

Genetic Structure and Distribution of Four Pathogenicity Islands (PAI I₅₃₆ to PAI IV₅₃₆) of Uropathogenic *Escherichia coli* Strain 536

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For the uropathogenic *Escherichia coli* strain 536 (O6:K15:H31), the DNA sequences of three pathogenicity islands (PAIs) (PAI I₅₃₆ to PAI III₅₃₆) and their flanking regions (about 270 kb) were determined to further characterize the virulence potential of this strain. PAI I₅₃₆ to PAI III₅₃₆ exhibit features typical of PAIs, such as (i) association with tRNA-encoding genes; (ii) G+C content differing from that of the host genome; (iii) flanking repeat structures; (iv) a mosaic-like structure comprising a multitude of functional, truncated, and nonfunctional putative open reading frames (ORFs) with known or unknown functions; and (v) the presence of many fragments of mobile genetic elements. PAI I₅₃₆ to PAI III₅₃₆ range between 68 and 102 kb in size. Although these islands contain several ORFs and known virulence determinants described for PAIs of other extraintestinal pathogenic *E. coli* (ExPEC) isolates, they also consist of as-yet-unidentified ORFs encoding putative virulence factors. The genetic structure of PAI IV₅₃₆, which represents the core element of the so-called high-pathogenicity island encoding a siderophore system initially identified in pathogenic yersiniae, was further characterized by sample sequencing. For the first time, multiple PAI sequences (PAI I₅₃₆ to PAI IV₅₃₆) in uropathogenic *E. coli* were studied and their presence in several wild-type *E. coli* isolates was extensively investigated. The results obtained suggest that these PAIs or at least large fragments thereof are detectable in other pathogenic *E. coli* isolates. These results support our view that the acquisition of large DNA regions, such as PAIs, by horizontal gene transfer is an important factor for the evolution of bacterial pathogens.

Pathogenicity islands (PAIs), as a distinct type of genetic element, were described for the first time for uropathogenic *Escherichia coli* strain 536 (O6:K15:H31) (2, 17), which is one of the model organisms of extraintestinal pathogenic *E. coli* (ExPEC) used for studies on ExPEC pathogenesis and the evolution of bacterial pathogens. The PAI type of genetic elements is characterized by a large size (>10 kb), the presence of virulence-associated genes, frequent association with tRNA-encoding genes or other *att* sites for temperate bacteriophages, and a G+C content different from that of the rest of the chromosome. These elements are frequently flanked by repeat structures and carry many fragments of other mobile and accessory genetic elements, such as bacteriophages, plasmids, and insertion sequence (IS) elements. Some PAIs are unstable regions and can spontaneously disappear from the chromosome. Therefore, PAIs are considered to have evolved from mobile genetic elements by horizontal gene transfer. It can also be assumed that these DNA regions, since their acquisition, underwent and will continue to undergo further evolutionary changes, resulting in an ongoing evolution of bacterial pathogens (14–16).

In addition to the PAIs of *E. coli* strain 536, several PAIs have been characterized for other ExPEC strains, and for many

of them at least partial DNA sequence information is available. Two PAIs have been identified for each of the uropathogenic isolates J96 and CFT073 (13, 30, 36) as well as for the sepsis isolate AL862 (23). One island has been described for the meningitis K1 isolate EV36 (5). Although some of these islands resemble each other, as they carry identical genes, they are markedly diverse with respect to size, genetic content and organization, chromosomal insertion site, and stability.

One aim of this study involved the characterization and sequence determination of already identified PAIs of uropathogenic *E. coli* strain 536 to obtain a detailed picture of these genetic elements. Additionally, to improve knowledge of the evolution and distribution of PAIs, the presence of PAI I₅₃₆- to PAI IV₅₃₆-specific sequences in different *E. coli* strains was investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A collection of 132 *E. coli* strains were used in this study and include the Institut für Molekulare Infektionsbiologie collection, which has already been used for the investigation of the flanking regions of determinants encoding S-family adhesins (8). Additional strains have been isolated during a long-term study of women with chronic urinary tract infections: 2A1, 5A1, 5A1U, 2A2, 16A2U, 16A3, 8B1, 16B1, 19B1, 8B2, 3B5, 1E1, 1E5, 8F4, 3N1, 3N2, and 3N5. All strains were grown in Luria-Bertani medium (33).

DNA techniques. Isolation of plasmid DNA and recombinant DNA was performed as previously described (33).

PCR. A description of the primers used in this study is available as supplementary material (<http://www.uni-wuerzburg.de/infektionsbiologie>). Screening for the presence of PAI I₅₃₆- to PAI IV₅₃₆-specific sequences was performed by

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PCR with primers specific for the individual region to be amplified. Chromosomal DNAs of *E. coli* strains 536 and MG1655 were used as positive and negative controls, respectively. DNA primers were purchased from MWG Biotech (Ebersberg, Germany). The *Taq* DNA polymerase used for the detection of genes in different *E. coli* strains was purchased from Qiagen (Hilden, Germany). Grouping into the main phylogenetic lineages of the ECOR strain collection was done by a triplex PCR described previously (6). The virulence assessment of the extraintestinal *E. coli* strains included a multiplex PCR specific for a set of typical virulence-associated genes of extraintestinal *E. coli* (20).

DNA sequencing and sequence analysis. Overlapping cosmid clones covering the entire regions of PAI I₅₃₆ to PAI III₅₃₆ and their 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 (29). 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 with 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 assembly and editing of the sequence data (35). The genetic structure of PAI IV₅₃₆ was confirmed by sample sequencing of the left and right borders and of several open reading frames (ORFs) located on PAI IV₅₃₆. The resulting sequences were compared with those of the already published high-pathogenicity islands (HPIs) of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (3, 4).

Homology searches were performed with the BLASTN, BLASTX, PSI-BLAST, and PHI-BLAST programs from the National Center for Biotechnology Information (1). Sequence annotation was performed by using Artemis software (32). Codon usage tables for PAI I₅₃₆ to PAI IV₅₃₆ were determined with Artemis and compared with codon usage tables for *E. coli* K-12, for different gram-negative and gram-positive bacterial pathogens, and for several bacteriophages of *E. coli* strain K-12, *E. coli* O157:H7 strain EDL933, *Salmonella* spp., and *Shigella* spp. (<http://www.kazusa.or.jp/codon/cgi-bin>).

Southern hybridization. Several PCR results from the screening for PAI I₅₃₆ to PAI IV₅₃₆ sequences in different *E. coli* isolates were confirmed by Southern hybridization of *Eco*RI-digested chromosomal DNA from the investigated *E. coli* isolates. After agarose gel electrophoresis, the *Eco*RI-digested *E. coli* genomic DNA was transferred to Biotodyne B nylon membranes (PALL, Roßdorf, Germany). The probes used for hybridization were obtained by PCR with the primer pairs used for PCR-based screening and the chromosomal DNA of *E. coli* strain MG1655 as a template. Hybridization and detection were carried out by using an enhanced chemiluminescence labeling and signal detection system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's recommendations.

Nucleotide sequence accession numbers. The complete nucleotide sequences of PAI I₅₃₆ to PAI III₅₃₆ were submitted to the EMBL database under accession numbers AJ488511 (PAI I₅₃₆), AJ494981 (PAI II₅₃₆), and X16664 (PAI III₅₃₆).

RESULTS AND DISCUSSION

Genetic features of PAIs of *E. coli* strain 536. Three PAIs of *E. coli* strain 536 (PAI I₅₃₆ to PAI III₅₃₆) comprising about 270 kb were subcloned and sequenced. The genetic organization of PAI IV₅₃₆, which represents the core element of the HPI initially described for *Yersinia*, was confirmed by PCR and by sample sequencing. The general characteristics of PAI I₅₃₆ to PAI IV₅₃₆ of uropathogenic *E. coli* strain 536 (e.g., chromosomal insertion site, association with tRNA-encoding genes, size, and encoded virulence factors) are compiled in Fig. 1 and Table 1. The genetic organization of PAI I₅₃₆ to PAI IV₅₃₆ is depicted in Fig. 2. A detailed list of the known and putative ORFs identified on these islands is available as supplementary material (<http://www.uni-wuerzburg.de/infektionsbiologie>).

Generally, PAI I₅₃₆ to PAI III₅₃₆ are mosaic-like structures consisting of many DNA fragments which show the highest homology on the nucleotide level to chromosomal regions of other pathogenic *E. coli* strains (e.g., O157:H7 strains EDL933 and Sakai and uropathogenic O6 strain CFT073) and of *Shigella flexneri* (*she* and SHI-2 PAIs). Many fragments of PAI I₅₃₆

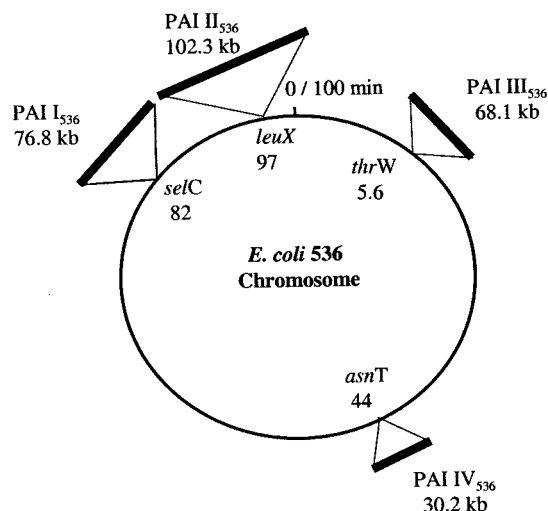


FIG. 1. Comprehensive map of PAI I₅₃₆ to PAI IV₅₃₆ of uropathogenic *E. coli* strain 536. The map is based on the chromosome of *E. coli* strain MG1655. PAIs are indicated according to their chromosomal insertion sites next to tRNA-encoding genes.

to PAI III₅₃₆ are also highly homologous to regions of different virulence plasmids of *E. coli* (pColV, pB171, pO157, and pAPEC-1), *Shigella* spp. (pWR100 and pWR501), and *Yersinia* spp. (pMT1 and pYVe227). Other fragments represent as-yet-unidentified DNA sequences without homology on the DNA level. These different regions are interspersed by each other and consist of already known functional and nonfunctional or truncated ORFs, of as-yet-unidentified putative ORFs with homology on the amino acid level to already known proteins, or of putative ORFs with unknown functions. A marked fraction of ORFs located on these PAIs is derived from mobile accessory genetic elements, such as bacteriophages, plasmids, and IS elements. Interestingly, PAI IV₅₃₆, which represents a broad-host-range PAI present in many different enterobacteria, comprises only functional ORFs. The ORFs detected on PAI I₅₃₆ to PAI IV₅₃₆ can be functionally grouped as shown in Table 2.

In the following text, ORFs are designated in a numerical way according to their localization and order on a PAI (as indicated by the index), starting downstream of the tRNA-encoding gene, which serves as the chromosomal insertion site for the respective PAI. Consequently, the nonfunctional bacteriophage P4-like integrase-encoding ORF on PAI I₅₃₆ which is located downstream of *selC* is designated ORF 1_{I-536}.

Characteristics of PAI I₅₃₆. PAI I₅₃₆ is associated with the tRNA-encoding gene *selC*. This island is 76,843 bp in size, is flanked by 16-bp direct repeats, and has a G+C content of 46%. The nonfunctional bacteriophage integrase gene (ORF 1_{I-536}) immediately downstream of *selC* exhibits high homology to other *intP4*-like genes described for PAIs as well as to that of phage ϕ R73 located at this tRNA-encoding gene. In addition to already known virulence-associated genes located on PAI I₅₃₆, such as the alpha-hemolysin-encoding gene cluster, the annotation of PAI I₅₃₆ sequences revealed two as-yet-unidentified putative adhesin determinants with no homology on the nucleotide level. The deduced amino acid sequences together with their genetic organization indicated that putative

TABLE 1. Main features of PAI I₅₃₆ to PAI IV₅₃₆ of *E. coli* strain 536

PAI	Chromosomal insertion site (tRNA)	Size (kb)	G+C content (%)	Known or putative virulence factor(s)	Integrase gene	Direct repeats (bp)
I ₅₃₆	<i>selC</i>	76.8	46	Alpha-hemolysin, F17-like fimbriae, ^a CS12-like fimbriae ^a	P4-like ^b	16
II ₅₃₆	<i>leuX</i>	102.2	46	Hek adhesin, P-related fimbriae, alpha-hemolysin, hemagglutinin-like adhesin ^a	P4	18
III ₅₃₆	<i>thrW</i>	68.1	47	S fimbriae, <i>iro</i> siderophore system, HmuR-like heme receptor, ^a Sap adhesin, Tsh-like hemoglobin protease ^c	SfX	47
IV ₅₃₆	<i>asnT</i>	30.2	57	Yersiniabactin siderophore system	P4-like ^b	

^a Putative ORF or operon shows no homology on the DNA level but does with respect to the deduced amino acid sequence and genetic structure.

^b Integrase-encoding gene is nonfunctional due to internal stop codons or a mutated start codon.

^c Tsh-like hemoglobin protease-encoding gene is located in the 7-kb region outside of PAI III₅₃₆, which is absent from the *E. coli* K-12 strain MG1655 genome.

ORF 15_{I-536} to ORF 18_{I-536} and ORF 37_{I-536} to ORF 42_{I-536} represent gene clusters coding for F17- and CS12-like adhesins, respectively. Interestingly, the gene product encoded by putative ORF 18_{I-536} shows homology to the F17a fimbrial subunit as well as to the uroepithelial cell adherence protein UcaA of *Proteus mirabilis* (EMBL accession no. CAA54703), indicating that fimbriae containing this subunit may indeed be involved in urinary tract infections. It will be important to determine whether these putative determinants are expressed and how the encoded fimbrial adhesins, whose homologues have so far been described only for enterotoxigenic *E. coli*, contribute to the virulence of extraintestinal *E. coli*. Another adhesin-like protein may be encoded by putative ORF 47_{I-536}, which is preceded by two putative ORFs (ORF 45_{I-536} and ORF 46_{I-536}) which may encode an ATP-binding cassette transporter. These ORFs have in common the facts that they also show no homology on the DNA level but that the deduced amino acid sequences are homologous to proteins (NMB0586, NMB0587, and NMB0588) encoded by three adjacent genes in *Neisseria meningitidis* strain MC58. Other interesting ORFs which have not been described so far are putative ORF 2_{I-536} and ORF 3_{I-536}. These overlapping ORFs are similar in size, and their deduced amino acid sequences (314 and 310 amino acids, respectively) are 43 and 39% identical to that of modification methylase NgoFVII of *Neisseria gonorrhoeae*, which consists of 374 amino acids. However, both ORFs show only 78% identity on the DNA level over the first 89 nucleotides, thus excluding the possibility of gene duplication. As the methylation status of chromosomal DNA is also regulated in response to different stimuli and affects gene expression, it will be interesting to investigate whether these modification methylase-encoding ORFs (in case they are expressed) also influence the gene expression of *E. coli* strain 536. The importance of the *dam*-encoded methylase for general gene expression and expression of virulence-associated genes in *Salmonella* and *E. coli* was previously reported (18, 19).

Characteristics of PAI II₅₃₆. PAI II₅₃₆ is associated with the tRNA-encoding gene *leuX*. This island is 102,200 bp in size, is flanked by 18-bp direct repeats, and has a G+C content of 46%. The functional bacteriophage integrase gene (ORF 1_{II-536}) immediately downstream of *leuX* exhibits the highest homology to the *intB* gene of bacteriophage P4 located at this tRNA-encoding gene in *E. coli* K-12. Already known virulence determinants located on PAI II₅₃₆ are the *pf* determinant (ORF 6_{II-536} to ORF 17_{II-536}), which codes for the P-related fimbrial adhesin, and another alpha-hemolysin-encoding gene cluster (ORF 22_{II-536} to ORF 25_{II-536}). Other putative viru-

lence-associated genes present on this PAI are ORF 4_{II-536}, which codes for the Hek adhesin described for *E. coli* (EMBL accession no. AY040859), and two putative ORFs (ORF 40_{II-536} and ORF 41_{II-536}) without homology on the DNA level. The encoded gene product of ORF 40_{II-536} shows homology to filamentous hemagglutinin-like adhesins of *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Yersinia pestis*, and ORF 41_{II-536} shows homology to a conserved ORF which is located upstream of the hemagglutinin-encoding one and which is required for secretion of the adhesins. Another interesting as-yet-unidentified putative ORF is ORF 35_{II-536}, whose encoded gene product shows homology to a fragment of modification methylase HgiDII of *Herpesosiphon aurantiacus* (EMBL accession no. P25265). The right-hand direct repeat structure is not immediately followed by *E. coli* K-12-specific sequences representing the conserved *E. coli* chromosomal backbone but by another 4-kb DNA region which is not present in *E. coli* K-12 strain MG1655. In this region, only one putative ORF coding for a hypothetical protein of *E. coli* O157:H7 strain Z5892 is located. This 4-kb DNA stretch is then followed by the *E. coli* K-12 chromosomal backbone starting with *yjhS*.

Characteristics of PAI III₅₃₆. PAI III₅₃₆ is associated with the tRNA-encoding gene *thrW*. This island is 68,124 bp in size and has a G+C content of 47%. The functional bacteriophage integrase gene (ORF 1_{III-536}) immediately downstream of *thrW* exhibits the highest homology to the *int* gene of bacteriophage SfX, which recognizes this tRNA-encoding gene as a chromosomal insertion site. This PAI is flanked by 47-bp direct repeats, as a truncated *thrW* gene located at the right-hand end of PAI III₅₃₆ can, together with the functional copy of *thrW* at the left-hand end, serve as a direct repeat structure. However, approximately 7 kb of DNA is located between the truncated *thrW* gene at the right-hand end of PAI III₅₃₆ and the transition into the *E. coli* K-12-like chromosomal backbone. This DNA stretch also represents foreign DNA, absent in *E. coli* strain MG1655, which contains an ORF with homology to the hemoglobin protease-encoding genes of pColV-K30 and pAPEC-1 (EMBL accession no. AJ223631 and AF218073, respectively). The Tsh hemoglobin protease has been shown to be involved in the virulence of, e.g., avian-pathogenic *E. coli* (10). As previously described (8), the right junction site of this composite structure includes sequences with homology to fragments of integrase genes of different *S. flexneri* and *E. coli* bacteriophages, followed by DNA sequences with homology to fragments of the *insB* and *insA* genes of the Iso IS1 element. These results demonstrate that the chromosomal region between *thrW* and *yagU* represents a composite structure of PAI

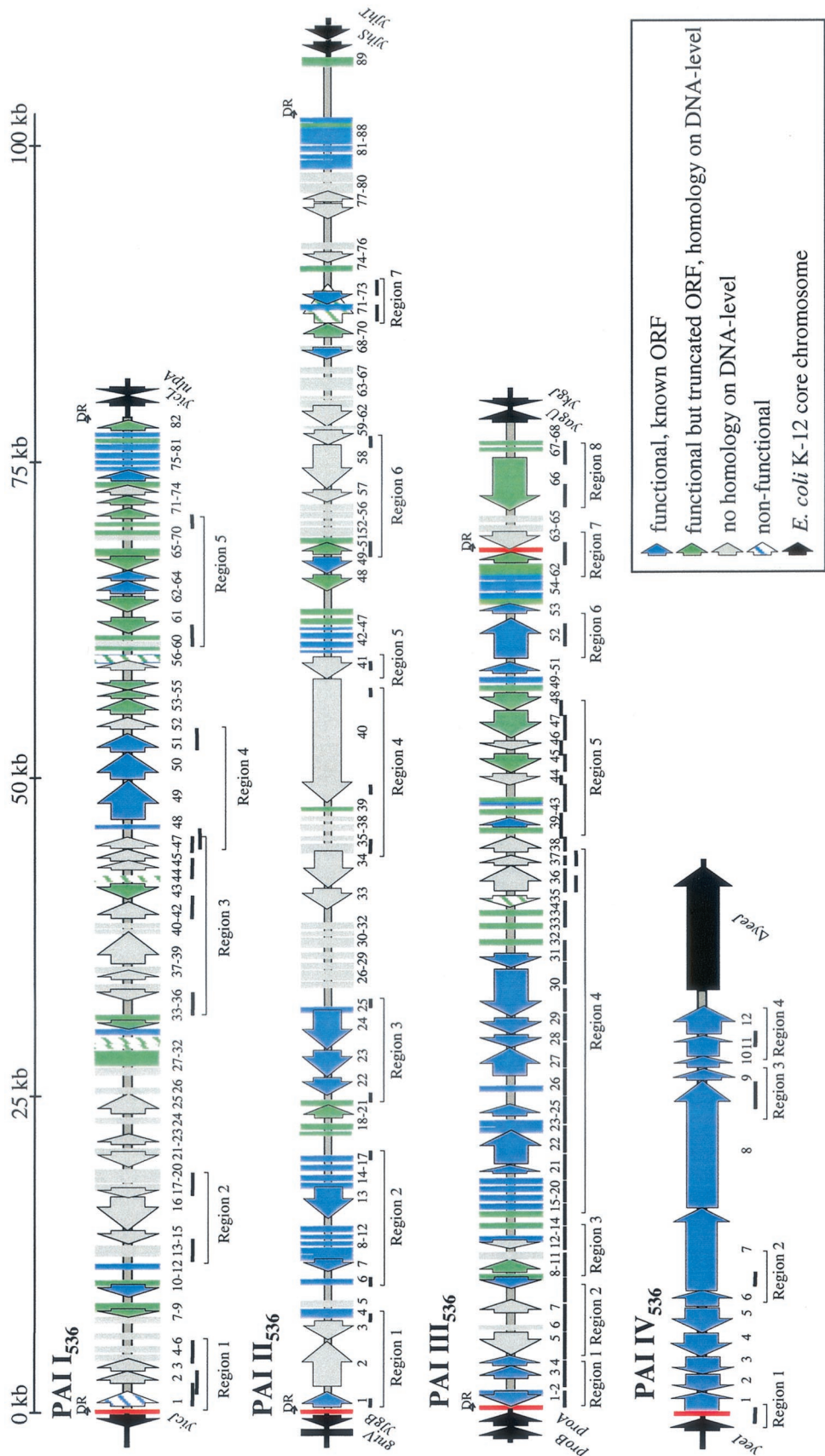


FIG. 2. Comparison of the genetic organization of PAI I₅₃₆ to PAI IV₅₃₆. Known or putative ORFs are grouped according to the following characteristics: blue, functional, known ORFs; green, truncated ORFs with a start codon and a stop codon; grey, as-yet-unidentified ORFs without homology on the DNA level. Nonfunctional ORFs (e.g., due to internal stop codons or frameshifts) are indicated by hatched symbols. ORF numbers are indicated below the corresponding ORF symbols. Functional or truncated tRNA-encoding genes are marked in red. Direct repeat (DR) structures flanking PAIs are indicated. Thick black lines below the PAIs represent regions of PAI I₅₃₆ PAI IV₅₃₆ which were detected by PCR. Several PAI-specific PCRs were grouped into PAI regions.

TABLE 2. Functional categories of known and putative ORFs located on PAI I₅₃₆ to PAI IV₅₃₆

Known or putative function	No. of ORFs that are:		
	Intact	Truncated	Nonfunctional ^a
Related to mobile genetic elements	14	33	3
Toxin	8		
Adhesin	37	2	
Iron acquisition	14	3	
Other virulence-associated function	1	3	
Enzymes	2	10	
Regulation	3	5	
Other functions	7	17	2
Hypothetical or unknown ^b	31	48	2
Without homology	4		

^a Due to internal stop codons or frameshifts.

^b Putative ORFs or their encoded products show homology on the DNA or protein level.

III₅₃₆ and other horizontally acquired sequences that could be considered to be flanked by sequences derived from *Shigella* bacteriophages which integrate into highly homologous *attP* sites next to the tRNA-encoding gene *thrW*. The occurrence of truncated tRNA-encoding genes within PAIs or DNA regions presumably acquired by horizontal gene transfer has also been observed for PAI II of CFT073 (30) and indicates the importance of these hot spots for chromosomal integration of foreign DNA sequences.

In addition to virulence determinants carried on PAI III₅₃₆, such as the S fimbrial adhesin-encoding gene cluster *sfa* (ORF 17_{III-536} to ORF 25_{III-536}) and the siderophore system-encoding gene cluster *iro* (ORF 27_{III-536} to ORF 31_{III-536}), sequence analysis of the entire region of PAI III₅₃₆ revealed the presence of other known genes coding for virulence factors of *E. coli*; an example is ORF 52_{III-536}, with homology to *sap*, which codes for an autotransporter-adhesin and which is located on the she PAI of *S. flexneri* 2a (EMBL accession no. AF200692). The as-yet-unidentified putative ORF 36_{III-536} encodes a HmuR-like heme receptor which has been described for *Y. pestis* (EMBL accession no. Q56989). Only short fragments of putative ORF 47_{III-536} and ORF 48_{III-536}, coding for lysine decarboxylase (CadA) and cadaverine/lysine antiporter (CadB) homologues, respectively, show homology on the DNA level to the corresponding *cadA* and *cadB* genes of *E. coli* or *Salmonella enterica* serovar Typhimurium, indicating either their acquisition by horizontal gene transfer or the occurrence of multiple DNA rearrangement processes resulting in these *cadA* and *cadB* variants. Interestingly, ORFs with high homology to these genes are also located on PAI II of CFT073 (30). Whereas CadA and CadB have been proposed to be involved in acid tolerance mechanisms of *Salmonella* (12), the encoding genes are part of the so-called "black hole" in *Shigella* and enteroinvasive *E. coli*, which describes a large chromosomal deletion in these bacteria (25). The loss of this chromosomal region promotes the virulence of *Shigella* and enteroinvasive *E. coli*, as the product of lysine decarboxylase activity, cadaverine, blocks the action of *Shigella* enterotoxins (26).

Characteristics of PAI IV₅₃₆. PAI IV₅₃₆ is associated with the tRNA-encoding gene *asnT*. This island represents the core element of the so-called HPI of pathogenic *Yersinia* species. It has been completely sequenced for several *Yersinia* strains (3,

4, 11). The left and right junction sites of PAI IV₅₃₆ have also been sequenced before (34). We therefore verified the genetic organization of this PAI in *E. coli* strain 536 by sample sequencing of PCR products obtained with primers described elsewhere (22). The obtained sequences showed between 97 and 100% identity on the nucleotide level to other HPI-specific sequences of *Y. pseudotuberculosis*. Sequence determination of the left and right junction sites of PAI IV₅₃₆ revealed only minor differences in the right-hand end of PAI IV₅₃₆ compared to previous results. According to our sequence analysis, the 5' end of PAI IV₅₃₆ shows 98% identity to the sequence published earlier but comprises a 1,002-bp intergenic region between *fyuA* and $\Delta b1978$ instead of 1,226 bp, as described by Schubert and colleagues (34). According to these results, we conclude that by analogy to the HPI of *Y. pseudotuberculosis*, PAI IV₅₃₆ is about 30.2 kb in size and has a G+C content of 57%. Flanking repeat structures are absent in PAI IV₅₃₆, which contains the gene cluster required for biosynthesis of the siderophore system yersiniabactin.

Genes of mobile genetic elements present on PAI I₅₃₆ to PAI IV₅₃₆. Besides the characteristic bacteriophage integrase-encoding genes located immediately downstream of the tRNA-encoding gene serving as a chromosomal insertion site for these PAIs, a considerable fraction of ORFs on PAI I₅₃₆ to PAI III₅₃₆ are remnants of various IS elements and transposons of different origins. The majority represent functional or nonfunctional transposase-encoding genes. Intact IS elements or transposons, however, have not been identified on these PAIs. Twenty-six fragments of IS elements have been detected on PAI I₅₃₆ to PAI III₅₃₆. They belong to different types of IS elements, including IS1, IS2, IS3, IS4, IS10, IS50R, IS100, IS110, IS629, IS630, IS679, IS911, IS1328, and IS1353. Many of the corresponding families of IS elements not only are restricted to enterobacteria but also are present among other bacteria (24). These IS elements are frequently located on virulence plasmids. Interestingly, at least one copy of each of the ORFs encoding the two subunits of the IS100 transposase is located on PAI I₅₃₆ to PAI III₅₃₆. Furthermore, several ORFs (copies of L0007 to L0010) of prophage CP-933 of *E. coli* O157:H7 strain EDL933 are always detectable on PAI I₅₃₆ to PAI III₅₃₆. Genes of prophage CP-933 are also present on PAIs of ExPEC strains CFT073 and AL862 (13, 23, 30). In addition, the putative ORF 63_{I-536} exhibits the highest homology to a fragment of an intron-associated reverse transcriptase/maturase-encoding ORF. The gene products of the adjacent putative ORF 52_{II-536} and ORF 53_{II-536}, which share no sequence homology, are homologous to the quaternary ammonium compound resistance protein QacE of *Brucella melitensis* (EMBL accession no. AE009544) and *E. coli* (EMBL accession no. AF205943), respectively. In *E. coli*, *qacE* is known to be part of intron In53, a class 1 plasmid- and composite transposon-located integron (27). These data indicate that these PAIs in their present state result from repeated recombination events, including integration of different mobile genetic and other accessory genetic elements. PAI IV₅₃₆ contains no remnants of IS elements.

Codon usage of ORFs located on PAI I₅₃₆ to PAI IV₅₃₆. A comparison of codon usage tables for every PAI demonstrated that several codons occur with markedly different frequencies in these islands and in the chromosomal *E. coli* K-12-specific

TABLE 3. Detection of PAI I₅₃₆- to PAI IV₅₃₆-specific sequences in different *E. coli* isolates

<i>E. coli</i> strains (n) ^a	% Positive specific PCRs for the indicated region of PAI:																							
	I ₅₃₆					II ₅₃₆					III ₅₃₆					IV ₅₃₆								
	1	2	3	4	5	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	1	2	3	4
UTI (62)	3.2	20.9	3.2	4.8	3.2	43.5	24.2	24.2	22.6	66.1	22.6	12.9	1.6	9.7	59.7	64.5	11.3	40.3	11.3	72.6	82.3	85.5	85.5	85.5
Human and animal MNEC and SEPEC (28)	0	14.3	0	0	0	32	25	7.1	21.4	35.7	25	10.7	0	7.1	7.1	46.4	7.1	28.6	7.1	64.3	85.7	89.3	89.3	89.3
ECOR (28)	7.1	14.3	3.6	7.1	7.1	21.4	17.9	7.1	16	47.4	5.3	0	0	0	35.7	39.3	0	32.1	0	53.6	75	78.6	78.6	78.6
Diarrheagenic pathogens (18)	0	0	0	0	0	11	0	0	0	0	5.6	0	0	0	0	0	0	17	0	0	27.8	27.8	16.7	27.8

^a UTI, urinary tract infection; MNEC, meningitis-causing *E. coli*; SEPEC, sepsis-causing *E. coli*; ECOR, *E. coli* reference collection.

backbone. Details are available as supplementary material (<http://www.uni-wuerzburg.de/infektionsbiologie>). The codon usage of PAI I₅₃₆ to PAI III₅₃₆ differs from that of PAI IV₅₃₆. The usage of codons such as ATA, ACA, AGA, and AGG is increased by at least a factor of 2 in these PAIs, whereas that of codon GCG is more than twofold lower than that in *E. coli* strain MG1655. This tendency is visible in several bacterial species, such as *S. flexneri*, *Shigella sonnei*, *Salmonella enterica*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Staphylococcus aureus*, and *Bacillus subtilis*, as well as in many bacteriophages of enterobacteria, such as 933W, ES18, P4, SfV, SfVI, and 7887. In PAI IV₅₃₆, codons AGG and CCC are used at least twice as much as in *E. coli* strain MG1655, whereas the usage of codons ACT, AAA, and GGT is more than twofold lower in PAI IV₅₃₆. These differences are indicative of the (more or less recent) acquisition of these PAIs by horizontal gene transfer.

Analysis of the presence of PAI I₅₃₆- to PAI IV₅₃₆-specific sequences in different *E. coli* isolates. Based on the DNA sequences of PAI I₅₃₆ to PAI IV₅₃₆, we selected DNA primer combinations in order to detect specific sequences of these islands in a set of 132 different *E. coli* strains including many ExPEC and intestinal pathogenic *E. coli* isolates as well as several nonpathogenic *E. coli* strains (Fig. 2). Detailed results of this extensive screening for multiple PAIs, which complete recently published results on the presence of PAI III₅₃₆ homologues in *E. coli* isolates (8), are available as supplementary material (<http://www.uni-wuerzburg.de/infektionsbiologie>). As shown in Table 3, PAI IV₅₃₆-specific sequences showed a higher percentage of occurrence among the different *E. coli* strains tested in this study than sequences specific for PAI I₅₃₆ to PAI III₅₃₆. PAI III₅₃₆-specific sequences were more frequently detectable than sequences specific for PAI II₅₃₆. PAI I₅₃₆-specific DNA regions were detected at the lowest frequency. PAI I₅₃₆- to PAI III₅₃₆-specific sequences were usually not detectable in intestinal pathogenic *E. coli* isolates, indicating that PAI I₅₃₆ to PAI III₅₃₆ represent a part of the ExPEC-specific gene pool. However, with a few exceptions due to local sequence differences, 17 to 28% of the intestinal pathogenic isolates were positive for all PAI IV₅₃₆-specific PCRs. Between 67 and 92% of the ExPEC isolates were positive for all PAI IV₅₃₆-specific PCRs, suggesting that they harbor the entire HPI core element. In contrast, the other three PAIs were not always completely detectable in some of the strains. In these instances, only certain PAI I₅₃₆- to PAI III₅₃₆-specific PCRs gave positive PCR results for the different strains used in this study.

As already speculated, strains harboring determinants cod-

ing for members of the S family of fimbrial adhesins were shown to possess a common module of different forms of a PAI, depending on the different S adhesin types (8). The PCR screening based on the sequence of the complete region of PAI III₅₃₆ confirmed this finding, as only some of the urinary tract infection isolates were positive for all PAI III₅₃₆-specific PCRs. However, the chromosomal insertion site for their PAI III₅₃₆ equivalent is so far unknown.

For some strains tested, all PAI I₅₃₆-, PAI II₅₃₆-, or PAI III₅₃₆-specific sequences (with the above-mentioned exception for PAI III₅₃₆) were detectable. Positive results in at least 13 specific PCRs for PAI I₅₃₆ to PAI III₅₃₆ strongly suggested that these strains contain the complete islands, as described for *E. coli* strain 536. Strains in which the complete PAIs of uropathogenic *E. coli* strain 536 have been detected are compiled in Table 4. As shown by a previously described triplex PCR (6), all strains belong to major phylogenetic group B2, and the majority carry the same repertoire of virulence-associated genes as *E. coli* strain 536. The approximate genome sizes of these strains were determined by pulsed-field gel electrophoresis after *CeuI* digestion. The restriction patterns obtained indicated that, with the exception of those of strains RZ454 and RZ532 (4.6 Mb), the genome sizes are similar, ranging between 4.9 and 5.1 Mb. These results indicate that one or even multiple PAIs which have been described for uropathogenic *E. coli* strain 536 are also detectable in other ExPEC isolates. For the first time, we provide evidence by an extensive analysis of specific sequences of multiple PAIs in 132 different *E. coli* strains that identical PAIs, other than the HPI core element, most likely are present in different *E. coli* strains. This evidence indicates that these large chromosomal regions have been transferred by horizontal gene transfer.

PAIs and their importance for the evolution of ExPEC. The results of this study increase knowledge on the variability of the genetic structures of PAIs in ExPEC. PAI IV₅₃₆ represents a broad-host-range PAI also present in many other *E. coli* pathotypes and different enterobacteria and differs with respect to G+C content and genetic organization from PAI I₅₃₆ to PAI III₅₃₆, which have similar G+C contents and structural features and which have so far been detected only in ExPEC. However, the latter islands share several ORFs which are also present on many PAIs of other ExPEC isolates, such as bacteriophage CP-933-related sequences. This fact may be indicative of continuously ongoing DNA rearrangements among PAI sequences resulting in evolution in ExPEC strains of different PAI variants deriving from an ancestral PAI which also consisted of phage CP-933. Although many PAIs of ExPEC strains

TABLE 4. Characterization of different ExPEC isolates presumably containing complete PAIs of *E. coli* strain 536 or large fragments thereof

Strain	Serotype ^a	Source ^b	Genome size (Mb) ^c	Characteristics ^d	Presence of entire PAI ^e		
					I ₅₃₆	II ₅₃₆	III ₅₃₆
536	O6:K15:H31	UTI	4.9	<i>malX papAH papC papEF papGIII fimH fyuA sfalfocDE sfaS hlyA kpsMTK5</i>	+	+	+
ECOR 52	ND	Feces	5	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc focG cnf1 iutA hlyA kpsMTK5</i>	+	-	-
ECOR 60	ND	UTI	5.1	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc focG cnf1 iutA hlyA rfc kpsMTK5</i>	+	-	-
RZ479	O6:K ⁺ :H ⁻	UTI	5.1	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 hlyA kpsMTK5</i>	+	+	+
2980	O18ac:K5	Feces	5	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS hlyA kpsMTK5</i>	-	+	-
HK24	ND	Sepsis	5	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 hlyA kpsMTK5</i>	-	+	+
HK8	ND	Sepsis	5	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 iutA hlyA kpsMTK5</i>	-	-	+
RZ422	O6:K14:H ⁻	UTI	4.9	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc focG cnf1 cdtB traT hlyA kpsMTK5</i>	-	-	+
RZ451	O6:K18/22:H31	UTI	5	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 traT hlyA kpsMTK5</i>	-	-	+
RZ505	O6:K14:H	UTI	5.1	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 hlyA kpsMTK5</i>	-	-	+
RZ532	O6:K ⁺ :H31	UTI	4.6	<i>malX papAH papC papEF papGII/III fimH fyuA sfalfoc sfaS cnf1 hlyA kpsMTK5</i>	-	-	+

^a ND, not determined.

^b UTI, urinary tract infection.

^c As determined by pulsed-field gel electrophoresis after *CeuI* digestion.

^d As determined by multiplex PCR; designation of PCR products is according to McCormick et al. (26).

^e Chromosomal insertion site of PAI III₅₃₆ homologues is unknown. +, present; -, not present.

superficially resemble each other with respect to the presence and/or genetic linkage of certain virulence determinants, there is nevertheless considerable variability in PAI composition and structural organization (8, 13, 21, 23, 30, 31, 36). The majority of homologous DNA sequences present on PAIs, such as fragments of IS elements and other accessory genetic elements, allow recombination and consequently PAI rearrangements and deletion and/or acquisition of foreign DNA that has been acquired by horizontal gene transfer. This feature facilitates rapid and continuous DNA rearrangements and remodeling of PAIs. In addition, some PAIs of ExPEC represent unstable genetic elements which can be deleted from the chromosome (2, 9, 28). It is assumed that such deleted PAIs, or at least large parts thereof, could be transferred to suitable recipients and thus contribute to the ongoing evolution of pathogenic bacteria (7, 15, 28).

In this study, we provide evidence that large regions of PAI I₅₃₆ to PAI III₅₃₆ can be detected in different ExPEC isolates, thus arguing for en bloc acquisition of these DNA regions by horizontal gene transfer. In summary, the study of the genetic organization of PAIs of uropathogenic *E. coli* strain 536 revealed the existence of several putative virulence determinants so far unknown in *E. coli*. A comparison of PAI I₅₃₆ to PAI III₅₃₆ with other PAIs of ExPEC supports a model in which PAI mobilization and horizontal gene transfer as well as continuous reorganization of PAIs by recombination, for example, mediated by multiple fragments of accessory DNA elements present on one or multiple PAIs, contribute to the ongoing evolution of ExPEC variants.

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