The Pathogenicity Island-Associated K15 Capsule Determinant Exhibits a Novel Genetic Structure and Correlates with Virulence in Uropathogenic *Escherichia coli* Strain 536

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The K15 capsule determinant of uropathogenic *Escherichia coli* strain 536 (O6:K15:H31) is part of a novel 79.6-kb pathogenicity island (PAI) designated PAI V₅₃₆ that is absent from the genome of nonpathogenic *E. coli* K-12 strain MG1655. PAI V₅₃₆ shows typical characteristics of a composite PAI that is associated with the *pheV* tRNA gene and contains the *pil* fimbriae determinant as well as genes coding for a putative phosphoglycerate transport system, an autotransporter protein, and hypothetical open reading frames. A gene cluster coding for a putative general secretion pathway system, together with a *kps*_{K15} determinant, is localized downstream of a truncated *pheV* gene (*'pheV*) also present in this chromosomal region. The distribution of genes present on PAI V₅₃₆ was studied by PCR in different pathogenic and nonpathogenic *E. coli* isolates of various sources. Analysis of the 20-kb *kps* locus revealed a so far unknown genetic organization. Generally, the *kps*_{K15} gene cluster resembles that of group 2 and 3 capsules, where two conserved regions (regions 1 and 3) are located up- or downstream of a highly variable serotype-specific region (region 2). Interestingly, recombination of a group 2 and 3 determinant may have been involved in the evolution of the K15 capsule-encoding gene cluster. Expression of the K15 capsule is important for virulence in a murine model of ascending urinary tract infection but not for serum resistance of *E. coli* strain 536.

The production of extracellular polysaccharides is a common feature of many bacteria (58). Polysaccharides are also components of the bacterial capsule, which often constitutes the outermost layer of the cell and mediates the interaction between the bacterium and its immediate environment. Additionally, capsules play a crucial role for the survival of bacteria in hostile environments. More than 80 chemically and serologically distinct capsules have been described for *Escherichia coli* (25). According to their genetic organization, regulation of expression, and biosynthetic mechanism, four capsule groups (groups 1 to 4) have been distinguished (57).

Group 1 and 4 capsules resemble those expressed by *Klebsiella* and *Erwinia* spp., and their determinants are located within a highly polymorphic region at 45 min of the *E. coli* K-12 chromosome, which contains genes required for biosynthesis of a number of cell surface-associated polysaccharides (6).

The organization of gene clusters coding for group 2 and 3 capsules resembles those of *Neisseria meningitidis* and *Haemophilus influenzae*, respectively. They exhibit a conserved modular genetic organization consisting of three regions (Fig. 1). A highly variable antigen-specific region (region 2) that encodes the enzymes required for polysaccharide biosynthesis is flanked by two conserved regions (region 1 and 3) coding for proteins involved in the secretion of capsule components.

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 (6, 8, 45, 57). In the case of group 2 capsules, two additional genes, *kpsF* and *kpsU*, belong to the gene cluster. Whereas the role of KpsF is unknown, the KpsU protein is a CMP-3-keto-3-deoxy-mannooctulosonate synthetase, which is a ligase necessary for successful translocation of sugars through the inner membrane (45). The majority of the known group 2 and 3 capsule-encoding gene clusters are located within the genome at a position corresponding to 64 min of the *E. coli* K-12 chromosome and are associated with the tRNA genes *serA* and *pheV*, respectively (41, 42, 55). tRNA loci frequently serve as chromosomal insertion sites of pathogenicity islands (PAIs). This type of genetic element has been recently reviewed (20, 21). For uropathogenic *E. coli* (UPEC) strain 536 (O6:K15:H31), the genetic organization and gene content of four PAIs have been characterized in detail. Important virulence factors of UPEC are encoded on these PAIs, e.g., alpha-hemolysin, P- and S-fimbrial adhesins, and the siderophore system yersiniabactin (16). In addition to these PAI-borne virulence-associated genes, smaller chromosomal DNA regions within the genome of strain 536 have been characterized that code for virulence-associated factors that are also important for pathogenesis, e.g., the hemin receptor (ChuA) (38), the enterobactin siderophore system, and a lipopolysaccharide (LPS) of serogroup O6. The K15 capsule locus of strain 536, however, has not been characterized so far.

To identify so far unknown genomic regions of UPEC strain

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FIG. 1. Structural similarities between the K15 capsule determinant and group 2 and 3 capsule gene clusters. (A) Genetic organization of group 2 and 3 capsule gene clusters. Two conserved regions (regions 1 and 3) encode enzymes required for capsule transport through the inner membrane and are localized up- and downstream of a highly variable serotype-specific region (region 2). (B) In UPEC strain 536, the genes *kpsFEDUC'*_{K5} of region 1 are highly homologous to region 1 of group 2 capsules, whereas the *kpsS* and *kpsC_{K10}* genes exhibit a higher homology to the corresponding genes of group 3 capsules. The nonfunctional ORF65 that exhibits a high homology to transposase-encoding ORFs and interrupts region 1 is indicated by a hatched arrow.

536 which are absent in nonpathogenic *E. coli* strain MG1655, the suppression subtractive hybridization (SSH) approach has been used before (24). One of the SSH fragments obtained showed homology to the capsule export protein-encoding gene *kpsC* of K10 capsules. Southern blot hybridization of genomic fragments of UPEC strain 536 separated by pulsed-field gel electrophoresis with a *kpsC_{K10}*-specific probe indicated that this SSH fragment was associated with the *pheV* tRNA-encoding gene. The fact that PAIs carrying capsule gene clusters have also been mapped near the tRNA genes *serA* and *pheV* in other *E. coli* strains (6, 11, 52) suggested that the K15 capsule determinant of strain 536 was localized on a PAI, designated PAI V₅₃₆, as well. One aim of this study was the characterization and sequence determination of this PAI. Beside the structural analysis, functional studies have also been performed to elucidate the role of PAI V₅₃₆ and the K15 capsule in uropathogenesis.

In addition, the prevalence of PAI V₅₃₆-specific sequences was investigated with different pathogenic and nonpathogenic *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. A collection of 136 *E. coli* strains was used in this study and includes the so-called IMIB (Institut für Molekulare Infektionsbiologie) collection which has already been used for the investigation of the distribution of PAI I₅₃₆ to IV₅₃₆ of UPEC strain 536 (15, 16). Bacterial mutants, plasmids, and cosmids used or constructed in this study are listed in Table 1. The strains were grown in Luria-Bertani (LB) broth (49). When necessary, the antibiotics ampicillin (100 µg/ml), tetracycline (5 µg/ml), kanamycin (40 µg/ml), chloramphenicol (20 µg/ml), and streptomycin (100 µg/ml) or

sucrose (7% wt/vol) were added to the medium at the concentrations indicated in parentheses.

DNA techniques. Qiagen products (Hilden, Germany) were used to isolate plasmid and cosmid DNA and to purify DNA fragments or PCR products. For cloning experiments, the Expand Long template PCR system (Roche Diagnostics, Mannheim, Germany) and the Dap Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) were used. For the screening of bacterial strains or the genomic cosmid library of strain 536 and for the verification of mutants, *Taq* DNA polymerase (Qiagen) was used. Primers were obtained from Sigma-Genosys (Taufkirchen, Germany) while restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany) or Promega (Mannheim, Germany). For Southern blot and colony blot hybridization, DNA or bacterial colonies were transferred to Nytran Supercharge nylon membranes (Schleicher & Schuell BioSciences, Dassel, Germany). Hybridization with and detection of horseradish peroxidase-labeled probes was performed with the ECL labeling and signal detection system (Amersham Biosciences, Freiburg, Germany). Inactivation of PAI V₅₃₆-localized genes was carried out by allelic exchange by using the suicide vector pCVD442 as described before (35, 36) or by using linear DNA fragments as previously described (14). The complete list of primers used for gene inactivation is available as supplemental material at the homepage of the *Enterobacteria* research group of the Institute for Molecular Biology of Infectious Diseases (<http://www.uni-wuerzburg.de/infektionsbiologie/imi-start.htm>).

RNA techniques. A small quantity of total RNA was isolated from *E. coli* by using the RNeasy purification kit (Qiagen). Large quantities of total RNA were extracted from 10 ml of bacterial cultures which were immediately mixed with an equal volume of crushed ice and harvested by centrifugation at 4°C. The cells were resuspended in 0.6 ml of an ice-cold buffer containing 10 mM KCl, 5 mM MgCl₂, and 10 mM Tris (pH 7.4) and then immediately added to 0.6 ml of hot lysis buffer (0.4 M NaCl, 40 mM EDTA, 1% sodium dodecyl sulfate) containing 1% β-mercaptoethanol and 100 µl of water-saturated phenol. This mixture was incubated at 100°C for 40 s. Cell debris was removed by centrifugation for 10 min at room temperature, and the supernatant was then extracted five times with phenol-chloroform, ethanol precipitated, washed, and dried. The dried RNA pellet was dissolved in sterile diethyl pyrocarbonate-treated water.

For reverse transcription-PCR (RT-PCR), 15 µg of RNA was digested with 0.5 U of RNase-free DNase I (Roche Diagnostics)/µl for 1 h at 37°C. After purification with RNeasy mini columns (QIAGEN), 2 to 5 µg of RNA was mixed with 3 µg of random hexamer primers (Amersham Biosciences) and heated for 10 min at 70°C. After cooling down on ice for 5 min, cDNA synthesis was performed with the Super Script II RNase H reverse transcriptase system (Gibco BRL, Eggenstein, Germany) at 46°C. After 1 h, the reaction was stopped by alkaline hydrolysis of RNA with 0.25 M NaOH (15 min, 65°C). The neutral pH of the sample was restored by the addition of 50 mM Tris-HCl (pH 7.5) before the cDNA was used as a template for PCRs.

DNA sequencing and sequence analysis. Overlapping clones of a genomic cosmid library were screened by PCR and colony blot hybridization. Cosmid clones (pCos6b38, pCos24/23, pCos25/52, and pCos2/13) covering the entire PAI V₅₃₆ and its flanking regions of *E. coli* strain 536 were sequenced as follows. Small insert libraries (2 to 2.5 kb) were generated by mechanical shearing of cosmid DNA (40). After end repair with T4 polymerase, the fragments were ligated into the prepared pTZ19R vector. Isolated plasmids were sequenced from both ends by using dye-terminator chemistry and analyzed on ABI-377 automated DNA sequencers (Applied Biosystems, Weiterstadt, Germany). After assembly, the remaining gaps were closed by primer walking on the plasmid clones. The Phrap software implemented in the STADEN software package was used for assembling and editing the sequence data (51). The nucleotide sequence of the complete PAI V₅₃₆ was submitted to the EMBL database. Homology searches were performed with the BLASTN, BLASTX, and PSI- and PHI-BLAST programs of the National Center for Biotechnology Information (3). The percent G+C plot of the *kps_{K15}* determinant was calculated with a window size of 150 bp with VectorNTI (InforMax Inc., Frederick, Md.).

Production of an antiserum directed against the K15 capsule. Serum against the K15 capsule was produced according to the method described by Kiesewalter and Selmann (28). Briefly, bacteria (*E. coli* strain 536) were incubated overnight in 50% ethanol at 4°C to denature protein antigens. After washing, bacteria were diluted to 2 × 10⁸ CFU/ml in phosphate-buffered saline (PBS). Rabbits were immunized with 0.2, 0.5, 1.0, and 2.0 (twice) ml of bacterial suspensions at 4-day intervals. Four days following the last inoculation, serum samples were taken and tested by tube agglutination. Afterwards, the rabbits were sacrificed. Their sera were collected and diluted 1:10 in sterile saline containing 0.3% phenol as a preservative. The removal of nonspecific antibodies (e.g., against LPS or flagellar sequential epitopes) was achieved by absorption with live and boiled cells of the encapsulated but K15 capsule-negative UPEC strain RZ532 (O6:K⁺:H31). In

TABLE 1. Mutants, plasmids, and cosmids used in this study

Bacterial strain, cosmid, or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
536	UPEC isolate (O6:K15:H31), Sm ^r	7
536Δ <i>kpsSC'</i> _{K5}	536, partial deletion of capsule region 1, Sm ^r , Km ^r	This study
536ΔR2	536, deletion of capsule region 2 mutant, Sm ^r , Cm ^r	This study
536Δ <i>kpsMT</i>	536, deletion of capsule region 3 mutant, Sm ^r , Cm ^r	This study
536Δ <i>kpsC'</i> _{K5}	536, deletion of <i>kpsC'</i> _{K5} , Sm ^r , Cm ^r	This study
536Δ <i>kpsC</i> _{K10}	536, deletion of <i>kpsC</i> _{K10} , Sm ^r , Cm ^r	This study
536Δ <i>kps</i> _{K15}	536, deletion of the complete K15 <i>kps</i> locus, Sm ^r , Cm ^r	This study
536Δ <i>gsp</i>	536, deletion of <i>gspLKJIHGFED</i> genes, Sm ^r , Cm ^r	This study
IMI402	Enterotoxigenic <i>E. coli</i> isolate 34344f (O6:K15:H ⁻)	IMIB strain collection
IMI403	Enterotoxigenic <i>E. coli</i> isolate C9221a (O6:K15:H16)	15
IMI415	<i>E. coli</i> isolate E642 (O6:K15)	IMIB strain collection
SM10λpir	<i>thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA::RP4-2-Tc::Mu Km^r</i>	35
SY327	F ⁻ <i>araD</i> Δ(<i>lac pro</i>) <i>argE</i> (Am) <i>recA56</i> Rif ^r <i>nalA</i> λpir	35
Plasmids and cosmids		
pKD46	Arabinose-inducible λ Red recombinase expression plasmid (<i>oriR101 repA101^{ts} P_{araB}-gam-bet-exo Ap^r</i>)	14
pKD3	Template plasmid for amplification of a <i>cat</i> cassette flanked by FLP recognition sites (FRT) (<i>oriRγ</i> , Ap ^r Cm ^r)	14
pKD4	Template plasmid for amplification of a <i>kan</i> cassette flanked by FLP recognition sites (FRT) (<i>oriRγ</i> Ap ^r Kan ^r)	14
pCP20	Plasmid with temperature-sensitive replication for temporal production of the FLP recombinase (FLP ⁺ λ c1857 ⁻ λ p _R Rep ^{ts} Cm ^r Ap ^r)	10
pBeloBAC11	Bacterial artificial chromosome cloning vector	29
pCVD442	Suicide vector for gene inactivation (<i>sacB ori</i> ColE1 Ap ^r)	18
pCos6b38	Cosmid clone harboring partial sequences of PAI V ₅₃₆ , Ap ^r	24
pCos25/52	Cosmid clone harboring partial sequences of PAI V ₅₃₆ , Ap ^r	This study
pCos24/23	Cosmid clone harboring partial sequences of PAI V ₅₃₆ , Ap ^r	This study
pCos2/13	Cosmid clone harboring downstream sequences of PAI V ₅₃₆ , Ap ^r	This study

brief, a 0.5-g aliquot of wet bacteria was suspended in 1 ml of the diluted serum and the suspension was incubated for 1 h at 37°C and overnight at 4°C. The next day, bacterial cells were harvested, resuspended in saline, and boiled for 1 h. Following centrifugation, the pellet was resuspended in the same serum and suspensions were incubated again for 1 h at 37°C and subsequently overnight at 4°C. After centrifugation, aliquots of serum were stored at -20°C. Due to this procedure, the resulting sera exhibited only minimal immunogenicity to minor protein antigens. The efficient detection of the K15 antigen was proven by enzyme-linked immunosorbent assay (ELISA).

ELISA. 96-well plates (CML-CEB, Nemours, France) were coated overnight with 0.2 ml of bacterial suspension (10⁹ CFU/ml) at 4°C. The following day, plates were washed with PBS plus 0.5% Tween 20 (washing buffer) and then blocked with PBS containing 2% bovine serum albumin (Sigma) for 1 h at 37°C. Anti-K15 serum was diluted in PBS plus 0.5% bovine serum albumin and incubated with antigen-coated plates for 90 min. Serial dilutions were conducted across the plates. After three washes, plates were probed with commercial anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Dako) dissolved in citric acid buffer containing H₂O₂. The 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.

Immunofluorescence microscopy. One milliliter of overnight cultures was washed once with saline and resuspended in water, and 20 μl was dropped on a glass slide. After drying (37°C for 20 min), a 1:4 dilution of the absorbed K15 antiserum was added. After 1 h of incubation at 37°C, the glass slide was carefully washed twice with PBS. Texas Red-labeled anti-rabbit immunoglobulin G (Dianova, Hamburg, Germany) was added, and the slide was incubated for 1 h at 37°C. After incubation, the slides were washed in PBS and analyzed by epifluorescence microscopy.

Serum bactericidal test. Bacteria grown in LB medium were washed with saline and diluted to 10⁶ cells/ml. One hundred-microliter aliquots were mixed with an equal volume of fresh human serum and incubated at 37°C for 4 h in microtiter plates. Samples were taken at 0-, 0.5-, 1-, 2-, 3-, and 4-h time points. The viable cell count was determined by plating aliquots of the sample onto LB plates and overnight incubation at 37°C. Assays were performed with normal and

heat-inactivated serum (56°C for 30 min). Triplicates were used for each strain, and every assay was performed 3 times.

Ascending urinary tract infection (UTI) model. Intravesical infections of 2- to 3-day-old suckling CFLP mice (Gödöllő, Hungary) were performed as previously described (39). Six to 14 infant mice were injected per strain, and the assays were repeated four times. Bacteria were grown overnight at 37°C in LB medium, harvested by centrifugation, washed once with saline, and normalized to the required inoculum density (10⁷/ml) in PBS. This bacterial suspension (0.025 ml) containing 0.05% Pontamin sky blue dye (Searle Pharmaceuticals, High Wycombe, United Kingdom) was introduced into the bladder directly through the abdominal wall. The stain, which had no toxic or antibacterial effects, served as an indicator of successful inoculation. Mice that survived infection were sacrificed at 21 days postinfection. The bladder and both kidneys were removed and 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. To prove that in this model mortality is due to urosepsis originating from an ascending infection which starts from the bladder and reaches the blood system through the kidneys, 3-day-old mice were infected as described above with wild-type strain 536. At different time points postinfection (1, 2, 4, 6, 8, 10, 12, 18, and 24 h), 2 mice were sacrificed. Following sterilization of the skin with iodine solution, the heart, kidneys, and bladder were removed in this order and the organs were homogenized separately (in 2 ml of 0.9% NaCl) and plated onto LB agar plates containing streptomycin. Viable counts were determined following a 24-h incubation at 37°C. According to this experiment, bacteria were permanently detectable in growing numbers in the bladder. They could only be exceptionally detected in the kidneys earlier than 4 h postinfection. From this time point on, their number increased constantly. In the hearts (blood) of the sacrificed mice, bacteria appeared as late as 12 h postinfection. This is indicative of a primary ascending route of infection with a subsequent entry of bacteria into the bloodstream in this model system.

Nucleotide sequence accession number. The nucleotide sequence of PAI V₅₃₆ has been deposited in GenBank under accession number AJ617685.

FIG. 2. Genetic structure of PAI V₅₃₆ and its downstream region. Important PAI regions are indicated as shown at the bottom of the figure. The localization of tRNA-encoding genes is indicated as well as that of the DNA regions included into the PCR screening approach for the detection of PAI V₅₃₆-specific sequences.

RESULTS

Genetic features of PAI V₅₃₆. A 105,801-bp chromosomal region of *E. coli* strain 536 comprising the complete PAI V₅₃₆ and its downstream region was subcloned and sequenced. The nucleotide sequence of PAI V₅₃₆ is available under the GenBank accession number AJ617685. This PAI is associated with the tRNA-encoding gene *pheV* located at 64 min of the *E. coli* K-12 strain MG1655 chromosomal map (Fig. 2). A truncated imperfect copy of this tRNA gene (*'pheV*) which comprises the last 22 bp of this gene's 3' end and lacks one G at position 71 of *pheV* is located 48,708 bp downstream of the intact *pheV* gene. Similarly to PAI III₅₃₆, the *pheV*-associated PAI V₅₃₆ represents a composite PAI, consisting of 58 open reading frames (ORFs) which are flanked by a 22-bp imperfect direct repeat (DR) structure (*pheV* and its truncated copy) as well as a 30,987-bp DNA region downstream of *'pheV* that also contains the determinants required for K15 capsule biosynthesis and for a type II secretion system. The composite island is 79,695 bp in size and exhibits an overall G+C content of 47.7%. Transition into the chromosomal backbone shared with *E. coli* K-12 strain MG1655 and UPEC O6 strain CFT073 occurs again upstream of gene b2970. In addition, the conserved *E. coli* chromosomal backbone is interrupted 17,969 bp further downstream between the *E. coli* K-12 genes b2981 and b2983. The IS5 transposase-encoding gene (b2982) present in the *E. coli* K-12 strain MG1655 is replaced by an 8,418-bp DNA stretch described for UPEC strain CFT073 (GenBank accession no. AE016766) and *Shigella flexneri* 2a (GenBank accession no. AE016988 and AE015315) that contains genes required for glycolate utilization as well as several hypothetical ORFs.

Several fragments of PAI V₅₃₆ are highly homologous to chromosomal regions of other extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* strains (e.g., strains CFT073, EDL933, and 239 KH 89), *S. flexneri* 2a (*she* and SRL PAIs), and *Salmonella enterica* serovar Typhimurium LT2. Moreover, PAI V₅₃₆ also contains fragments found on different virulence plasmids of *E. coli* (pB171 and pSFO15) and *Shigella* spp. (pWR501 and pWR301) as well as on the *Yersinia pestis* plasmids pMT1 and pCD1. PAI V₅₃₆ exhibits typical features of a PAI, e.g., a mosaic-like structure and the presence of multiple functional and fragmented mobile genetic elements. Several virulence-associated factors are encoded on this PAI, including the K15 capsule (ORF60 to 74), the re-

cently described Pix fimbria-encoding gene cluster (ORF6 to 13) which belongs to the P adhesin family (33), and an auto-transporter protein (ORF48) with the highest similarity to Sap, a putative autotransporter adhesin encoded on the *she* PAI of *S. flexneri* (GenBank accession no. AF326777 and AAL08472) (32). In addition to the corresponding virulence-associated determinants, a gene cluster (ORF16 to 19) is located on PAI V₅₃₆ that codes for a putative phosphoglycerate transport system (*pgt*) also present in *S. enterica* serovar Typhimurium LT2 and other UPEC strains (19, 56). A complete list of the putative ORFs located on PAI V₅₃₆ is available as supplemental material.

Transcriptional analysis of *pgtC*, *pixB*, and *pixC* expression by RT-PCR demonstrated that *pgtC* was transcribed under in vitro conditions (37°C, LB batch culture), as was *pixB*, a hypothetical negative regulator of the *pix* operon. Due to the expression of *pixB*, the structural genes of the *pix* operon were not transcribed, as no *pixC* transcript was detected by RT-PCR (data not shown).

Mobile genetic elements present on PAI V₅₃₆. Mobile genetic elements play an important role in recombination, resulting in genetic rearrangements and deletions. Insertion sequence (IS) elements and prophages are typical components of PAIs, and their high numbers indicate how frequently recombination occurs within PAIs. As a typical feature of PAIs, a P4-like bacteriophage integrase-encoding gene (ORF1) is located downstream of the *pheV* gene, the chromosomal insertion site of PAI V₅₃₆. The same type of integrase gene is reported to be present on different PAIs integrated at the same tRNA-encoding gene of different *E. coli* strains (1, 30, 43, 53, 56). In addition, several putative transposase-encoding and other IS element-associated ORFs of IS2, IS21, IS100, IS110, and IS911, as well as the R6 transposase-encoding gene of PAI II_{CFT073}, are present on PAI V₅₃₆.

Characterization of the K15 capsule determinant of *E. coli* strain 536. The *kps*_{K15} gene cluster required for K15 capsule biosynthesis was localized about 1 kb downstream of *'pheV*. The genetic organization of the capsule determinant of UPEC strain 536 combines features of both group 2 and 3 capsules (Fig. 1 and 3A). Region 1 of the *kps*_{K15} gene cluster encodes enzymes required for the transport of capsule components and consists of five ORFs (*kpsF*, *-E*, *-D*, *-U*, and *-C*). This region is highly homologous to those of K1 and K5 capsule determinants. However, the *kpsC* gene (*kpsC'*_{K5}) is truncated and the

FIG. 3. Genetic organization of the K15 capsule determinant. (A) Regions 1 to 3 of the *kps*_{K15} gene cluster are indicated by different shades of gray. The nonfunctional ORF65 is indicated by a hatched arrow. The locations of two putative integration host factor (IHF) binding sites within the *kpsF* promoter region and that of the JUMPstart (just upstream of many polysaccharide-associated gene starts) region located upstream of *kpsM* are indicated, as are the DNA regions whose transcription has been studied by RT-PCR. Fractions of the *kps*_{K15} gene cluster that could be amplified from the corresponding cDNA are marked with plus signs, and those whose transcripts were not detectable are marked with a minus sign. (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.

kpsS gene is missing relative to its K1 and K5 counterparts. Downstream of *kpsC'*_{K5}, an inactivated putative ORF (ORF65) is localized that is highly similar to the putative ORF c1248 of UPEC strain CFT073, coding for a putative transposase (NP_753162).

Interestingly, a *kpsS* gene and another complete *kpsC* gene were identified downstream of the nonfunctional ORF65 on the complementary strand. This *kpsC* gene (*kpsC*_{K10}) was homologous to *kpsC* of the *E. coli* K10 capsule type, a well-studied representative of group 3 capsules (12). *kpsS* exhibited significant similarity to the corresponding gene of *Aeromonas hydrophila* (GenBank accession no. AF375657). Due to the nucleotide sequence homology and gene order of these two genes, this DNA stretch can be considered a fragment of region 3 of a group 3 capsule determinant. The presence of a nonfunctional putative transposase-encoding ORF between *kpsC'*_{K5} and *kpsC*_{K10} indicates that region 1 of the K15 capsule determinant resulted from recombination between a group 2 and 3 capsule gene cluster involving IS element(s). Upstream of *kpsS* and *kpsC*_{K10}, region 2 of the K15 capsule locus was located, consisting of five ORFs (ORF68 to 72), without any homology on the nucleotide level. The deduced amino acid sequences of two genes (ORF71 and 72) were similar to those of a putative glycosyltransferase of *Actinobacillus* (AAC26630) and a mannosyltransferase B of *Aquifex aeolicus* strain VF5 (NP_213361), respectively. Region 3 of the K15 capsule determinant was located upstream of region 2. The encoded KpsM and KpsT proteins were shown to be highly homologous (>70% identity) to corresponding proteins of other group 2 capsules.

Analysis of the distribution of PAI V₅₃₆-specific sequences among various *E. coli* strains from different sources. To study the distribution of PAI V₅₃₆-specific sequences among 137 nonpathogenic and pathogenic *E. coli* isolates as well as to find out whether an identical repertoire of genetic information can be detected in other *E. coli* strains, PCRs were designed for the

amplification of selected regions of PAI V₅₃₆. These regions include the *pixC*, *pgtC*, and *gspD* genes as well as parts of regions 1 to 3 of the *kps*_{K15} determinant (Fig. 2). The complete list of PCR screening results is available as supplemental material at the homepage of the *Enterobacteria* research group of the Institute for Molecular Biology of Infectious Diseases (<http://www.uni-wuerzburg.de/infektionsbiologie/imi-start.htm>).

A large group of ExPEC and intestinal pathogenic *E. coli* strains as well as nonpathogenic, commensal *E. coli* strains has been screened, including the strains of the IMIB collection. The results obtained (Table 2) demonstrate that, generally, PAI V₅₃₆-specific sequences were distributed more frequently 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 rarely been detected among the strains included in this study. Within this group of strains, only three strains including UPEC isolate 536 have been shown to carry the K15 capsule determinant. 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 the notion that PAI-associated DNA regions are also detectable in commensal isolates, indicating that the commensal flora represents the natural reservoir for ExPEC strains.

Transcription analysis of the K15 capsule-encoding locus. To analyze the operon structure of the K15 capsule determinant, RT-PCRs were performed. Both *kpsC* variants (*kpsC'*_{K5} and *kpsC*_{K10}) were shown to be transcribed (see Fig. 1 in the supplemental material). According to the amplification of cDNAs representing different parts of the K15 capsule determinant, it can be assumed that region 3, region 2, and *kpsC*_{K10} and *kpsS* of region 1 represent a single transcription unit (Fig. 3B). The JUMPstart motif (CAGTGTATTGGTAGCTGTTA

TABLE 2. Detection of PAI V₅₃₆-specific sequences in various *E. coli* isolates

Source or type of <i>E. coli</i> strain (<i>n</i>)	% Presence of PAI V ₅₃₆ -specific region (gene):						
	1 (<i>pixC</i>)	2 (<i>pgtC</i>)	3 (<i>kpsC</i> _{K5})	4 (<i>kpsC</i> _{K10})	5 (ORF68 to 69)	6 (<i>kpsMT</i>)	7 (<i>gspD</i>)
UTI strains (67)	8.9	47.8 ^a	53.7	17.9	3.0	4.5	74.6
Human and animal MNEC and SEPEC isolates ^b (28)	17.9	42.9	60.7 ^a	3.6	3.6	3.6	96.4 ^a
Diarrheagenic <i>E. coli</i> pathogens (18)	0	0 ^a	5.6 ^a	5.6	0	5.6	50
Nonpathogenic strains (24)	4.2	25.0	33.3	12.5	0	0	58.3
Total (137)	8.8	36.5	45.3	12.4	2.2	3.6	73.0

^a Significantly different from the frequency of occurrence among nonpathogenic strains (chi-square probe, *P* < 0.05).
^b MNEC, meningitis-causing *E. coli*; SEPEC, sepsis-causing *E. coli*.

AGCCAAGGGCGGTAGCGTA) including the *ops* element required for efficient transcription of long operons (5) is localized upstream of *kpsM*. It is already known that region 1 of group 2 capsules is organized as a single transcription unit (57). Consequently, the genes *kpsF-kpsC'*_{K5} of the K15 capsule gene cluster may also constitute one transcription unit. Two putative integration host factor binding sites have been detected upstream of *kpsF*.

Inactivation of the K15 gene cluster and identification of the functional *kpsC* gene. To confirm that the detected putative *kps* determinant represents the functional K15 capsule-encoding gene cluster of strain 536, different mutants have been generated in which one of the regions of the *kps* determinant has been inactivated. For this purpose, the DNA regions comprising *kpsS/kpsC*_{K10}, ORF1 to 5, or *kpsMT* have been deleted as well as the complete *kps*_{K15} determinant of strain 536. Furthermore, to find out which *kpsC* gene was functional and required for expression of the K15 capsule determinant, either *kpsC'*_{K5} or *kpsC*_{K10} was inactivated. The mutants generated were tested for K15 capsule production by ELISA (Fig. 4). According to these experiments, partial deletion of one of the regions always resulted in a markedly reduced expression of

the K15 capsule. Capsule expression of these mutants was comparable to that of UPEC strain RZ532 (O6:K⁺:H31), which does not express a K15 capsule, to that of the complete *kps*_{K15} deletion mutant and of the strain 536 *rfaH* mutant, in which the transcriptional antiterminator required for *kps* expression has been inactivated (17, 39). Complementation of strain 536 Δ *kps*_{K15} with a bacterial artificial chromosome clone carrying the complete *kps*_{K15} gene cluster restored capsule expression. These results confirm that the investigated gene cluster is the functional capsule determinant of *E. coli* strain 536. The loss of capsule production upon inactivation of region 2 of the K15 *kps* gene cluster was also visualized by immunofluorescence microscopy. ELISA with an anti-K15 serum indicated that inactivation of *kpsC*_{K10} resulted in reduced K15 capsule production, whereas the *kpsC'*_{K5} mutant exhibited the same encapsulated phenotype as wild-type strain 536 (Fig. 4). This demonstrates that *kpsC*_{K10} is required for K15 capsule biosynthesis, whereas *kpsC'*_{K5} has been inactivated due to truncation.

Role of the K15 capsule and the PAI V₅₃₆-encoded general secretion pathway for serum resistance and urovirulence. Serum resistance assays demonstrated that nonencapsulated mu-

FIG. 4. Detection of K15 capsule expression of *E. coli* strain 536 and various derivatives. (A) Detection of K15 capsule by ELISA with a K15 capsule-specific antiserum. The binding of a preabsorbed polyclonal K15 capsule-specific antibody was visualized by using an anti-rabbit antibody conjugated with horseradish peroxidase. The optical density was measured at 490 nm. Values marked with an asterisk are statistically significant (*P* < 0.001) compared to the values of the wild type as calculated by the Student *t* test. (B) Detection of K15 capsule expression by immunofluorescence microscopy. The binding of a preabsorbed polyclonal K15 capsule-specific antibody was visualized by a Texas Red-labeled secondary antibody and examined by epifluorescence microscopy. Upper row, phase contrast images; lower row, fluorescence images; left column, UPEC strain 536 (K15, encapsulated); middle column, UPEC strain 536 Δ *kps*_{K15} (nonencapsulated); right column, *E. coli* K-12 strain MG1655 (negative control).

FIG. 5. Influence of the K15 capsule on serum resistance of UPEC strain 536 and various capsule-negative derivatives. Bacteria were incubated in 50% serum obtained from a healthy individual. The graph shows means $\pm$ standard errors of the means of values originated from three similar assays. The values obtained for the *rfaH*-negative mutant are significantly different ($P < 0.01$) between the time points from 1 to 4 h (Student's *t* test).

tants of strain 536 in which one of the regions of the *kps*_{K15} determinant has been partially deleted were able to grow in 50% human serum as well as the encapsulated wild-type strain. In contrast, the strain 536 *rfaH* mutant does not express K15 capsule and LPS and was not able to grow in human serum (Fig. 5). This indicates that, in contrast to LPS, the K15 capsule is not required for serum resistance of strain 536. Serum resistance of the PAI V₅₃₆-encoded general secretion pathway mutant strain 536 Δ *gsp* was not impaired relative to that of the wild type.

To analyze whether the K15 capsule contributes to virulence of UPEC strain 536, different *kps*_{K15} mutants that have been tested for K15 capsule expression before (Fig. 4) were examined in a murine model of experimental ascending UTI. According to the results of the experiments, the virulence of the different nonencapsulated mutants was markedly reduced relative to that of the encapsulated wild-type strain and the encapsulated *kpsC'*_{K5} mutant (Table 3). Consequently, the K15 capsule contributes to the urovirulence of strain 536. Inactivation of the general secretion pathway determinant localized on PAI V₅₃₆ did not affect capsule expression (see Fig. 2 in the supplemental material) and the virulence of the corresponding mutant (Table 3).

DISCUSSION

Four PAIs have so far been described in detail for UPEC strain 536 (O6:K15:H31) (16). In this study, the genetic organization of PAI V₅₃₆ was characterized and prominent gene clusters of this PAI have been subjected to functional characterization. The composite PAI V₅₃₆ exhibits typical features of PAIs, such as (i) the association with a tRNA-encoding gene, (ii) the presence of different virulence-associated genes (the *pil* fimbria determinant [33] and the K15 capsule-encoding gene cluster), and (iii) the presence of DR structures formed by the

TABLE 3. Contribution of K15 capsule and PAI V₅₃₆-encoded general secretion pathway (*gsp*) to urovirulence of *E. coli* strain 536

Strain	Characteristics	No. of infected mice	Mortality rate (%)
536 wild type	K15 capsule ⁺ , GSP ⁺	21	100
536 Δ <i>kpsSC</i>	K15 capsule ⁻ , GSP ⁺	27	14.8 ^a
536 Δ R2	K15 capsule ⁻ , GSP ⁺	20	10.0 ^a
536 Δ <i>kpsMT</i>	K15 capsule ⁻ , GSP ⁺	24	8.3 ^a
536 Δ <i>kps</i> _{K15}	K15 capsule ⁻ , GSP ⁺	21	19.0 ^a
536 Δ <i>kpsC'</i> _{K5}	K15 capsule ⁺ , GSP ⁺	12	91.7
536 Δ <i>kpsC'</i> _{K10}	K15 capsule ⁻ , GSP ⁺	13	15.4 ^a
536 Δ <i>gsp</i>	K15 capsule ⁺ , GSP ⁻	12	91.7

^a Significant difference from wild-type strain 536 (chi-square probe, $P < 0.001$).

last 22 bp of the 3' end of *pheV* and its truncated copy '*pheV*'. Similarly, PAI III₅₃₆ also represents a composite island with flanking DR structures formed by the last 47 bp of the 3' end of *thrW* and its truncated copy '*thrW*' (16). In the case of PAI V₅₃₆, the G+C content of 47.7% and the fact that this DNA region is absent in nonpathogenic *E. coli* strain MG1655 underline the fact that it has been acquired by horizontal gene transfer. In addition, several regions of PAI V₅₃₆ are highly homologous to regions of (formerly) mobile DNA elements, i.e., virulence plasmids or PAIs, bacteriophages, and other accessory DNA elements such as IS elements. In UPEC strain 536, a bacteriophage P4-like integrase gene is localized immediately downstream of the *pheV* tRNA gene. P4-like integrase genes seem also to be the typical integrase genes present on *pheV*-associated PAIs of other *E. coli* strains (1, 30, 43, 52, 53, 56).

Analysis of the gene content of PAI V₅₃₆ revealed that the putative K15 capsule determinant is located downstream of '*pheV*'. Several other *kps* gene clusters have been reported to be localized on *pheV*-associated PAIs (52, 56) or next to *pheV* (11), and it is anticipated that group 2 and 3 capsule loci are chromosomally inserted between *pheV* and *yghD* (6). Comparison of the genetic structure of the PAI V₅₃₆-localized *kps* determinant with that of three other *E. coli* K15 strains present in the IMIB strain collection (IMI402, IMI403, and IMI415) by overlapping PCRs demonstrated that all K15 isolates tested share a *kps* locus with an identical genetic organization (data not shown). This underlines the notion that the *kps* determinant localized on PAI V₅₃₆ is indeed the functional K15 capsule-encoding gene cluster.

The K15 capsule locus exhibits a so far unknown genetic organization and combines structural features of group 2 and 3 capsule determinants (Fig. 1 and 3A). Group 2 and 3 capsule gene clusters have a segmental gene organization with two conserved regions (1 and 3) flanking the serotype-specific region 2 (12, 45). The most striking difference between the *kps*_{K15} gene cluster and a typical group 2 capsule locus is the inversion of the functional *kpsS* and *kpsC*_{K10} genes relative to the rest of the genes of region 1. *kpsS* and *kpsC*_{K10}, which more closely resemble corresponding genes of group 3 capsule operons, are separated from the truncated *kpsC'*_{K5} gene by a non-functional ORF with homology to the putative transposase-encoding ORF c1248 of UPEC strain CFT073. The encoded gene product is similar to a transposase of the IS630 family (NP_929067). This indicates that truncation of *kpsC'*_{K5} and the presence of an inversed orientation of *kpsS* and *kpsC*_{K10} rela-

tive to *kpsC'*_{K5} is due to recombination between group 2 and 3 capsule gene clusters and involved IS element(s). The concomitant loss of functional *kpsC*_{K5} and *kpsS*_{K5} genes has been complemented by the horizontally acquired *kpsS* and *kpsC*_{K10} genes. The KpsS and KpsC proteins are involved in capsule maturation when the synthesized polysaccharide is ligated to phosphatidyl-3-keto-3-deoxy-mannooctulosonate (45). Clarke and coworkers demonstrated that KpsC and KpsS of group 2 and group 3 capsules are homologous and functionally interchangeable (12). The fusion of segments of two unrelated *kps* determinants is further corroborated by a dramatic decrease of the G+C content immediately downstream of *kpsC'*_{K5}. The overall G+C content of the ORF65-*kpsS*-*kpsC*_{K10} region (37.8%) is markedly lower than that of the DNA stretch comprising the *kpsFEDUC'*_{K5} genes (50.9%). Region 2 exhibits the lowest G+C content (30.9%) within the *kps*_{K15} determinant (Fig. 3B). This is typical for genes encoding enzymes involved in polysaccharide biosynthesis (44). It has been hypothesized that acquisition of different region 2 sequences by homologous recombination between flanking regions 1 and 3 of two group 2 capsule gene clusters contributes to the diversity of group 2 capsules and that the insertion of new capsule genes into an existing group 2 gene cluster may lead to the generation of group 3 capsule determinants (6). This is supported by the genetic structure of the *kps*_{K15} locus indicative of genetic rearrangements and recombination between group 2 and 3 capsule determinants that result in the generation of a new capsule type.

The KpsM and KpsT proteins are similar to ABC (ATP-binding cassette) transporters (8, 22) that are believed to be the cytoplasmic membrane component of the capsule transport system. Further steps of capsule polysaccharide translocation through the outer membrane still remain to be elucidated. It has been speculated that this process may also involve a general mechanism such as the type II general secretion pathway (GSP). Interestingly, a GSP determinant was identified 5 kb downstream of the *kps*_{K15} locus on PAI V₅₃₆. To find out whether this gene cluster in the vicinity of the *kps*_{K15} determinant codes for proteins involved in the transport of capsule polysaccharides across the outer membrane, the GSP determinant has been inactivated. However, this inactivation had no effect on K15 capsule expression, serum resistance, and virulence of *E. coli* strain 536 (Table 3 and Fig. 5; see also Fig. 2 in the supplemental material).

It has been amply reported that group 2 capsules are more frequently distributed among uropathogenic isolates than among fecal isolates (26, 27, 50). This led to the speculation that group 2 capsules contribute to urovirulence according to their ability to impede phagocytosis (13, 34, 54). The capsule has also been reported to confer resistance to the bactericidal activity of serum (2, 13, 31, 47). Furthermore, a signature-tagged mutagenesis screen has previously suggested the role of the K2 capsule of UPEC strain CFT073 in UTI (4). However, other findings do not support a role of group 2 capsules as a virulence factor of UPEC and favor a role in systemic infections rather than in an ascending UTI (47, 48). It has been suggested that the capsule protects the organism by masking the cell surface and by serving as a physical barrier to prevent bacterial attachment to phagocytic cells like polymorphonuclear leukocytes and monocytes and, consequently, to protect the cells from internalization (9, 13, 23, 37, 54). To analyze

whether the K15 capsule contributes to pathogenesis, the virulence of capsule-deficient mutants of UPEC strain 536 have been compared to that of the corresponding wild-type strain in an experimental ascending UTI model with infant mice. The data obtained demonstrate that the loss of the K15 capsule is associated with a dramatic attenuation. It has been proven that, in the experimental infection model used in this study, mortality is due to urosepsis originating from an ascending UTI which starts from the bladder and reaches the blood system through the kidneys. This indicates that the K15 capsule contributes to the urovirulence of strain 536 (Table 3) and not only to a systemic infection as reported before (46, 48). However, the K15 capsule is not required for serum resistance, as the wild-type strain 536 and the different *kps*_{K15} mutants used in this study multiplied for several hours in human serum (Fig. 5).

In summary, the study of the genetic organization of PAI V₅₃₆ 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 V₅₃₆-localized genes will have to be further elucidated.

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