated into the chromosome, because 536-21 does not contain the *pir* gene. With the exception of one transconjugant where an incorrect integration process seem to have occurred, in all tested clones PAI II_{536} was found to be integrated into *leuX* thereby restoring the reading frame of this gene. This experiment underlines that *leuX* is the preferred integration site of PAI II_{536} not only in the uropathogenic strain but also in other strains such as *E. coli* K-12.

One pivotal question is the frequency of successful mobilisation. With our construct which enables PAI II_{536} to replicate, the frequency of successful conjugation was also not very high but detectable. With the detection system where exconjugants were detected on Cm and Nal blood agar plates, the frequency of successful exconjugants was as low as 10^{-8} and 10^{-9}. Several experiments were done with different dilutions and ratios of donor and recipient, and experiments were carried out at different temperatures. Positive clones resulted from those experiments where donor cells were in late logarithmic phase, which correlates with the results of Middendorf (2004). A more thorough statistic was done in the remobilisation experiments where a better selection system could be used (M9 lactose, Cm) with which the background with resistant donor mutant cells could be avoided. With this detection system the frequency of remobilisation was nearly 10^{-7} in those cases where the donor strain was SY327 with PAI II_{536} integrated into the chromosome in *leuX*. In contrast, the frequency of transfer was nearly 100-fold higher when SY327 carrying PAI II_{536} as a circular intermediate was used as a donor. These findings indicate that the successful excision of PAI II_{536} from the chromosome seems to be the limiting step for an effective transfer of the island.

Deletion frequency experiments of PAI II_{536} (Middendorf *et al.*, 2004) have already revealed that PAI II_{536} of the UPEC strain 536 had a much higher tendency for deletion at 20 ºC at the

beginning of the stationary phase and it was therefore expected that we also could detect a higher transfer at this temperature. Most of the exconjugants resulted indeed from matings at 20 °C, but some were also obtained at 30 and 37°C. Furthermore, the most exconjugants were detected from matings at 20 °C although many of them contained only part of PAI II_{536}. In summary, these observations support that, presumably due to the activity of the integrase gene, excision frequency of PAI II_{536} is the highest at 20 ºC, but that the conditions for transfer of the complete PAI were not ideal. The assumption that the integrase gene is more expressed at lower temperature was also supported by the results of the remobilisation experiments. With PAI II_{536} integrated into the core chromosome of SY327, successful transfer could mainly be detected at 20 ºC and 30 ºC. It was also clear from the results that temperature had no effect on the conjugation rate in those cases where PAI II_{536} was present in SY327 as a circular form. This indicates that the conjugation machinery works effectively at all the examined temperatures.

Taken together, we have successfully demonstrated that transfer of a complete PAI is possible by conjugation. The characterisation of PAIs in the last two decades has revealed their typical features and raised questions about their evolution. Striking similarities and association of PAIs with mobile genetic elements, such as prophages and DR sequences has suggested some mechanisms of acquisition.

The role of phage-mediated transduction for the horizontal gene transfer of PAIs such as the VPI from *V. cholerae* or SaPI1 from *S. aureus* has already been mentioned (see above). Analysis of the features of the LEE PAIs of three rabbit-specific EPEC strains 83/39 (O15:H-), 84/1101 (O103:H2), RDEC-1 and the human EPEC and EHEC strains E2348/69 and EDL933 has outlined another possible evolutionary process (Tauschek *et al.,* 2002). They studied the role of site-specific integrases in the integration of PAIs into the tRNA genes but they hypothesised that P4-like bacteriophage has not played a role in the transfer, only integrated into the tRNA locus as a starting step (Fig 36a). This bacteriophage created a favourable environment for the integration of other mobile elements such as IS elements and transposases that were associated with different virulence determinants. Evidences for this hypothesis can be collected by analysing the sequence data of different PAIs where virulence associated genes are often flanked by complete or truncated IS elements.

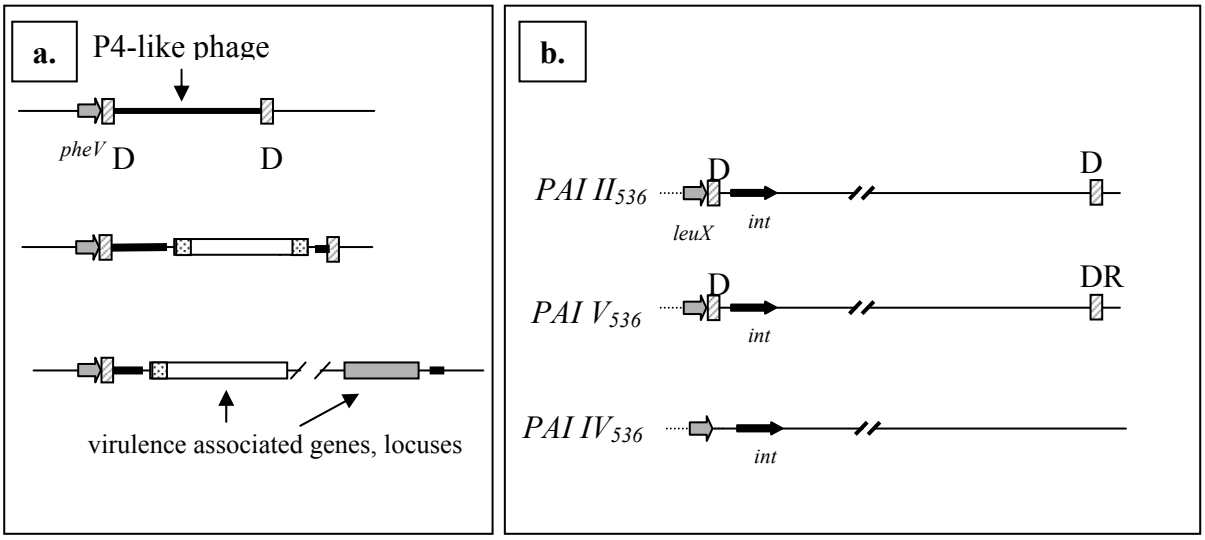


Fig. 36: Possible routes of PAI evolution. (**a**) Life of a PAI can start by the integration of prophages into the host's chromosome. Phage sequences help the integration of foreign elements and genes into the new part of the host's chromosome. In the other model a transferred and integrated PAI can go through a stabilisation process (**b**) as it can be observed in the case of different PAIs of strain 536. The stabilisation process involves the mutation and loss of DRs that are essential for the excision of a PAI from the core chromosome. After some time, it becomes a stable part of the host's chromosome. (DR – Direct Repeat, DR^x – Direct Repeat with mutation, *int'* - integrase)

Beside mobility genes and phage related sequences, another typical feature of pathogenicity islands is the presence of flanking direct repeats (DRs) (Fig. 36b). It was verified that these short sequences (10-50 bp) play a crucial role for the deletion process of PAIs (Blum *et al*., 1994). Deletion of PAI I_{536} and PAI II_{536} has been shown to occur by recombination between these flanking DRs that serve as recognition sites of bacteriophage integrases which are usually encoded on the same PAI. Sequence analysis and the island-probing approach (Rajakumar *et al*., 1997) of the five completely sequenced PAIs of UPEC strain 536 revealed, that there is a correlation between the instability of a PAI and the presence of flanking DRs (Middendorf *et al*., 2003). Four PAIs (PAI I_{536}, PAI II_{536}, PAI III_{536} and PAI V_{536}) out of five are able to be deleted by site-specific recombination, while PAI IV_{536} is stably integrated into the core chromosome.

The comparison of the deletion frequency of PAI II_{536}, which is the most unstable one ($\sim 2 \times 10^{-5}$) with that of PAI V_{536}, which is a relatively stable ($\sim 1 \times 10^{-6}$), and analysis of the remaining boundary regions between which the deletion occured led to the conclusion that the lower deletion frequency of PAI V_{536} could at least be partially due to the fact that the site-specific recombination between *pheV* (76 bp) and its truncated copy *phe'V* (22 bp) that also exhibits an internal 1 bp deletion is not as efficient as recombination between identical copies of *pheV*. The

most stable island of strain 536 is PAI IV_{536}, that completely lacks the flanking DRs. This island could be considered a stably integrated island (Middendorf *et al*., 2003), which is at an advanced homing stage.

It has been hypothesized that PAIs acquired by horizontal gene transfer and integrated into the genome of the host go through a homing process in which, if their genetic features turn out to be advantageous, they favour such mutations that render the islands stable (Hacker and Kaper, 2000). The two evolutionary models how PAIs may have been evolved are not one way streets. A single model cannot describe the formation of all PAIs, however, the role of some mechanisms involved in evolution of pathogenic bacterial variants is generally accepted.

In summary, the study of the genetic organization of PAI V_{536} and in particular that of the K15 capsule gene cluster of *E. coli* strain 536 supports the model that horizontal gene transfer and recombination play an important role for acquisition of additional genetic information and the generation of new variants of already existing determinants. Whereas the function of the K15 capsule has been successfully analyzed, the transport and assembly pathway of the capsule polysaccharides across the outer membrane as well as the functional characterization of other PAI V_{536}–localized genes will have to be further elucidated.

VII.
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VIII.
APPENDICES

A/1 - Acronyms and abbreviations of units and chemicals

A	amper
aa	amino acid
Ap/Ap^R	ampicillin/ampicillin resistence
APS	NH_4-persulfate
bp	base pair
°C	grad celsius
cat	chloramphenicol-acetyl-transferase
Da	dalton
dATP	desoxyadenosin-5'-triphosphate
dCTP	desoxycytosin-5'-triphosphate
dGTP	desoxyguanosin-5'-triphosphate
dH_2O	distilled water
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide
dTTP	desoxythymidin-5'-triphosphate
EDTA	ethylene-diamin-tetraacetate
et al.	et alili (and others)
EtBr	ethidium bromide
EtOH	ethanol
Fig.	figure
g	gram
h	hour
IPTG	isopropyl-β-D-thiogalactopyranosid
kb	kilobase (pairs)
Km/Km^R	kanamycin/kanamycin resistence
l	liter
LB	Luria Bertani broth
m	meter; milli (1×10^{-3})
M	molar, mega (1×10^{6})

mg	milligram
min	minute
ml	milliliter
OD	optical density
ON	overnight
ORF	open reading frame
PAA	polyacrylamid
PBS	phosphate buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SDS	sodium-dodecyl-sulfate
sec	second
Sm/Sm^R	streptomycin/streptomycin resistence
SSC	standart saline citrate
t	time
Tab.	table
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N`,N`-tetramethyldiamin
Tris	Tris-(hydroxymethyl)-aminomethan
U	enzyme unit (1 U=1 µmol substrate × min^{-1})
UV	ultraviolet irradiation
V	volt
v/v	volume/volume
W	watt
w/v	weight/volume
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-glucoside
YT	yeast triptone

μ	micro (1×10^{-6})
μg	microgram
μl	microliter

A/2 - Abbreviations of biological expressions

Afa	afimbrial adhesin
CNF	cytotoxic-necrotizing factor
EHEC	enterohaemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
Fim	type 1 fmbriae
GEI	genomic island
hly	haemolysin
HPI	high pathogenicity island
ICE	integrative and conjugative element
Iha	putative non-fimbrial adhesin
Int	integrase
IPEC	intestinal pathogenic *E.coli*
IS	insertion element
Iuc	aerobactin
kps	capsule locus
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
ORF	open reading frame
oriV	origin of replication (vegetative)
oriT	origin of transfer
PAI	pathogenicity island
PCR	polymerase chain reaction
PFGE	pulse field gelelectrophoresis
PAP	pyelonephritis associated pilus
PRF	pyelonephritis related fimbria
REPEC	rabbit specific enteropathogenic *E. coli*
RT-PCR	Reverse Transcription PCR
Sap	Ag43-homologue
SFA	S-fimbriae

SSH	suppression subtractive hybridisation
Trs	transposase
Tn	transposon
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
VPI	*Vibrio* pathogenicity island

A/3 - Oligonucleotids used in this work

Cutting sites in the primers are shown in bold . For primers not designed in this study, reference is given.

Annealing temperatures were always calculated according to the following formula:

(G+C) / length of oligo x 41 + 69,3 - 650 / length of oligo .

Designation	Sequence (5` - 3`)	Comment and reference
SuperCos uni (F)	CGGCCGCAATTAACCCTCAC	5‘-sequencing primer für pSuperCos1 cosmid vector
SuperCos rev (R)	GCGGCCGCATAATACGACTCACT	3‘-sequencing primer für pSuperCos1 cosmid vector
Genrev 6buni	TATCCGCTCTGGGTATAAGC	5‘-end of the right end („kps side“) of pCos6b38
Genrev 6brev	TTATCGCAGATAACTGGCGG	3‘-end of the right end („kps side“) of pCos6b38
K5reg3down	CCATTACGACCAATAAAGGCAA	K5 specific primer
K5reg3up	CTTATCGTCTGGATGGCAGGT	,,
b2981fw	ATTCATGTTCGGTAAACGGCACAAATC	connects GSP and Saframycin synthase gene
Safrev	TGACGCCACGGGGAAAACGGG	,,
yghs_F	CTTCGCAGATAACGCCCAAAAGGTG	7kb sequencing primer
yghs_R	GGATGTGATGGTTCAGGTAAATCGAC	,,
pitB_F	GATGCCGATAATCGCACCAATCAAGG	,,
pitB_R	CAATCAATGGTTTTCATGACACGGCGAA	,,
gsp_F	TTCACCGCCTTTATCCCAGATAAGAAG	,,
gsp_R	CCACAAGTGGCAATGCGTTGAATTTG	,,
hybF_F	GATAATTTCAACGGCGCTCTGGCAAA	,,
hybF_R	GACGGTGTTTCGCAATATCTCTCCTG	,,
hybD_F	CGCTACCGCAATCAGGTTCACTTC	,,
hybD_R	CAACCAAAGCCTCCGAAATGCTGAAA	,,
hybA_F	GATAGTGGTATTCGCTGCCAGGCT	,,
hybA_R	GGTGTGGAACGTCTCGATAAAGGC	,,
b2998_F	GTCAGAATACCCGCTTCCCAGG	,,
b2998_R	CGGGATTAAAAGCAACGCATGCCG	,,
b3001_F	GCAAAATAACGGCGTGGGCTGTATTG	,,
b3001_R	GCCATTTGCGCCATTGATTGTCCAC	,,
b3004_R	CTGAAGATAGGTCTGGTCGGCGA	,,
K12_Eforw	CAGGCAGGAGATTCGCTCT	screening on the left side of pCos6b38
K12_Erev	TGATTCCCCTTTACTCTCTCG	,,
	Primers for the mutants of the *kps locus*	
kpsSCfw_XbaI	AAAA**TCTAGA**CTCAGGCCACTAAAATCAGCTATG	Inactivation of *kpsSC*

kpsSCrev_SacI	ATTA**GAGCTC**GGGGATTATTTAGCTGACTCGTTTG	”
Kan_BstBI	AACGG**TTCGAA**GACACCCTCATCAGTGCCAAC	”
KanBsiWI	GGAAT**CGTACG**CCGTCAAGTCAGCGTAATGCTC	”
$kpsC_{K5}$fwCm	TGCGCCCTGTTCCGCAAGAAGTTGATGCTATCGCCG TGTGGGGACATCGTCCCAGCGCGGCGAAACCAGTC GCCATCGCCAAAGCAGCGGG*GCTTGTCAGGGGGGC GGAG*	Inactivaton of $kpsC_{K5}$
$kpsC_{K5}$revCm	AGTACAGCGACGTGAAAAACTCAGCCGGTTTGTTG CCGTTTGCAAGAAAGGTTTCAGGATCGCTGACTTCC ACAGCGTTAAGCCTGGCGC*ACCCTGCCCTGAACCGA CGA*	”
CK5_elfw	GGAGTCCATGCAGGAGCAG	Checking the $kpsC_{K5}$ mutant
CK5-elrev	CAGTGTTAACTGGCTGTGATTAAGC	”
$kpsC_{K10}$fwCm	AATAATTAAATTTAATCCGCCAGCGTCTCCAACGAT TTAGACCTACGACTGGAGTCTTATTTTGCCCTGCCA TAAGATGAATTAATTGTTC*GCTTGTCAGGGGGGCGG AG*	Inactivaton of $kpsC_{K10}$
$kpsC_{K10}$fwCm	TATACAAATTCAAAAGGGATTTTAGCTAAGAAATA TTATATACAATCCATCCTAAATATGGAATTGCGTTA TTTAAAAAAACAACAAACCC*ACCCTGCCCTGAACCG ACG*	”
CK10_elfw	CATCACCACCATTGAAATTAACTTTTATAAC	Checking the $kpsC_{K10}$ mutant
CK10_elrev	GTAATTTAGCCGCTGAGAATAATGTC	”
R2_pKD4_fw	CTTCAATTGCAGAATATGATATATGACGATGTTTAA AATTTTTCAGTTCCTGATGTTCACAAATAGGCATAC GTAAACTACAAATATAC*GTGTAGGCTGGAGCTGCTT C*	Inacivation Region2
R2_pKD4-rev	TACGTCTCCCTATGAAGGAGGCCCTGCATGTCTACC TGAAGCTCTTGCAGCAGGTTGTTTAGTTTTTTCATCT CGTAGTGGTATGGCTGAAGATCT*CATATGAATATCCT CCTTAGTTCCTATTCC*	”
kpsMfw_Cm	ATGGCAAGAAGTGGATTTGAAGTCCAGAAAGCCGC CGTTCATGCTCTATTTTTACGTGAGCTTAGAACA*GT GTAGGCTGGAAGCTGCTTC*	Inactivation of Region 3
kpsTrev_Cm	TTGAATATAATCTAGCAACAAATTTCACATTCTCTC GTCCTGTCAGACTTCCTTGAAAACCGC*CATATGAATA TCCTCCTTAGTTCCTATTCC*	”
K15totpKD_ fw	GCTGGCATACGTGACTCCAGCTCAACCGGAGATAA TCACCATTGTTATTTTTCTATTTAATAATAATCTCCT GAAAAATAGATATAGCA*GTGTAGGCTGGAAGCTGCT TC*	Knocking out the whole *kps locus*
K15totpKD_ rev	GCCCAATGGTGAGTGCGGAACTGTCAAACCCGCTC ACCGGGCTGAGTTCCGATGTAGCAAGAATTATCTCC CTCCAGACCACCACGGAAT*CATATGAATATCCTCCTT AGTTCCTATTCC*	”
K15fw_ell	GCCTGATGATTGCCTCTTTTCC	Checking the total kps mutants
K15rev_ell	CACAAATTGATGTCAGTGCTGCTG	”
gspD_Cm	TGGAGCGCAGCGGCAACAGCCAGGTTTTCTATCTCA AATACAGCAAAGCCGAAGATCTGGTCGATGTACTG AAGCAGGTCAGCGGCACGC*GCTTGTCAGGGGGGCG GAG*	Inactivation of the Type II GSP
gspL_Cm	CCATTGCTGGAGACATTCATCAGCAACGTCAGGCC AGATGCCGCACTCCACGTCGGCGGTACGACGCTGC CAACGGATGGGAGCGGAAGC*ACCCTGCCCTGAACCG*	”

	ACGA	
	Primers for RT-PCR	
papBrev	GGAGCCTGAGCAACCGCAT	RT-PCR *pixB*
papBfw	GGATTGTTATCTAAGGATAGATATACCTC	”
SfpCfwRT	GGGTTCCTCCGTTCATTCCTC	RT-PCR *pixC*
SfpCrevRT	CCGTTCACCATTCAGGACCT	”
pgtPfwRT	GGAGTGATGAGTAGCCTTGCC	RT-PCR *pgtP*
pgtPrevRT	GCACGATCTCCATAGTCTGAAC	”
C5in_fw	CCCGTCATTCGTCTGGAAGATG	RT-PCR $kpsC_{K5}$
C5in_rev	CAGGTATGCTGCGGCAAAAAGTTCTT	”
12000	AATCAATCGTTCAATACACTTATGGTAAC	binds in *kpsS*
13000rRT	GAACAATTAATTCATCTTATGGCAGGG	binds in $kpsC_{K10}$
ISfw	GCCTTTTTAATCAGCCAGTTATCCGA	RT-PCR for IS1230B transposase
ISrev	GGTTCTCCCTAAGATAATTTTGTTTTAACTATG	”
13165fRT	CTGAACTAAGGCTTCGAATCCACT	RT-PCR $kpsC_{K10}$
14167rRT	GTTGGGTTTGAAGCATTGCTCTTG	”
orf5RTrev	GACATTATTCTCAGCGGCTAAATTAC	RT-PCR Region2
orf4RTfw	CAAATGGGCATTCGGCAAATACATC	”
kpsMRTfw	GAACTAGGTTTGGCAAATATCGCTTG	RT-PCR betwee Region1 and 2
orf1RTrev	CTGCGTATGATTTGGGTTCAATACTAA	”
gspDfwRT	GATCGTCCATCAGACCGCCAA	RT-PCR *gspD*
gspDrevRT	CAGTGTGATTGTCAGTGGTGAC	”
	Primers for the mobilisation experiments	
Cm_fw_PstI	**CTGCAG**GCTTGTCAGGGGGGCGGAG	Cm castte in pGP704
Cm_rev_PstI	**CTGCAG**ACCCTGCCCTGAACCGACGA	”
pir_fw_SacI	TTT**GAGCTC**CCGTCAAGTCAGCGTAATGCTC	
pir_revStop_ EcoRI	TTT**GAATTC***TCA*AGCTTCATCATCTTTATCGCCAGA	PIR *STOP codon* is built in
paiII_1XhoI	TTT**CTCGAG**GGGAAGCACGATATGCAGCC	labelling PAI II with homologue recombination with pSG704
paiII_1SacI	TT**GAGCTC**GATATTTTTTGGCTGCTTCAGCTTTACG	”
paiII_2SacI	TTT**GAGCTC**GGACAAGAACACAAAATCACTCTACT GA	”

paiII_2XhoI	TTT**CTCGAG**CCCGGCTTCATCGACAATGA	,,
ATT1	GAGGTACCAGCGCGGTTTGATC	checking the integration into the λ attachment site (*E. coli*)
ATT2	CAGATGGCGCAATGCCATCTGG	,,
17kD up	CCCGGCTGAACAGACGATT	screening PCRs for PAI II_{536}
17kD in	GCAGCGGAGAGTCATTGTC	,,
hlyDup	CGCGATAATCCGCTACATC	,,
hlyDin	GGGTATGGCTGTCACTGCA	,,
hec_down1	CACACTGAAAGGCCGCAGC	,,
hec_down2	GTACAGCGTGCCGTTCTGC	,,
dsdXin	GCTGTATCCCGACATCAGC	,,
dsdAup	GCCACATCATTCTCCCGTA	,,
ORFAin	CATCCCCTTCACTACAGGA	,,
Na-Anti_pdo	CAGCGATGCTGGACCGTAT	,,
PaiII_1fw	CAACCGTAGAAATACGTGCCG	overlapping PCRs of PAI II_{536}
PaiII_1rev	CCAGTATGAGGCAAACCCTAAAG	,,
PaiII_2fw	GACAATAGCTGTCCATACGGTG	,,
PaiII_2rev	CAATGCTGGCCATATCCATCAG	,,
PaiII_3fw	GACTCACTACTGGGAAGGTC	,,
PaiII_3rev	GATGGTCATGTGCAGGGAGG	,,
PaiII_4fw	GTGATGTCACTGGCGGTAATAATC	,,
PaiII_4rev	GGCGATGACCATGATACGGTA	,,
PaiII_5fw	CGGAATACTGAACTGCGGATAA	,,
PaiII_5rev	GCTGGATGCCAATCAATGATCG	,,
PaiII_6fw	GCATCCATCTCCGTTCACAG	,,
PaiII_6rev	GCATGGCTGGTTGTTCCTAAAC	,,
PaiII_7fw	CCTCCTTTGGACTGAAGTTCA	,,
PaiII_7rev	GCACAGGCGCTCTTTTATTGTTTG	,,
PaiII_8fw	GGACACATGGCAGGGTCTG	,,
PaiII_8rev	GGAACACGTCTTCTTGTTGACA	,,
PaiII_9fw	CAGCGATTTGTCACCACCTG	,,
PaiII_9rev	GTACTTGACGCGGCGGACA	,,

PaiII_10fw	GCTTCTGAAAAACGGGTGAAGTC	,,
PaiII_10rev	CATGGCGCATCATGAAATCATCA	,,
PaiII_11fw	CTCTCGCGTATATTCAGCAAAAAC	,,
PaiII_11rev	CGTCACATCGGATAACATTCGG	,,
PaiII_12fw	CTGATATCTCTTTCAGACTTCAGAAC	,,
PaiII_12rev	GTCACCTCAAGAACGTCTAACC	,,
PaiII_13fw	CTGGCACCTATGGATCAGGT	,,
PaiII_13rev	GTTCAGCAACTGAAGGTTCATACT	,,
PaiII_14fw	GATGACCATCAGTGTTTCCGCT	,,
PaiII_14rev	CGGGGATTTAAGTATTGGTCAGTT	,,
PaiII_15fw	GCAACAATCTGACCTGCAAGCAT	,,
PaiII_15rev	GGATGATGAGCTTCAGGTTCAG	,,
PaiII_16fw	TGCCGTACAGCTTGTCATTACC	,,
PaiII_16rev	CTGGGTACTGCACTTTCCTCA	,,
PaiII_17fw	CTGACATTGCCACCAGATTTTTGT	,,
PaiII_17rev	GGTGTAATGCGCTAACCTGTTTC	,,
PaiII_18fw	CTACAAATGTTCAATATGGTGGGTATATC	,,
PaiII_18rev	CGCTGTTGCCACTGGATTAATG	,,
PaiII_19fw	GCCATCCACTACATATCATGCC	,,
PaiII_19rev	CGACGGGTTTCTATGCTGAG	,,
PaiII_20fw	CCTCAACTGGAGCAATTTTCTGTC	,,
PaiII_20rev	GGACTTGGATCACTGAAGCTTTAC	,,
PaiII_21fw	CCACAAGCTGTTGATTTTGGTACG	,,
PaiII_21rev	GCGATAGTGGGCAATTTGCTATTG	,,
PaiII_22fw	CGTAAACGTCCTCCAGAATTTATATC	,,
PaiII_22rev	GGACGATGGCGATATGTCTG	,,
PaiII_23fw	GTTCTTCATTTCTACGTTGCTTTGTC	,,
PaiII_23rev	CCTACCAGAGATAACCCATGG	,,
PaiII_24fw	CGGTTTCTCCTGAACATAACTTTG	,,
PaiII_24rev	GGTGAAGTCCGTAACCAGAATG	,,
PaiII_25fw	GCCTGTTTTTGCTGCTGTTCAC	,,

PaiII_25rev	GCAGGACTAAAGTTGCAGAGC	,,
PaiII_26fw	CGCTTTCGCCCCGATTTCTA	,,
PaiII_26rev	ATGACCGTCGTACTGTGGAC	,,
PaiII_27fw	GTCAGCCCGCTTTTCTTCTG	,,
PaiII_27rev	GCTCCGTCGTATACCGATGA	,,
PaiII_28fw	CGGTCAAGAAAATACGATGAGCC	,,
PaiII_28rev	GAACGACAGCAAAATCCTCTCC	,,
PaiII_29fw	CAGCACCTGCGCCGTCA	,,
PaiII_29rev	GCGATGCCACGGTGAAAACC	,,
PaiII_30fw	CCGGTATTACTGAATGTCCCG	,,
PaiII_30rev	GAACATGAAGACAGCACTGACC	,,
PaiII_31fw	CCTGAAGCAGAACATCATCCAG	,,
PaiII_31rev	CGGCTGATATCCTGAGACTG	,,
PaiII_32fw	CTCGCTTCCACGGACGTTG	,,
PaiII_32rev	GTTGGCAGTGCTGAAAGCAG	,,
PaiII_33fw	GGTCAGAATGTCCTCAGTGAG	,,
PaiII_33rev	CAATGAAATTGACAGGAGAGATACC	,,
PaiII_34fw	CCACTGGCATGATTTTTACCCTG	,,
PaiII_34rev	CGGGCACATTCCTGCTGTC	,,
PaiII_35fw	GGATACTGGGCGATGAGCG	,,
PaiII_35rev	GAGCACGGTGAGCGAAATAG	,,
PaiII_36fw	CCGCATTAGGTGACTTTACACG	,,
PaiII_36rev	GACGCCGTACTGACCGATG	,,
PaiII_37fw	CCAGGTTTGTTATCGAGGTAAGG	,,
PaiII_37rev	GGCGCTATCGACTACGTCC	,,
PaiII_38fw	GGCAGTATATCGATTCGGCGA	,,
PaiII_38rev	GCTTCCCAGCCTGTCACTTC	,,
PaiII_39fw	GGGCATCTTCAAAGTCAAAGCC	,,
PaiII_39rev	CGCCCGTCTGTTTTTCAGTTTC	,,
PaiII_40fw	GGGGCATCAAGGTCGCTATTT	,,
PaiII_40rev	CAGAACCGCAGCCAGCCAT	,,

PaiII_41fw	GCTGCGATGCGGATCCAC	,,
PaiII_41rev	GGTTACCGCAATGGTGAAAGG	,,
PaiII_42fw	GCTTTTACTGCGCCGACATCA	,,
PaiII_42rev	CGTTGCACGCGGCTATCTG	,,
PaiII_43fw	CGATGGATACATTCGGGTTTAGC	,,
PaiII_43rev	GCAACAGCGACATCATCCTG	,,
PaiII_44fw	CTCTCTCTTCAGCCAGTCATC	,,
PaiII_44rev	GCCAAAATCTGATCCCCAGC	,,
PaiII_45fw	GCAACTACGCCATTGGTTTGTC	,,
PaiII_45rev	GAAAAACTGGCACGTCATCAACG	,,
PaiII_46fw	GAAGGCTGCCATTCGGGTATA	,,
PaiII_46rev	CTGTACTGACTCGTCAGCACT	,,
PaiII_47fw	CTTGAGATTCAGCAAGGTGGC	,,
PaiII_47rev	GGAATCCCCTAATGCTGGTG	,,
PaiII_48fw	GTATAACGGGATGAAAGTGGGG	,,
PaiII_48rev	GTTGAGAATGTCGGGAATGGTAC	,,
PaiII_49fw	GGATGTGTATCAGACAAAGCAATG	,,
PaiII_49rev	TTTCTGGCGAATTTCTTCAGGAAG	,,
PaiII_50fw	CAGCCATTTTTCCCTCTCCG	,,
PaiII_50rev	CCTGACCATCTTCCGTCATG	,,
PaiII_51fw	CTGCTGTTCACTGTGGCATC	,,
PaiII_51rev	GAGTGGCAACCAGTTGAGACT	,,
PaiII_52fw	CGCATAATTCCACCACACCTTC	,,
PaiII_52rev	GGCTGGTCGGTACGCAC	,,
PaiII_53fw	GGCAGGCATTTCACTGTGTGA	,,
PaiII_53rev	CGAAGGCCGGACTCGAACA	,,
K12R (rev)	ATCCTGCGCACCAATCAACAA	K12 specific, Kuhnert *et al*.,1995
K12L (fw)	TTCCCACGGACATGAAGACTACA	,,
K12IS-L (fw)	CGCGATGGAAGATGCTCTGTA	,,
orf4bico	GGAATGAATGCCACTCCATTATTGACAGAAATG	536 (K15) specific primer
orf5bico	GATCAAACGAGTCAGCTAAATAATCCCCAC	536 (K15) specific primer

M803b	GCCTGGAGTGTGACAAAGGTTAC	*leuX* flanking prim., Middendorf
M805c	GATGTTCACCAAGGTGGGCGT	,,
leu 2	ACCAAGCGCTGCAAAAAGAT	concatamer PAI II_{536}
Concat 1	CCGGATTGGATCTATCGCGA	,,
yjgB1	ACTTTATCGGCACCCATCG	downstr from *leuX*, Grozdanov
yjgB2	GCATGAGGTGATTGGGCG	,,

A/4 – ORFs of PAI V_{536} and the downstream sequences

Position, length, orientation and homology are shown in the first four columns, while identity is indicated to known proteins or genes in the further columns of the table.

ORF	from to	length-b.	ori.	protein similarity to / function	identitity	length/ aa.	accessionnumber
pheV	1 to 76	76	>				
ORF1	274-1539	1266	>	CP4 integrase	418/420 (99%)	421	AAK16198
ORF2	2082-2279	198	>	putative IS110 transposase	46/57 (80%)	398	CAD33753
ORF3	2695-2886	192	>	transposase like protein	46/67 (68%)	124	AAL72476
ORF4	3156-3938	782	<	put. diguanylate cyclase/phosphodiesterase (*S. typhimurium* LT2) EAL domain	20/41 (48%)	183	AAL23457
ORF5	3991-4983	993	<	PixG -minor subunit	201/330 (60%)	330	CAC41983
ORF6	5006-5284	279	<	PixF -minor subunit	53/87 (60%)	86	CAC41982
ORF7	5608-6171	564	<	PixJ -member of assembly machinery	95/187 (50%)	187	CAC41981
ORF8	6208-6939	732	<	PixD -periplasmic chaperon	228/243 (93%)	243	CAC41980
ORF9	7008-9578	2571	<	PixC -pore forming outer membrane protein	729/834 (87%)	835	AF228759
ORF10	9639-10226	588	<	PixH -minor subunit	156/195 (80%)	190	CAC41977
ORF11	10312-10845	534	<	PixA -pilus rod	114/177 (64%)	174	CAC41977
ORF12	10890-11165	276	<	PixB - regulatory protein	32/72 (44%)	104	CAD42031
ORF13	11698-12414	717	>	R6 transposase	3 parts (86%)	488	AAC61728
ORF14	12620-12802	183	>	selenium-binding protein-like, *Arabidobsis thaliana*	21/56 (37%)	849	BAB09998
ORF15	12811-14067	1257	<	PgtA, phosphoglycerate transport activator	403/417 (96%)	418	AE016771
ORF16	14048-16057	2010	<	PgtB, phosphoglycerate transport: protein for signal transmission	614/669 (91%)	669	AE016771
ORF17	16054-17325	1272	<	PgtC, phosphoglycerate transport regulatory protein PgtC	401/423 (94%)	435	AE016771
ORF18	17670-19034	1365	>	PgtP, phosphoglycerate transporter	406/454 (89%)	454	AE016771
ORF19	19077-19442	366	>	phosphoglycerate mutase *Bacillus stearothermophilus*	23/68 (33%)	511	T46865
ORF20	19470-19853	384	>	hypothetical transposase (insC), IS2	50/103 (48%)	136	NP_418692
ORF21	19982-20995	1014	<	putative cytoplasmic protein	327/337 (97%)	337	NP_752277
ORF22	21007-22323	1317	<	putative L-fucose permease	379/438 (86%)	437	AE016756
ORF23	22351-23298	948	<	putative sugar kinase, ribokinase family	295/306 (96%)	306	AE016756
ORF24	23576-24358	783	>	putative regulatory protein, deoR family	259/260 (99%)	261	AE016756
ORF25	24535-24708	174	>	putative acetolactate synthase I, valine sensitive subunit	56/68 (82%)	96	NP_290309
ORF26	25185-25379	195	>	hypothetical protein	62/181 (34%)	169	NP_286729
ORF27	25427-26160	734	<	hypothetical protein	129/135 (95%)	188	NP_752257
ORF28	26280-26957	678	<	ORFB protein, IS911	225/225 (100%)	225	CAD33743
ORF29	27146-27448	303	<	Transposase insN for insertion sequence element IS911	100/100 (100%)	100	CAD33744
ORF30	28037-28462	426	<	conserved hypothetical protein	136/141 (96%)	141	AE015311
ORF31	28459-28848	390	<	hypothetical protein	119/129 (92%)	129	AE016756
ORF32	29348-30370	1023	>	ORFA protein, transposase, IS100	340/340 (100%)	340	CAD42088
ORF33	30370-31149	780	>	ORFB protein, transposase, IS100	259/259 (100%)	259	AAC82752
ORF34	31193-31999	807	<	conserved hypothetical protein	185/185 (100%)	199	NP_752253
ORF35	32003-32389	387	<	putative haemolysin expression modulating protein	58/69 (84%)	74	AE016766
ORF36	33228-34019	792	>	hypothetical protein sim. to yagM	250/263 (95%)	263	NP_752251
ORF37	34403-34609	207	>	hypothetical protein	67/68 (98%)	86	NP_755525

ORF38	34697-35305	609	>	hypothetical protein	198/202 (98%)	202	NP_752249
ORF39	35343-35549	207	>	putative ATPase component of ABC transport system (*Coryneb. glutamicum*)	26/89 (29%)	543	NP_600769
ORF40	35583-35807	225	>	istB protein, IS21	18/20 (90%)	265	BVECIT
ORF41	36065-36277	213	>	hypothetical protein	58/59 (98%)	61	AE016755
ORF42	36324-36626	303	>	hypothetical protein	95/96 (98%)	122	AF447814
ORF43	36775-37212	438	<	putative proline-rich protein	119/125 (95%)	141	AE016762
No ORF	37213-37747						
ORF44	37748-38374	627	>	DNA double-strand break repair, ATPase	39/123 (31%)	886	RA50_SULAC
ORF45	38514-39188	675	<	hypothetical protein	40/152 (26%)	251	NP_569516
ORF46	39294-40355	1062	>	putative YeeP protein, / GTP binding prot.	269/290 (92%)	290	AAL08471
ORF47	40650-43571	2922	>	putative adhesin / autotransporter Ang43	840/973 (86%)	1005	NP_286746
ORF48	43606-43875	270	>	hypothetical protein ykfF	68/77 (88%)	79	AE016771
ORF49	43966-44784	819	>	hypothetical protein yafZ	262/272 (97%)	272	AE016769
ORF50	44876-45361	486	>	hypothetical protein yfjX	146/161(90%)	161	AE005313
ORF51	45362-45853	492	>	putative yeeS protein	145/158 (91%)	319	AE016759
ORF52	45922-46143	222	>	YeeT protein	70/73 (96%)	73	AAK16199
ORF53	46154-46585	432	>	YeeU protein	134/141 (95%)	142	AE016771
ORF54	46675-47052	378	>	similar to protein L0007, encoded within prophage CP-933L, YeeV	117/124 (94%)	124	NP_290242
ORF55	47049-47537	489	>	unknown protein encoded within prophage CP-933L, L0008, YeeW	141/162 (87%)	162	NP_290243
ORF56	47504-47746	243	>	similar to protein L0009, encoded within prophage CP-933L	66/69 (95%)	80	CAD66209
ORF57	47831-48673	843	>	similar to proteins L0010 and L0012	267/280 (95%)	280	AE015313
DpheV	48784-48805	22(76)	>				
ORF58	48911-49204	294	<	hypothetical protein, (putative transposase -*S. flexneri*)	50/51 (98%)	51	AE016766
ORF59	49709-49920	212	<	no homology			
ORF60	49997-50980	984	>	KpsF, polysialic acid capsule export protein	325/327 (99%)	327	KSF5_ECOLI
ORF61	51052-52200	1149	>	KpsE, polysaccharide export protein	350/382 (91%)	382	KSE5_ECOLI
ORF62	52224-53900	1677	>	KpsD, polysialic acid transport protein precursor	538/558 (96%)	558	KSD1_ECOLI
ORF63	53910-54650	741	>	KpsU, CMP-KDO synthase(3-deoxy-manno-octulosonate cytidylyltransf.)	241/246 (97%),	246	KSU5_ECOLI
ORF64	54647-56095	1449	>	KpsC, capsule polysaccharide export protein	384/453 (84%)	675	KSC5_ECOLI
ORF65	56133-57170	1038	>	transposase IS630 family, *Photorabdus luminescens*	245/341 (71%)	343	NP_929308
ORF66	57325-58179	855	<	KpsS (*Aeromonas hydrophila*)	243/393 (61%),	437	AF375657
ORF67	58612-60741	2130	<	KpsC, capsule export protein	496/716 (69%),	710	AF127177
Rep1	60732-60787						
ORF68	61095-62042	948	<	unknown protein (*Mannheimia haemolytica*)	40/128 (31%)	480	AF170495
ORF69	62331-62969	639	<	unknown protein *Synechocystis* sp. PCC 6803	43/157 (27%),	181	NP_441374
ORF70	62966-63922	957	<				
ORF71	63922-64911	990	<	mannosyltransferase B (*Aquifex aeolicus*)	62/252 (24%),	374	NP_213361
ORF72	64908-65798	891	<	put. glycosyltransferase (*Actinobacillus*), Glycos_transf_Group1/gnl/CDD7651	90/313 (28%),	321	AAC26630
Rep2	66029-66078						
Rep3	66602-66639						
Rep4	66765-66821						
ORF73	66793-67461	669	<	KpsT, polysialic acid transport protein -ABC transporter	159/216 (73%),	224	KST5_ECOLI
ORF74	67458-68234	777	<	KpsM, polisyalic acid transport protein	183/258 (70%),	258	KPM2_ECOLI
ORF75	69290-69826	537	<	GspM, hypothetical type II secretion protein	169/178 (94%),	178	AF426313

ORF76	69828-70997	1170	<	GspL, hypothetical type II secretion protein	354/387 (91%),	392	AAL10701
ORF77	71003-71980	978	<	GspK, hypothetical type II secretion protein	285/325 (87%),	325	AAL10700
ORF78	71977-72546	606	<	GspJ, hypothetical type II secretion protein	182/187 (97%),	187	AF426313
ORF79	72579-72950	372	<	GspI, hypothetical type II secretion protein	106/123 (86%),	123	AF426313
ORF80	72947-73477	531	<	GspH, hypothetical type II secretion protein	172/176 (97%),	176	AF426313
ORF81	73514-73969	456	<	GspG, hypothetical type II secretion protein	151/151 (100%),	151	AF426313
ORF82	73986-75209	1224	<	GspF, hypothetical type II secretion protein	341/407 (83%),	407	AF426313
ORF83	75209-76702	1494	<	GspE, hypothetical type II secretion protein	476/497 (95%),	497	AF426313
ORF84	76702-78552	1851	<	GspD, hypothetical type II secretion protein	606/616 (98%),	616	AF426313
ORF85	78792-79622	831	<	GspC, hypothetical type II secretion protein	254/276 (92%)	276	AF426313
ORF86	79769-80179	411	<	YghG, hypothetical lipoprotein precursor (b2971)	121/136 (88%)	136	YGHG_ECOLI
ORF87	80245-81054	810	<	Leader peptidase pppA (prepilin peptidaseA)	248/269 (92%)	269	PPPA_ECOLI
ORF88	81194-85759	4566	<	Putative lipoprotein acfD homolog precursor	1269/1431 (88%),	1520	ACFD_ECOLI
ORF89	85910-86110	201	>	lpgD (EIEC, Shigella)	9/27 (31%)	538	AAK53908
ORF90	86244-87926	1683	<	GlcA, putative glycolate permease	493/560 (88%)	560	NP_417449
ORF91	88281-90452	2172	<	GlcB, malate synthase G	706/723 (97%)	723	NP_417450
ORF92	90474-90878	405	<	GlcG, hypothetical protein	118/118 (100%)	134	NP_417451
ORF93	90883-92106	1224	<	GlcF, glycolate oxidase F, iron-sulfur subunit	424/443 (95%)	761	NP_417452
ORF94	92117-93169	1053	<	GlcE, glycolate oxidase subunit	339/350 (96%)	350	GLCE_ECOLI
ORF95	93169-94668	1500	<	GlcD, glycolate oxidase subunit	498/499 (99%)	499	NP_417453
ORF96	94919-95683	825	>	hypothetical protein	270/271 (99%)	274	AAA69147
ORF97	95690-96832	1143	<	hypothetical protein YghO	379/380 (99%)	402	AE016766
ORF98	97204-98924	1721	>	putative saframycin MX1 synthetase B	293/322 (90%)	576	AE016766
ORF99	98921-99835	915	>	conserved hypothetical protein	288/298 (96%)	298	AEO15315
ORF100	99867-100115	249	>	AcpP, putative acyll carrier protein	81/82 (98%)	82	AEO15315
ORF101	100115-101287	1173	>	similar to hypothetical transferase	358/390 (91%)	390	AE016766
ORF102	101322-102401	1080	<	hypothetical protein / predicted Permease YjgP_YjgQ family	333/359 (92%)	386	AE016766
ORF103	102398-103469	1072	<	hypothetical protein / predicted permease	212/225 (94%)	356	AE016766
ORF104	103500-104063	564	<	YtfJ precursor/predicted transcription regulator	174/187 (93%)	187	AE016766
ORF105	104072-104896	825	<	hypothetical protein with ATP binding similarities	267/274 (97%)	274	AE016766
ORF106	104896-105630	735	<	conserved hypothetical protein, putative membrane protein	225/244 (92%)	449	AEO15315

A/5 – Result of the screening PCRs made from the IMI uropathogenic strain collection

Strains were tested with primers from the *pix*, *pgt*, *kps* and *gsp* geneclusters.

PAI V-specific PCRs

Strain	Origin	Serotyp	Strainnr.	Characteris.	M436/ M805c	*sfpC* fw/*sfpC* rev	*pgtC* fwRT/*pgtC* revRT	ellCK5fw/ellCK5rev	RTfw13165/RTrev14167	orf4bico/orf5bico	*kpsM* RTfw/*kpsM* RTrev	*gspD* fwRT/*gspD* revRT
						sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*	*gspD*
O6:K5-strains												
DSM 6601		O6:K5:H1				-	-	+	+	-	-	+
RZ439	UTI	O6:K5:H1				-	+	+	-	-	-	+
RZ441	UTI	O6:K5:H1				-	+	+	-	-	-	+
RZ443	UTI	O6:K5				-	-	+	+	-	-	+
RZ468	UTI	O6:K5:H1				-	+	+	-	-	-	+
RZ475	UTI	O6:K5:H1				-	+	+	-	-	-	+
RZ495	UTI	O6:K5				-	+	+	-	-	-	+
RZ500	UTI	O6:K5:H1				-	+	+	+	-	-	+
RZ525	UTI	O6:K5:H1				-	+	+	+	-	-	+
RZ526	UTI	O6:K5:H1				-	+	+	+	-	-	+
n=10; positive from						**0**	**8**	**10**	**5**	**0**	**0**	**10**
Percent						**0%**	**80%**	**100%**	**50%**	**0%**	**0%**	**100%**
Different O6-Strains												
RZ446	UTI	O6:K53:H1				-	+	+	-	-	-	+
RZ422	UTI	O6:K14:H-				-	+	-	-	-	-	+
RZ505	UTI	O6:K14:H				-	+	-	-	-	-	+
RZ436	UTI	O6:K13:H1				-	-	+	+	-	-	+
RZ454	UTI	O6:K2:H-				-	+	+	-	-	-	+
RZ458	UTI	O6:K2:H1				-	-	+	-	-	-	+
RZ451	UTI	O6:K18/22/H31				-	+	-	-	-	-	+
RZ479	UTI	O6:K+:H-				-	+	-	-	-	-	+
RZ532 I	UTI	O6:K+:H31				-	+	-	-	-	-	+
RZ532 II	UTI	O6:K+:H31				-	+	-	-	-	-	+
RZ411	UTI	O6:K-:H1			-	-	-	+	-	-	-	-
n=11; positive from						**0**	**8**	**4**	**1**	**0**	**0**	**7**
Percent						**0%**	**73%**	**36%**	**9%**	**0%**	**0%**	**64%**

PAI V-specific PCRs

Strain	Origin	Serotyp	Strainnr.	Characteris.	M436/ M805c	*sfpC* fw/*sfpC* rev	*pgtC* fwRT/*pgtC* revRT	ellCK5fw/ellCK5rev	RTfw13165/RTrev14167	orf4bico/orf5bico	*kpsM* RTfw/*kpsM* RTrev	*gspD* fwRT/*gspD* revRT
						sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*	*gspD*
Strains from the Jena-Study												
1E2	stool		J256			-	-	-	-	-	-	-
1E5	stool		J259			-	-	-	-	-	-	-
2A1	stool		J76			-	-	-	-	-	-	+
2A2	stool		J77			-	-	-	-	-	-	-
2E2U	urine		J179			-	+	+	-	-	-	+
2E1U	urine		J177			-	-	+	+	-	-	+
3B5	stool		J128		+	-	-	-	-	-	-	-
3D5	stool		J420			+	-	+	-	-	-	+
5A1	stool		J231			-	-	-	-	-	-	+
5A1U	urine		J281			-	-	-	-	-	-	-
8B1	stool		J171			-	-	-	-	-	-	+
8B2	stool		J172			-	-	-	-	-	-	-
13A1	stool		J207			-	+	+	-	-	-	-
16A2U	urine		J268			-	+	+	+	-	-	+
16A3	stool		J221			-	-	-	-	-	-	-
1G1	stool		J580			-	-	-	-	-	-	+
1G1U	urine		J587			-	+	+	-	-	-	+
1H1	stool		J705			-	+	+	-	-	-	+
1H1U	urine		J712			-	+	+	-	-	-	+
3N1	stool		J747			-	-	+	-	-	-	+
3N2	stool		J748			-	-	-	-	-	-	+
3N5	stool		J751		-	-	-	-	-	-	-	+
8F4	stool		J757		- !	-	-	-	-	-	-	-
16B1	stool		J719		-	-	-	-	-	-	-	-
19A1	stool		J863		? !	-	-	-	-	-	-	+
19B1	stool		J869		-	-	-	-	-	-	-	-
20A1	stool		J876		-	+	+	-	-	-	-	+
20A1U	urine		J882		-	+	+	-	-	-	-	+
22A2	stool		J961		-	+	+	-	-	-	-	+
22B2U	urine		J981		-	+	+	-	-	-	-	+
n=30; positive from						**5**	**10**	**9**	**1**	**0**	**0**	**19**
Percent						**17%**	**33%**	**30%**	**3%**	**0%**	**0%**	**63%**

PAI V-specific PCRs											
Strain	**Origin**	**Serotyp**	**Strainnr.**	**Characteris.**	**M436/ M805c**	***sfpC* fw/*sfpC* rev**	***pgtC* fwRT/*pgtC* revRT**	**ellCK5fw/ellC K5rev**	**RTfw13165/R Trev14167**	**orf4bico/orf5 bico**	***kpsM* RTfw/*kpsM* RTrev**
						sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*
Classic UTI-Strains											
536	UTI					+	+	+	+	+	+
536-21						+	+	+	+	+	+
536-114					-	+	+	+	+	+	+
536-225					+	+	+	+	+	+	+
J96	UTI				-	-	+	-	+	-	-
J96-M1					-	-	+	-	+	-	-
764					-	-	-	+	+	+	+
764-2					-	-	-	+	-	-	-
EB-35	UTI		JH154 old Hackercoll.		+	-	+	+	+	-	-
A284						-	-	-	-	-	-
AD110			JH130			-	+	+	-	-	-
2980			JH144			-	-	+	+	-	+
F18	stool					-	+	+	-	-	-
F18 Col-						-	+	+	-	-	-
4405/1	stool		GB58			-	+	+	-	-	-
7521/94-1	stool		GB148			-	-	+	-	-	-
S5	controlstrain		IMI29			-	-	-	-	-	-
CFT073						-	+	+	+	-	-
n=18; positive from						**4**	**12**	**12**	**10**	**5**	**6**
Percent						**22%**	**67%**	**67%**	**56%**	**28%**	**33%**

Animalpathogenic E. coli-Strains											
W1825	Turkey, sepsis					-	-	-	-	-	-
AC1= 781	chicken				-	-	-	-	-	-	-
RB9	sheep	GB131				-	-	-	-	-	-
n=3; positive from						**0**	**0**	**0**	**0**	**0**	**0**
Percent						**0%**	**0%**	**0%**	**0%**	**0%**	**0%**

PAI V-specific PCRs

Strain	Origin	Serotyp	Strainnr.	Characteris.	M436/ M805c	*sfpC* fw/*sfpC* rev	*pgtC* fwRT/*pgtC* revRT	ellCK5fw/ellCK5rev	RTfw13165/RTrev14167	orf4bico/orf5bico	*kpsM* RTfw/*kpsM* RTrev	*gspD* fwRT/*gspD* revRT
						sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*	*gspD*
ECOR-straincollection												
ECOR1 (group A)						-	-	-	-	-	-	-
ECOR7 (group A)						+	+	-	-	-	-	+
ECOR23 (group A)						-	-	-	-	-	-	-
ECOR25 (group A)						-	-	-	-	-	-	-
ECOR28 (group B1)						-	-	-	-	-	-	-
ECOR32 (group B1)						-	-	-	-	-	-	+
ECOR58 (group B1)						-	-	+	-	-	-	+
ECOR71 (group B1)						-	-	-	-	-	-	-
ECOR51 (group B2)						-	-	+	+	-	-	+
ECOR52 (group B2)						-	+	-	-	-	-	-
ECOR53 (group B2)						-	+	+	+	-	-	+
ECOR57 (group B2)						-	+	+	-	-	-	+
ECOR60 (group B2)						-	-	+	-	-	-	+
ECOR66 (group B2)						-	+	+	-	-	-	+
ECOR40 (group D)						-	-	-	-	-	-	-
ECOR48 (group D)						-	-	-	-	-	-	-
ECOR50 (group D)					shorter	-	-	+	-	-	-	+
ECOR31 (group E)						-	-	-	-	-	-	+
ECOR42 (group E)						-	-	-	-	-	-	+
ECOR63						-	-	-	+	-	-	+
ECOR64						-	-	+	-	-	-	+
ECOR65						-	-	-	-	-	-	+
ECOR62						-	+	+	-	-	-	+
ECOR61						-	-	+	-	-	-	+
ECOR55						-	-	-	-	-	-	-
ECOR56						-	-	+	-	-	-	-
ECOR59						-	-	-	-	-	-	+
ECOR54						-	-	+	-	-	-	+
n=19/28; positiv from						**1/1**	**5/6**	**5/7**	**2/3**	**0**	**0**	**11/18**
Percent						**5%/4%**	**26%/21%**	**26%/25%**	**11%/11%**	**0%**	**0%**	**58%/64%**

PAI V-specific PCRs

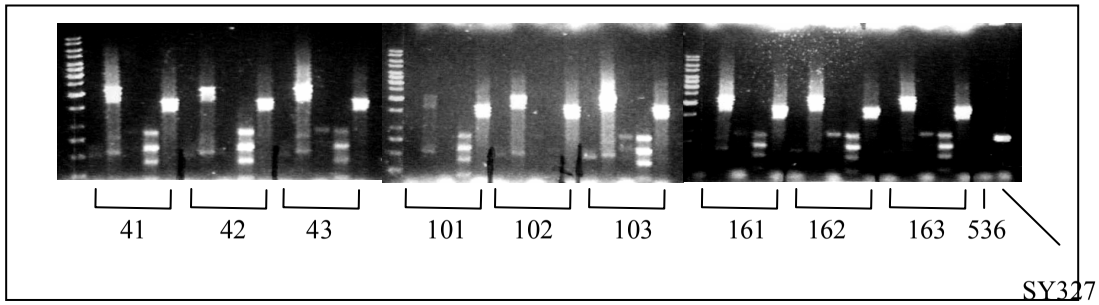
Strain	Origin	Serotyp	Strainnr.	Characteris. M436/ M805c	*sfpC* fw/*sfpC* rev	*pgtC* fwRT/*pgtC* revRT	ellCK5fw/ellCK5rev	RTfw13165/RTrev14167	orf4bico/orf5bico	*kpsM* RTfw/*kpsM* RTrev	*gspD* fwRT/*gspD* revRT
					sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*	*gspD*
Humanpathogen newbornmeningitis (NBM)- and sepsis-strains											
IHE3034	NBM				-	-	+	-	-	-	+
IHE3036	NBM				+	+	+	-	-	-	+
IHE3080	NBM			-	-	-	+	-	-	-	+
RS176					-	-	-	-	-	-	+
RS218					-	-	+	-	-	-	+
RS226					-	-	+	-	-	-	+
Ve1140	NBM		Coni Meier		-	+	+	-	-	-	+
Ve239	NBM		Coni Meier		-	+	-	-	-	-	+
B10363	NBM		Coni Meier		-	+	+	-	-	-	+
B13155	NBM		Coni Meier	-	-	-	+	-	-	-	+
B616	Sepsis		Coni Meier		-	-	+	-	-	-	+
E642 (O6:K15)			JH115		+	+	+	+	+	+	+
E351 (O6:K45)			JH112		+	+	-	-	-	-	+
E457 (O6:K5)					-	-	+	-	-	-	+
BK658			JH131		-	-	+	-	-	-	+
n=15; positiv from					**2**	**6**	**3**	**1**	**1**	**1**	**15**
Percent					**13%**	**40%**	**20%**	**7%**	**7%**	**7%**	**100%**
Human bloodisolates (H. Karch)											
HK1 2656/93			JH535		-	+	-	-	-	-	+
HK2 1939/93			JH536		-	-	+	-	-	-	+
HK4 9855/93			JH537	longer	+	+	+	-	-	-	+
HK8 269/93			JH541		-	+	-	-	-	-	+
HK17 2770/93			JH548		-	-	-	-	-	-	+
HK19 2882/93			JH550		+	-	+	-	-	-	+
HK24 10413/93			JH554	shorter	-	+	-	-	-	-	+
HK25 10968/93			JH555		-	-	-	-	-	-	+
HK54 10209/93			JH570	-	-	+	+	-	-	-	-
HK58 4549/93			JH573	-	-	+	+	-	-	-	+
n=10; positive from					**2**	**6**	**4**	**0**	**0**	**0**	**9**
Percent					**20%**	**60%**	**40%**	**0%**	**0%**	**0%**	**90%**

PAI V-specific PCRs

Strain	Origin	Serotyp	Strainnr.	Characteris.	M436/ M805c	*sfpC* fw/*sfpC* rev	*pgtC* fwRT/*pgtC* revRT	ellCK5fw/ellCK5rev	RTfw13165/RTrev14167	orf4bico/orf5bico	*kpsM* RTfw/*kpsM* RTrev	*gspD* fwRT/*gspD* revRT
						sfpC	*pgtC*	*kpsC* (K5)	*kpsC* (K10)	*kps* (region 2)	*kpsMT*	*gspD*
Other E. coli-groups												
933W	EHEC		Inge M. 28			-	-	-	-	-	-	-
86-24	EHEC		Inge M. 29		-	-	-	-	-	-	-	-
SF493/89	EHEC	O157:H-				-	-	-	-	-	-	-
3574/92	EHEC					-	-	-	-	-	-	-
2907/97	EHEC					-	-	-	-	-	-	+
5729/96	EHEC					-	-	-	-	-	-	+
3697/97	EHEC					-	-	-	-	-	-	-
ED 142	EHEC					-	-	-	-	-	-	+
E2348/69	EPEC		Inge M. 19		-	-	-	-	-	-	-	+
179/2	EPEC					-	-	-	-	-	-	+
156 A	EPEC					-	-	-	-	-	-	+
37-4	EPEC					-	-	-	-	-	-	-
C9221a	ETEC		Uli			-	-	-	+	-	+	+
EDL1284	EIEC		Inge M. 18		-	-	-	-	-	-	-	-
76-5	EIEC					-	-	-	-	-	-	-
DPT065	EaggEC		Zhang 88			-	-	-	-	-	-	+
5477/94	EaggEC					-	-	+	-	-	-	-
17-2	EaggEC					-	-	-	-	-	-	+
n=18; positive from						**0**	**0**	**1**	**1**	**0**	**1**	**9**
Percent						**0%**	**0%**	**6%**	**6%**	**0%**	**6%**	**50%**
Controlls												
MG1655					-	-	-	-	-	-	-	-
PCR-Mix without Template						-	-	-	-	-	-	-
all n =												
135/144 ; positive from						**14/14**	**55/56**	**48/50**	**21/22**	**6**	**8**	**101/108**
Percent						**10%/10%**	**41%/39%**	**36%/35%**	**16%/15%**	**4%/3%**	**6%/6%**	**75%/75%**

A/6/1 – Remobilisation experiments from strain SY327 / 77 into the recipient 536/21

Starting conditions of conj.			OD 600			CFU	Results of the conjugation													
	Donor:Recipent ratio		77->0,735	-6	20				CFUs		Ratio aft	No. of recipients	CFUs of					No. of conjugants	Efficiency of
	77:536/21		536/21->0,735	-6	55		77 (Cm/Nal)		536/21 (Sm)		conjug.	in 1ml	transconjugants					in 1ml	conjugation
A37	1800:300	2,2:1					-7	110	-7	50	2,2:1	5000000000	-1	lot	-2	270		270000	**5,4x10-5**
B30							-8	21	-8	4	5,2:1	4000000000	-1	lot	-2	480		480000	**1,2x10-4**
C20							-7	150	-7	22	6,8:1	2200000000	-1	380	-2	28		28000	**1,2x10-5**
D37	1900:100	6,9:1					-7	365	-7	9	40,5:1	900000000	-1	300	-2	38		38000	**4,2x10-5**
E30							-8	24	-6	83	28,9:1	830000000	-1	590	-2	70		70000	**8,4x10-5**
F20							-7	140	-7	17	8,2:1	1700000000	-1	170	-2	17		17000	**1x10-5**
G37	2000:30:00	24,2:1					-7	364	-7	6	60,6:1	600000000	-1	246	-2	26		26000	**4,3x10-5**
H30							-8	26	-6	75	34,6:1	750000000	-1	430	-2	45		45000	**6x10-5**
I20							-7	208	-7	26	8,0:1	2600000000	-1	50	-2	5		5000	**1,9x10-5**



Remobilization experiments were carried out with the donor strains SY327 / 77 (A/6/1) and SY327 / 23 (A/6/2) where the mobilised PAI II_{536} was present as a stabile circular form and with the donor strain SY327 / 54 where the mobilised PAI II_{536} was integrated into the chromosome. In all cases the recipient strain was 536-21. In each experiment the donor and recipient strains were mixed in different concentrations and exact cell numbers were determined. Cell ratio was calculated before and after conjugation. Conjugation efficiency was calculated (last column). Transconjugants were selected on M9 minial medium with lactose and Cm. All the transconjugants were 536-21 where PAI was conjugated. Some transconjugants were also checked for integration into the tRNA gene *leuX*. 41,42, 43 –A/6/1; 101, 102, 103 – A/6/2; 161, 162, 163 – A/6/3.

A/6/2 - Remobilisation experiments from strain SY327 / 23 into the recipient 536/21

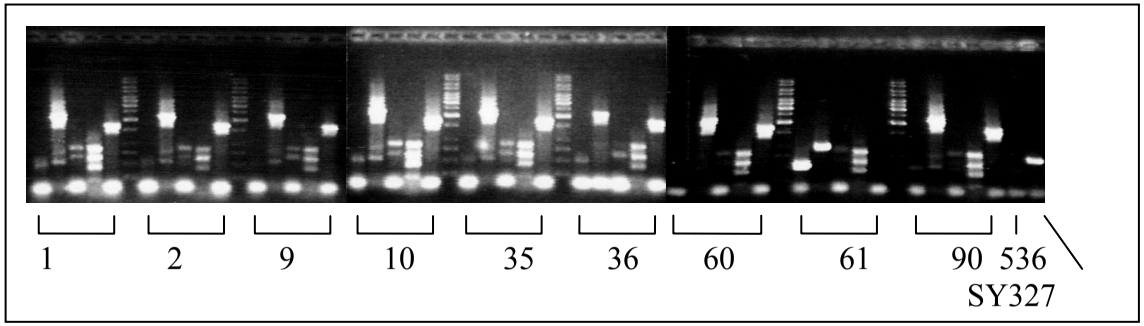
Starting conditions of conj.			OD 600		CFU	**Results of the conjugation**															
	Donor:Recipent ratio		23->0,67	-6	22			CFUs		Ratio aft.	No. of recipients			CFUs of					No. of conjugants	Efficiency of
NoTemp	23:536/21		536/21->0,73	-6	55	23 (Cm/Nal)		536/21 (Sm)		conjug.	in 1ml			transconjugants					in 1ml	conjugation
A37	1800:500	1,44:1				-7	305	-7	13	23,5:1	1300000000	T	2	-1		-2			20	**1x10-9**
B30						-8	19	-7	41	4,6:1	4100000000	T		-1	3	-2			300	**7,3x10-8**
C20						-8	2	-7	100	1,0:5,0	10000000000	T		-1		-2			0	**0**
D37	1900:100	7,6:1				-7	426	-7	11	38,7:1	1100000000	T		-1		-2			0	**0**
E30						-8	31	-7	8	38,7:1	800000000	T	1	-1		-2			1	**1x10-9**
F20						-8	21	-7	105	2,0:1,0	10500000000	T	2	-1	33	-2			3300	**3,1x10-6**
G37	2000:20:00	40,0:1				-7	295	-7	2	147,5:1	200000000	T		-1		-2			0	**0**
H30						-8	21	-7	6	35,0:1	600000000	T	4	-1		-2			40	**6,6x10-8**
I20						-8	9	-7	75	1,2:1	7500000000	T		-1	15	-2	5		5000	**6,6x10-7**

Starting conditions of conj.			OD 600		CFU	**Results of the conjugation**															
	Donor:Recipent ratio		23->0,75	-6	34			CFUs		Ratio aft	No. of recipients			CFUs of					No. of conjugants	Efficiency of
NoTemp	23:536/21		536/21->0,66	-6	32	23 (Cm/Nal)		536/21 (Sm)		conjug.	in 1ml			transconjugants					in 1ml	conjugation
A37	1800:500	2,2:1				-8	60	-6	205	30,1:1	2050000000	T		-1		-2			0	**0**
B30						-8	19	-7	55	3,5:1	5500000000	T		-1	17	-2			1700	**3x10-7**
C20						-8	8	-7	55	1,45:1	5500000000	T		-1	10	-2	1		1000	**1,8x10-7**
D37	1900:100	6,9:1				-8	57	-6	40	142,5:1	400000000	T		-1		-2			0	**0**
E30						-8	35	-7	12	29,1:1	1200000000	T		-1	3	-2			300	**2,5x10-7**
F20						-8	13	-7	40	3,25:1	40000000000	T	1	-1	23	-2			2300	**5,75x10-7**
G37	2000:20:00	24,2:1				-8	80	-6	16	500,1:1	160000000	T		-1		-2			0	**0**
H30						-8	30	-7	2	150,1:1	200000000	T	1	-1	1	-2			100	**5x10-7**
I20						-8	3	-7	60	02:01	6000000000	T		-1	10	-2			1000	**1,6x10-7**

A/6/3 – Remobilisation experiments from strain SY327 / 54 into the recipient 536/21

Starting conditions of conj.			OD 600	CFU		Results of the conjugation														
	Donor:Recipent ratio		54->0,73	-6	17			CFUs		Ratio aft.	No. of recipients			CFUs of				No. of conjugants	Efficiency of
NoTemp	54:536/21		536/21->0,75	-6	55	54 (Cm/Nal)		536/21 (Sm)		conjug.	in 1ml			transconjugants				in 1ml	conjugation
A37	1800:500	1,1:1				-7	280	-7	10	28,0:1	1000000000	T	1	-1		-2		10	**1x10-8**
B30						-8	18	-7	42	180,0:42	4200000000	T		-1	9	-2		900	**2,14x10-7**
C20						-8	4	-7	110	1,0:2,75	11000000000	T		-1	9	-2	1	1000	**9x10-7**
D37	1900:100	5,8:1				-7	370	-7	18	20,5:1	1800000000	T		-1		-2		0	**0**
E30						-8	23	-7	11	20,9:1	1100000000	T	1	-1	3	-2		300	**2,7x10-7**
F20						-8	15	-7	95	1,6:1	9500000000	T		-1	20	-2	3	3000	**3,15x10-7**
G37	2000:20:00	30,9:1				-7	450	-7	5	90,0:1	5000000000	T	2	-1		-2		20	**4x10-8**
H30						-8	30	-7	42	7,1:1	4200000000	T		-1	1	-2	1	100	**2,3x10-8**
I20						-8	20	-7	100	2,0:1	10000000000	T		-1	60	-2	1	6000	**1x10-8**

Starting conditions of conj.			OD 600	CFU		Results of the conjugation														
	Donor:Recipent ratio		54->0,73	-6	34			CFUs		Ratio aft.	No. of recipients			CFUs of				No. of conjugants	Efficiency of
NoTemp	54:536/21		536/21->0,75	-6	32	54 (Cm/Nal)		536/21 (Sm)		conjug.	in 1ml			transconjugants				in 1ml	conjugation
A37	1800:500	2,2:1				-8	49	-6	165	29,6:1	1650000000	T		-1		-2		0	**0**
B30						-8	36	-7	32	11,2:1	3200000000	T		-1	17	-2	2	2000	**6,25x10-7**
C20						-8	12	-7	115	1,04:1	11500000000	T		-1	24	-2	1	2400	**2x10-7**
D37	1900:100	6,9:1				-8	60	-6	65	92,3:1	650000000	T		-1		-2		0	**0**
E30						-8	52	-7	15	34,6:1	1500000000	T	2	-1	3	-2	1	1000	**6,6x10-7**
F20						-8	10	-7	100	01:01	10000000000	T		-1	45	-2	1	4500	**4,5x10-7**
G37	2000:20:00	24,2:1				-8	45	-6	8	562,5:1	80000000	T		-1		-2		0	**0**
H30						-8	30	-7	3	100,1:1	300000000	T		-1		-2		0	**0**
I20						-8	17	-7	110	1,5:1	11000000000	T		-1	18	-2	4	4000	**3,63x10-7**



A/7 - Competitive growth experiments between 536-21 and SY327 containing different fragments from PAI II_{536}

	Starting conditions **X : 536-21**					after 24h incubation **X : 536-21**	
X		µl : µl	cell number	ratio		cell number	ratio
21	A	1800 : 500	$5,4x10^{-7} : 3x10^{-8}$	0,18:1		$2,1x10^{-8} : 9,6x10^{-9}$	0,022:1
	B	1900 : 100	$5,7x10^{-7} : 6x10^{-7}$	0,95:1		$6,1x10^{-8} : 2,1x10^{-9}$	0,3:1
	C	2000 : 20	$6x10^{-7} : 1,2x10^{-7}$	5:1		$5,3x10^{-8} : 8x10^{-8}$	0,66:1
22	A	1800 : 500	$8,1x10^{-8} : 3x10^{-8}$	2,7:1		$2,3x10^{-10} : 1x10^{-10}$	0,47:1
	B	1900 : 100	$8,5x10^{-8} : 6x10^{-7}$	14,2:1		$3,1x10^{-10} : 5,1x10^{-9}$	6:1
	C	2000 : 20	$9x10^{-8} : 1,2x10^{-7}$	75:1		$5x10^{-10} : 8x10^{-8}$	62:1
27	A	1800 : 500	$2,6x10^{-8} : 3x10^{-8}$	0,87:1		$2,3x10^{-10} : 7,7x10^{-9}$	3:1
	B	1900 : 100	$2,8x10^{-8} : 6x10^{-7}$	4,6:1		$2,4x10^{-10} : 1,7x10^{-9}$	13,8:1
	C	2000 : 20	$2,9x10^{-8} : 1,2x10^{-7}$	24:1		$2,4x10^{-10} : 6x10^{-8}$	39,5:1
28	A	1800 : 500	$7,2x10^{-7} : 3x10^{-8}$	0,24:1		$3x10^{-8} : 8x10^{-9}$	0,038:1
	B	1900 : 100	$7,6x10^{-7} : 6x10^{-7}$	1,2:1		$1,1x10^{-9} : 5,6x10^{-10}$	0,2:1
	C	2000 : 20	$8x10^{-7} : 1,2x10^{-7}$	6,6:1		$5x10^{-8} : 6x10^{-8}$	0,83:1
77	A	1800 : 500	$2,5x10^{-8} : 3x10^{-8}$	0,83:1		$1,5x10^{-10} : 4,5x10^{-9}$	3,3:1
	B	1900 : 100	$2,6x10^{-8} : 6x10^{-7}$	4,37:1		$1,7x10^{-10} : 7x10^{-8}$	24,5:1
	C	2000 : 20	$2,8x10^{-8} : 1,2x10^{-7}$	23:1		$1,7x10^{-10} : 2x10^{-8}$	86:1
SY3	A	1800 : 500	$1,1x10^{-7}:3x10^{-8}$	0,036:1		$1x10^{-9} : 3,8x10^{-10}$	0,026:1
27	B	1900 : 100	$1,1x10^{-7}:6x10^{-7}$	0,19:1		$2,9x10^{-9} : 4,3x10^{-10}$	0,067:1
	C	2000 : 20	$1,2x10^{-7}:1,2x10^{-7}$	1:1		$3,9x10^{-9} : 2,3x10^{-10}$	0,16:1

A/8 – Publications

Benedek O, Khan AS, Schneider Gy, Nagy G, Autar R, Pieters RJ, Emődy L.
Mapping the laminin binding site of Yersinia pestis plasminogen activator (Pla) via phage display.
In press- Accepted for publication in International Journal of Medical Microbiology

* **Schneider G, Dobrindt U, Bruggemann H, Nagy G, Janke B, Blum-Oehler G, Buchrieser C, Gottschalk G, Emody L, Hacker J.**
The pathogenicity island-associated K15 capsule determinant exhibits a novel genetic structure and correlates with virulence in uropathogenic Escherichia coli strain 536.
Infect Immun. 2004 Oct;72(10):5993-6001.

* **Hacker J, Hochhut B, Middendorf B, Schneider G, Buchrieser C, Gottschalk G, Dobrindt U.**
Pathogenomics of mobile genetic elements of toxigenic bacteria.
Int J Med Microbiol. 2004 Apr;293(7-8):453-61. Review.

Fekete PZ, Schneider G, Olasz F, Blum-Oehler G, Hacker JH, Nagy B.
Detection of a plasmid-encoded pathogenicity island in F18+ enterotoxigenic and verotoxigenic Escherichia coli from weaned pigs.
Int J Med Microbiol. 2003 Aug;293(4):287-98.

Szakal D D, Schneider G, Pal T.
A colony blot immune assay to identify enteroinvasive Escherichia coli and Shigella in stool samples.
Diagn Microbiol Infect Dis. 2003 Mar;45(3):165-71.

* **Dobrindt U, Blum-Oehler G, Nagy G, Schneider G, Johann A, Gottschalk G, Hacker J.**
Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic Escherichia coli strain 536.
Infect Immun. 2002 Nov;70(11):6365-72.

Nagy G, Dobrindt U, Schneider G, Khan AS, Hacker J, Emody L.
Loss of regulatory protein RfaH attenuates virulence of uropathogenic Escherichia coli.
Infect Immun. 2002 Aug;70(8):4406-13.

* : papers based on the topic of the PhD Thesis

A/9 – Curriculum Vitae

Name: György Schneider
Place of birth: Szeged, Hungary
Date of birth: 06. September, 1971
Address: 7632 Pécs, Zsuzsanna street 2., Hungary

Schools:

1985-89 Leöwey Klára Secondary School, Pécs (H)

1989-90 Intensiv language course (English)

1990-94 Janus Pannonius University of Pécs, Faculty of Biology and Chemistry; Qualification: *teacher of biology and chemistry*

1994-97 Eötvös Lóránd University, Budapest, Faculty of Microbiology Qualification: *microbiologist*

1997- Medical University of Pécs, Institute of Immunology and Medical Microbiology, PhD student

2000-04 Bayerische Julius-Maximilianus Universität, Würzburg, Research Center for Infectious Diseases, PhD student

2004- Medical University of Pécs, Institute of Immunology and Medical Microbiology