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Characterization of an *iroBCDEN* Gene Cluster on a Transmissible Plasmid of Uropathogenic *Escherichia coli*: Evidence for Horizontal Transfer of a Chromosomal Virulence Factor

Liisa Johanna Sorsa, Severin Dufke, Jürgen Heesemann, and Sören Schubert*

Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, 80336 Munich, Germany

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The chromosomal *iroBCDEN* gene cluster first described for *Salmonella enterica* is involved in the uptake of catecholate-type siderophore compounds. An orthologous gene cluster has recently been detected in *Escherichia coli* strains which cause extraintestinal disease. This *E. coli iroBCDEN* gene cluster has an impact on virulence and has been reported to be located in a pathogenicity island on the chromosome. In this study we characterized an *iro* gene cluster of a uropathogenic *E. coli* isolate which is located on a transmissible plasmid related to the R64 plasmid of *S. enterica*. This cluster is highly homologous to the chromosomal *iro* cluster of *E. coli*. When introduced into an *E. coli fepA cir fiu aroB* mutant, IroN, but not IroBCDE, mediated the utilization of structurally related catecholate siderophores, including 2,3-dihydroxybenzoyl-L-serine, 2,3-dihydroxybenzoyl-D-ornithine, 2,3-dihydroxybenzoic acid, and enterochelin. This study supports the idea of an ongoing horizontal transfer of putative virulence factors and the mobilization of single virulence gene clusters, which lead to a modular assembly of virulence determinants such as pathogenicity islands.

The virulent *Escherichia coli* strains that cause extraintestinal infections such as urinary tract infection (UTI), bacteremia, and meningitis are distinct from most intestinal commensal *E. coli* types and diarrheagenic *E. coli* types (14, 28, 37). These extraintestinal pathogenic *E. coli* (ExPEC) strains possess specialized virulence factors, including adhesins, siderophores, toxins, polysaccharide coatings (capsules and lipopolysaccharides), protectins, and invasins (18), which allow ExPEC strains to colonize host mucosal surfaces, invade host tissues, foil host defense mechanisms, and incite an injurious host inflammatory response (14, 18, 27, 28). These factors are primarily thought to be inherited vertically within evolutionary lineages but are also thought to be transferred horizontally between lineages, in some instances on plasmids or on pathogenicity-associated islands (PAIs) (6, 21, 26, 35). PAIs are large blocks of established or suspected virulence genes that are inserted into the *E. coli* genome and which may provide a mechanism for coordinate horizontal transfer of virulence genes between lineages within *E. coli* and even between species (6, 8, 16, 36). However, the detailed mechanism of horizontal transfer of PAIs and other chromosomal virulence determinants still remains to be elucidated.

As iron is limited in extraintestinal sites of infection, the acquisition of iron is a prerequisite for pathogens encountering the host. The ExPEC strains have multiple iron acquisition mechanisms, including siderophore-mediated iron uptake systems, which contribute to the fully virulent phenotype. The $iroBCDEN_{E.\ coli}$ gene cluster has recently been described for ExPEC strains, with $iroN_{E.\ coli}$ being orthologous to a catecholate siderophore receptor gene identified in *Salmonella* spp. (4, 31).

IroN expression was shown to be regulated by the ferric uptake regulator (Fur) and increased by incubating the respective *E. coli* strains in human urine, ascitic fluid, or blood (31). As with *Salmonella*, the *iroBCDEN* gene cluster of *E. coli* was found to be chromosomally located and in the archetypal uropathogenic *E. coli* strain 536 part of PAI III (4, 12). The recent work of Russo et al. demonstrated that $IroN_{E.\ coli}$ enables the uptake of the catecholate siderophore enterobactin in *E. coli* (32). Using a mouse infection model of ascending UTI, the authors of that study found that the presence of $iroN_{E.\ coli}$ contributed significantly to the virulence of *E. coli* (32).

In the present study, we applied a suppressive subtractive hybridization strategy to extraintestinal *E. coli* strains and detected a gene cluster exhibiting high homology to the *E. coli iroBCDEN* genes. Unlike the known $iroBCDEN_{E.\ coli}$ cluster, which is located on a chromosomal PAI of uropathogenic *E. coli*, the *iro* gene cluster described here is part of a transmissible plasmid related to *Salmonella enterica* serovar Typhimurium plasmid R64. The characterization of the border revealed the presence of several IS elements. Here we present evidence that *iroN* mediates the uptake of catecholate siderophores such as 2,3-dihydroxybenzoic acid (DHBA), 2,3-dihydroxybenzoyl-D-ornithine (DHBO), and enterochelin. The data of this study further emphasize the impact of horizontal transfer in the distribution of virulence factors and show that even parts of chromosomal PAIs can be mobilized individually and thus contribute to a modular organization of virulence determinants such as pathogenicity islands.

* Corresponding author. Mailing address: Max von Pettenkofer-Institut, Pettenkoferstr. 9a, 80336 Munich, Germany. Phone: 49 89 51605261. Fax: 49 89 51605248. E-mail: schubert@pk-i.med.uni-muenchen.de.

TABLE 1. Bacterial strains, plasmids, cosmids, and oligonucleotides used in this study

Strain, plasmid, cosmid, or oligonucleotide	Relevant characteristic(s), description, or sequence	Reference and/or source
Strains		
HE300	Positive for *papA papC fimH fyuA iroN iha bmaE gafD iss*	This study; UPEC isolate[a]
DH10B	F$^-$ *mcrA*Δ*(mrr-hsdRMS-mcrBC)* ϕ80d*lacZ*Δ*M15* Δ*lacX74 endA1 recA1 deoR (ara leu)7697 araD139 galU galK nupG rpsL* λ$^-$	Invitrogen
S17-1	Streptomycin resistance (Str^r); trimethoprim resistance (Tmp^r); λ*pir; hsdR pro recA*	23
XL-1 Blue MR′	Δ*(mcrA)183* Δ(mcrCB-hsdSMR-mrr)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
TH2	*supE44 hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 thi trpR624*	Takara Shuzo Co., Ltd.
TH2 spec	Spontaneous spectinomycin mutant	This study
H5058	*aroB malT tsx thi cir fiu fepA*	4
Plasmids and cosmids		
p300	Wild-type plasmid of *E. coli* HE300; Tc^r; Tp^r; carrying *iroBCDEN* genes	This study
SuperCos 1	Cosmid vector; 7.6 kb; Ap^r Km^r	Stratagene
pJS332	SuperCos 1 with 31.7 kb of p300 carrying *iroBCDEN* genes	This study
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^r Km^r	Invitrogen
pJS11	pCR4-TOPO with 196-bp fragment of subtractive cloning (AA107)	This study
pJS12	pCR4-TOPO with 3.8 kb of p300 carrying *iroEN* genes	This study
pJS13	pCR4-TOPO with 8.6 kb of p300 carrying *iroBCDE* genes	This study
pJS14	pJS12 with 0.8-kb Tp^r cassette in *iroN* gene	This study
pJS15	pJS12 with 0.8-kb Tp^r cassette in *iroE* gene	This study
Oligonucleotides (position [nt])		
ydfB.for (6808)	5′-ATATCACGGGAAAGGAAGCAC-3′	This study
ydfB.rev (7215)	5′-GTCCGGCAAGGTTTATCA-3′	This study
ORF14.for (12886)	5′-TGGTCAGGTTGCGGAGGCTAT-3′	This study
*iroN*1.for (13464)	5′-CCGACGATGATAATGACGAGA-3′	This study
iroN.rev (13806)	5′-TGTTCCGGTGGCACCCAGTTG-3′	This study
*iroN*2.for (14668)	5′-AGATAACATTGAGCCGGTTCC-3′	This study
iroE.for (15734)	5′-GCATTGACGCCTTTATCTTTC-3′	This study
iroD.rev (16680)	5′-CTGATTGACGGGATTGGTT-3′	This study
ORF20. rev (23257)	5′-GCGAAGGCGAGACTATCAGGA-3′	This study
iroB.for (22565)	5′-CGAGCGGAGGGTAGATGATGA-3′	This study

[a] UPEC, uropathogenic *E. coli.*

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and cosmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) medium, in Mueller-Hinton II, and in nutrient broth (NB). For iron depletion, NB was supplemented with 50 to 100 μM 2,2-dipyridyl (NBD). For plasmid maintenance, kanamycin (50 μg/ml), ampicillin (100 μg/ml), tetracycline (12 μg/ml), trimethoprim (75 μg/ml), and spectinomycin (50 to 200 μg/ml) were added to the culture medium as required. The *E. coli* CFT073 strain used in this study is a well-characterized archetypal uropathogenic *E. coli* (21, 24); the *E. coli* strain HE300 was isolated from a urine sample of a UTI patient at the diagnostic laboratory of the Max von Pettenkofer-Institut, Munich, Germany. A total of 50 additional *E. coli* strains from urine samples of patients suffering from UTI and 25 *E. coli* strains from stool samples of healthy individuals were obtained from the Max von Pettenkofer-Institut diagnostic laboratory and were used as reference strains in PCR and hybridization assays.

Recombinant DNA methods. Isolation of plasmids, cosmids, and genomic DNA as well as cloning of DNA fragments was performed using standard techniques (3). Using the SuperCos 1 Cosmid vector kit and the Gigapack III gold packaging extract (Stratagene, Heidelberg, Germany), a cosmid library of total DNA from *E. coli* strain HE300 was constructed according to the manufacturer's recommendations. PCR products representing parts of the *iroBCDEN* gene cluster were cloned into vector pCR4-TOPO as recommended by the manufacturer (Invitrogen, Karlsruhe, Germany). For DNA hybridization analyses, dot blot or colony blot assays were carried out by the method of Southern by transferring DNA onto a Hybond N+ membrane (Amersham Biosciences Europe, Freiburg, Germany) (3). The DNA probes were generated by PCR from plasmid pJS11 (fragment AA107: forward primer, T7; reverse primer, T3) or from genomic DNA of *E. coli* HE300 (*iroN*1.for and *iroN*.rev) and (*ydfB*.for and *ydfB*.rev). The PCR primers used in this study are shown in Table 1. Labeling, hybridization, and detection were carried out by means of the ECL random prime labeling and detection system (Amersham).

Bacterial matings. *E. coli* strains HE300, DH10B, and S17-1 were transformed with plasmid p300 and used as donor strains in bacterial mating assays. A spectinomycin-resistant derivative of *E. coli* TH2 was used as the recipient strain (TaKaRa Bio Inc., Shiga, Japan). Both donor and recipient strains were grown to the late exponential phase, washed, and mixed in a donor/recipient ratio of 1:1. Mating mixture (30 ml) was sedimented by centrifugation, resuspended in 200 μl of LB medium, deposited onto a blood agar plate (BD Biosciences Clontech GmbH, Heidelberg, Germany), and incubated overnight at 37°C. Cells were collected, resuspended by vigorous vortexing, and diluted on liquid medium. *E. coli* TH2 transconjugants were selected on LB agar plates containing antibiotics (tetracycline [12 μg/ml] and spectinomycin [200 μg/ml]). For calculation of transfer frequencies, donor, recipient, and transconjugant CFU were counted after mating disruption and plating of appropriate dilutions. Donor and recipient spontaneous resistance to selective antibiotics was also determined. *E. coli* HE300 spectinomycin-resistant strains arose at frequencies of $1:10^7$ or less, whereas resistance to tetracycline in *E. coli* TH2 alone was undetectable ($<1:10^9$).

Selective subtractive hybridization (SSH). Using *E. coli* CFT073 as the driver strain and *E. coli* HE300 as the tester strain, genomic subtractions were carried out by means of the PCR-Select bacterial genome subtraction kit (BD Biosciences Clontech). The genomic DNA from both strains was isolated and digested with *Rsa*I. The tester DNA was ligated separately with two sets of linkers provided by the manufacturer. These two pools were separately hybridized to excess DNA from the driver strain and then mixed together to allow hybridization of the tester sequences. PCR was performed using oligonucleotides com-

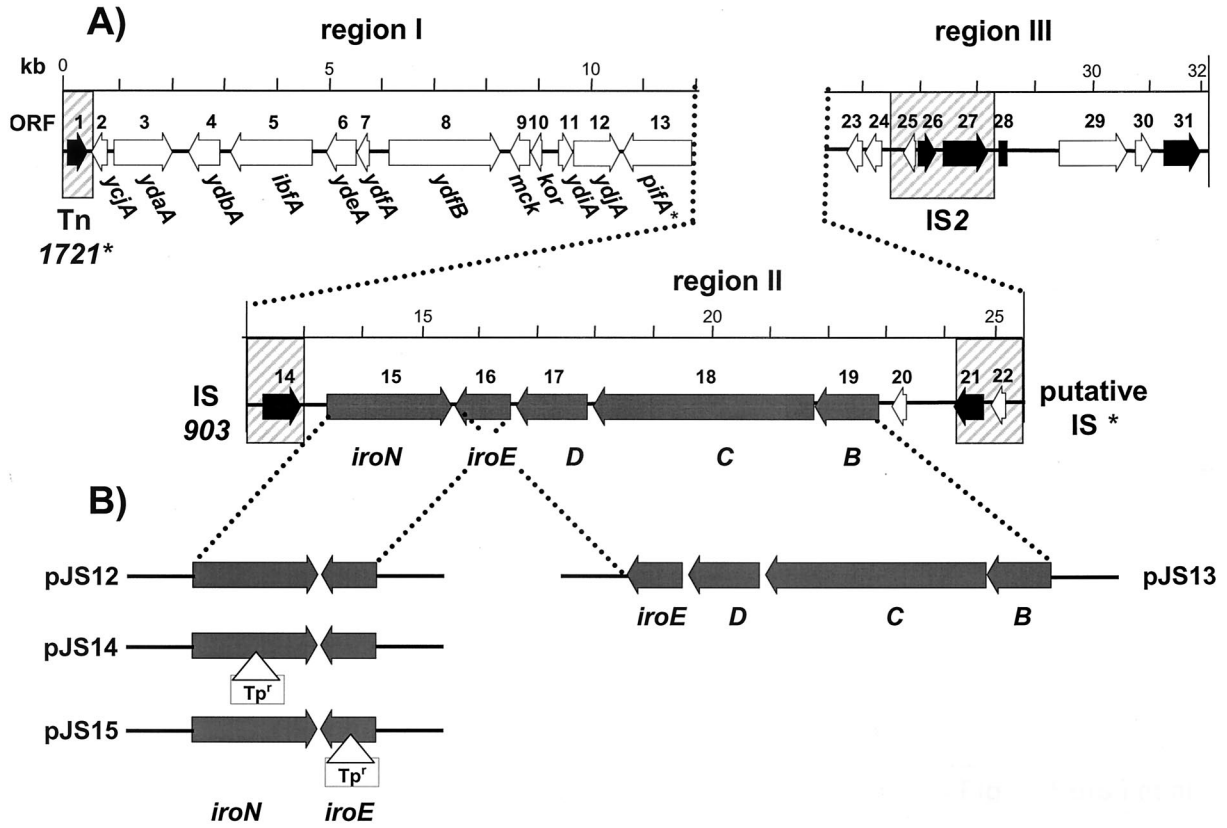

FIG. 1. Genetic organization of the 31,870-bp inserted DNA of pJS332 with three distinct DNA regions (I to III). (A) The locations and orientations of the ORFs described in this study are indicated by arrows. Genes of the *iroBCDEN* cluster are indicated by dark gray shading, black arrows indicate transposase genes, and IS elements and transposons are depicted as hatched boxes. Designations below the schemes represent genes with extensive homology to other bacterial alleles as shown in Table 2. (B) Structures of subcloned fragments of the *iro* gene cluster in pCR4-TOPO giving rise to pJS12 and pJS13 plasmids, as well as the structure of the mutated *iroN* and *iroE* genes in plasmids pJS14 and pJS15, respectively. Triangles indicate insertions of trimethoprim transposon to generate mutations of *iroN* and *iroE* genes.

plementary to the linker sequences to enrich for sequences that contained different linkers on both ends and thus had not been hybridized to the excess driver DNA. The PCR products were cloned directly into pCR4-TOPO vector (Invitrogen) following the manufacturer's instructions. Using the universal T7 and M13 oligonucleotide primers, sequencing of the cloned products was carried out.

DNA sequencing and phylogenetic analysis. DNA was sequenced using a BigDye Deoxy termination kit according to the manufacturer's instructions and a model 377 DNA sequencing system (Applied Biosystems, Weiterstadt, Germany). To sequence the pJS332 cosmid, a shotgun library was prepared from mechanically sheared DNA. Fragments with sizes of 1.5 to 2.0 kb were separated by agarose gel electrophoresis, end repaired, and cloned into the pZErO-2.1 vector (Invitrogen). DNAs of random plasmid clones were isolated, purified, and used as templates for shotgun sequencing reactions as described above. Management and analysis of nucleotide sequence data were performed using a Lasergene sequence analysis software system (DNASTAR, Inc., Madison, Wis.). Using the programs BlastN and BlastX (2) (http://www.ncbi.nlm.nih.gov) and FASTA (http://www.ebi.ac.uk/fasta3), homology searches were performed by comparing the sequences with the public DNA and protein databases. The DNA sequences were also compared with the sequences in the unfinished *E. coli* CFT073 genome DNA database (http://www.genome.wisc.edu; last updated on 2 June 2002).

Sequence analysis. A MegAlign program from a LASERGENE software package (version 5.01; DNASTAR, Inc.) was used to produce a multiple alignment of amino acid sequences, which included the inferred sequence of IroN determined here together with sequences retrieved from GenBank. Using a MEGA version 2.0 program (22), phylogenetic trees were constructed on the basis of pairwise estimates of the expected number of amino acid replacements per site with the aid of the neighbor-joining algorithm (33).

Construction of *iroN* and *iroE* mutants by EZ::TN transposon insertion. The DNA fragments carrying either *iroN* and *iroE* (fragment size, 3,776 bp; primers, ORF14.for and *iroD*.rev) or *iroB*, *-C*, *-D*, and *-E* (8,571 bp; *iroN*2.for and ORF20.rev) were amplified using LA-*Taq* DNA polymerase (TaKaRa Bio Inc.) with genomic DNA of *E. coli* HE300 as the template. The resulting PCR products were cloned into pCR4-TOPO vector (Invitrogen), resulting in plasmids pJS12 and pJS13 for *iroN* and *iroBCDE*, respectively (Table 1 and Fig. 1). Both plasmids were mutagenized in vitro using a DHFR-1 EZ::TN insertion kit (Epicentre Technologies, Madison, Wis.) and transformed into *E. coli* TH2, resulting in selection for ampicillin, kanamycin, and trimethoprim resistance. Insertion points were confirmed by restriction analysis and by sequencing with forward and reverse primers provided with the EZ::TN kit (Epicentre). Two plasmids derived from pJS12 with a dihydrofolate reductase 1 EZ::TN insertion in *iroN* and *iroE* genes were isolated and designated pJS14 and pJS15, respectively (Table 1 and Fig. 1).

RT-PCR. *E. coli* H5058 carrying pJS332 was grown to the logarithmic phase at 37°C in nutrient broth containing 50 μM 2,2′-dipyridyl. The RNA was isolated by means of an SV total RNA isolation system (Promega), and reverse transcriptase PCR (RT-PCR) was performed using an Access RT-PCR system (Promega) according to the recommendations of the manufacturer. Briefly, 2 μg of total RNA was treated with 10 U of RQ1 RNase-free DNase (Promega) for 60 min at 37°C followed by heat inactivation of the enzyme at 75°C for 6 min. For cDNA synthesis, the DNase-treated RNA sample was divided among two tubes, the sample and the respective negative control without reverse transcriptase. Avian myeloblastosis virus reverse transcriptase (Promega) was added to the sample together with 0.5 mM deoxynucleoside triphosphates and incubated at 48°C for 45 min. Three pairs of primers were chosen to amplify cDNA and detect transcription of the *iro* gene cluster: *iroN*1.for and *iroN*.rev for *iroN* (323 bp), *iroE*.for

and *iroD*.rev for *iroED* (928 bp), and *iroB*.for and ORF20.rev for *iroB*-ORF 20 (674 bp). PCR products were separated by electrophoresis on agarose gel.

Catecholate siderophore uptake assays. Enterobactin and 2,3-dihydroxybenzoyl-L-serine were purified from the growth supernatant of *E. coli* AN311 according to a published protocol (39). DHBA was purchased from Sigma Chemicals (Sigma-Aldrich, Munich, Germany), and DHBO was kindly provided by R. Reissbrodt (RKI, Werningerode, Germany). To investigate the functional expression of the identified *iroN-iroBCDE* gene cluster, the ability to promote the uptake of different catecholate siderophores was tested by a plate assay (30). *E. coli* strain H5058 (deficient in both catecholate siderophore uptake and enterobactin synthesis) was used as a tester strain for recomplementation of catecholate siderophore-mediated iron uptake (4). For this purpose, plasmids pJS12, pJS13, pJS14, and pJS15 were introduced into the *E. coli* H5058 strain and recombinant strains were poured into 10 ml of 0.6% NBD agar (NB with 100 μM 2,2′-dipyridyl). Filter paper disks impregnated with enterobactin, DHBS, DHBO, or DHBA (10 μl each of a 1-mg/ml solution) were placed onto the top agar, and after incubation overnight at 37°C, a visible halo around the filter papers appeared, indicative of growth support. Bacteria were stained using a 0.5% solution of 2,3,5-triphenyltetrazoliumchloride (TTC) (9).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been deposited in the GenBank database under accession number AY205565.

RESULTS

Detection of a virulence-associated locus by SSH. To identify new virulence-associated loci in uropathogenic *E. coli*, we performed SSH with genomic DNA of the uropathogenic archetypal *E. coli* strain CFT073 and the clinical *E. coli* isolate HE300 obtained from a patient suffering from severe UTI. A total of 25 subtracted DNA fragments with sizes ranging from 0.1 to 1.8 kb were obtained and subsequently subcloned into pCR4-TOPO vector (data not shown). In total, 13 of the subtractive clones were used as DNA probes in dot blot hybridizations of a reference strain collection to determine the prevalence of subtracted fragments among ExPEC strains. The collection consisted of 50 *E. coli* strains from blood cultures and urine samples of patients suffering from septicemia and UTIs, respectively. In addition, 25 *E. coli* strains from stool samples of healthy volunteers were included as control strains. One of the subtracted DNA fragments (AA107) revealed a high level of association with virulence, as it reacted with 23 out of 50 pathogenic *E. coli* strains but with only 1 out of 25 commensal strains ($\chi^2 = 13.51$; $P < 0.001$). Using the BlastN algorithm of the National Center for Biotechnology Information database at the website http://www.ncbi.nlm.nih.gov (1) as well as the FASTA algorithm of the EMBL database at the website http://www.ebi.ac.uk/fasta33 (22), we determined the nucleotide sequence of fragment AA107 and compared it for sequence homology to known genes. The subtracted fragment AA107 was shown to be a nucleotide fragment 196 bp in length with no significant homology to sequences in the databases.

To further characterize the DNA region adjacent to AA107, we constructed a cosmid library of uropathogenic *E. coli* strain HE300. Using the fragment AA107 as a DNA probe to screen some 800 cosmid clones by colony blot hybridization, eight cosmid clones were identified. We chose one of the cosmids, pJS332, for further studies and determined the complete sequence by a shotgun approach. To do this, we prepared a random plasmid library and obtained single sequence reads from about 600 clones. Given an average read length of 500 bp, approximately 300 kb of unique reads were generated.

Sequence analysis of cosmid pJS332. Cosmid pJS332 has 31,870 bp of inserted DNA that appears to be a composite of genes derived from plasmids, a chromosomal pathogenicity island, and additional diverse and unknown sources (Fig. 1 and Table 2). The overall G+C content of the pJS332 DNA region is 49.1%, which is about the average for the *E. coli* K-12 genome (50.8%). However, the G+C content differs significantly within pJS332 (23 to 72%), displaying peaks and troughs (Fig. 2). These peaks and troughs correspond to different functional units such as transposable elements, indicating a mosaic structure of pJS332 composed of elements of different origin. A total of 31 open reading frames (ORFs) larger than 150 nucleotides (nt), corresponding to a total coding region of 80.1%, were identified (Fig. 1 and Table 2). Seven translated ORFs were found to show no significant similarity or low similarity to protein sequences in the databases, while 24 putative polypeptides were very similar to proteins of *S. enterica* and *E. coli*. With regard to the nucleotide homology, the pJS332 DNA region exhibits a modular structure with three distinct regions (Fig. 1 and 2). Region I is located between positions 447 and 11972 and reveals 99.2% homology to 11,528 nt of *S. enterica* plasmid R64 (accession no. AP005147; nt 29968 to 41495). A 448-bp DNA fragment that represents the end of transposon Tn*1721* and that includes the 38-bp repeat unit (IRRII) precedes region I. Immediately downstream of IRRII, region I begins with ORF 2, which shows 100% identity to the *ycjA* gene of *S. enterica* serovar Typhimurium plasmid R64. Region I extends from ORF 2 (*ycjA*) to ORF 13 (*pifA**) (Table 2 and Fig. 1).

ORF 13 (*pifA**) represents the truncated 3′ part of the *pifA* gene of R64 and shares 100% identity with nt 893 to 2225. The interruption of *pifA* is caused by the insertion of a 1,058-bp DNA fragment with 98% identity to insertion sequence IS*903*. This fragment represents the border with region II and carries ORF 14, which shares 99% identity with the transposase IS*903*. Region II is located between positions 13078 and 23728 and harbors ORF 15 to ORF 20. It exhibits a high degree of homology to the *iroBCDEN* gene cluster found in the genome of uropathogenic *E. coli*. Adjacent to the 3′ end, region II is followed by a small 355-bp DNA mosaic comprised of four fragments of between 30 and 170 nt which is identical to the genomic DNA of *E. coli* CFT073 neighboring the 3′ end of the *iroBCDEN* cluster. The border of region II is composed of a truncated putative insertion element carrying ORF 21 and 22, the first of which encodes a conserved amino acid sequence, which is homologous to integrase core domains (Table 2 and Fig. 1).

Region III (located between positions 25287 and 31870) extends from ORF 23 to ORF 31 and reveals sequences related to insertion elements and plasmids or that encode putative products with no significant similarity to sequences in the GenBank database. The presence of a complete copy of insertion element IS*2* as well as two further putative transposase genes (ORF 28 and ORF 31) suggests region III to be a hot spot of insertional genetic elements (Fig. 1). The mosaic structure of region III (with DNA fragments of apparently different origins) is also reflected by the G+C content. It displays several troughs, which correspond to different transposable elements (Fig. 2). In particular, the three putative transposase genes (ORF 26, 27, and 31) are visible as deep troughs with a notably lower G+C content than their surroundings (Fig. 1 and 2). Moreover, region III is characterized by a low amount of

TABLE 2. Characteristics of ORFs and deduced amino acid sequences present in the sequenced DNA fragment

ORF	Gene	Product size (no. of amino acids)	ORF location (nt)[a]	Protein (description) to which ORF product exhibits homology	Source	Identity[b]		Accession no.
						%	Range (aa)	
1	*tnpA*	136	3–413	TnpA; transposase of Tn*1721*, truncated	*E. coli*	95	135	P51565
2	*ycjA*	107	791–468	YcjA; putative bacterial repressor protein	Plasmid R64; *S. enterica* serovar Typhimurium	100	107	BAB91595
3	*ydaA*	377	896–2029	YdaA; putative permease	Plasmid R64; *S. enterica* serovar Typhimurium	90	377	BAB91596
4	*ydbA*	205	2939–2322	YdbA	Plasmid R64; *S. enterica* serovar Typhimurium	96	205	BAB91597
5	*ibfA*	518	4686–3130	lbfA; putative ABC transporter	Plasmid R64; *S. enterica* serovar Typhimurium	95	518	BAB91598
6	*ydeA*	196	5539–4949	YdeA	Plasmid R64; *S. enterica* serovar Typhimurium	100	196	BAB91599
7	*ydfA*	85	5796–5539	YdfA; putative ABC transporter	Plasmid R64; *S. enterica* serovar Typhimurium	100	85	BAB91600
8	*ydfB*	712	6150–8288	YdfB; putative ABC transporter	Plasmid R64; *S. enterica* serovar Typhimurium	100	712	BAB91601
9	*mck*	133	8851–8450	Mck; involved in coordination of plasmid replication with cell division	Plasmid R64; *S. enterica* serovar Typhimurium	99	133	BAB91602
10	*kor*	76	9093–8863	Kor; involved in coordination of plasmid replication with cell division	Plasmid R64; *S. enterica* serovar Typhimurium	100	76	BAB91603
11	*ydiA*	96	9389–9679	YdiA; 93% identical to protein YebA on *E. coli* plasmid F	Plasmid R64; *S. enterica* serovar Typhimurium	100	96	BAB91604
12	*ydiA*	299	9669–10568	YdjA; 100% identical to protein YebB on *E. coli* plasmid F	Plasmid R64; *S. enterica* serovar Typhimurium	100	299	BAB91605
13	*pifA**	443	11949–10618	Truncated ORF; homologous to residues 299 to 714 of 741 residues of protein PifA; phage T7 exclusion protein	Plasmid R64; *S. enterica* serovar Typhimurium	100	443	BAB91606 P96329
14	*tnpA*	224	12299–12973	TnpA; transposase of IS*903*	Insertion sequence IS*903*	99	223	AAF30382
15	*iroN*	725	13385–15562	IroN; enterochelin and dihydrobenzoic acid receptor	PAI III of *E. coli* strain 536	99	725	CAC43424
16	*iroE*	318	16563–15607	IroE	PAI III of *E. coli* strain 536	99	318	CAC43425
17	*iroD*	409	17877–16648	IroD; putative ferric enterochelin esterase	PAI III of *E. coli* strain 536	99	409	CAC43426
18	*iroC*	1,261	21766–17981	IroC; putative ABC transporter	PAI III of *E. coli* strain 536	99	1,261	CAC43427.2
19	*iroB*	371	22895–21780	IroB; putative UDP-glucoronosyl and UDP-glucosyl transferase	PAI III of *E. coli* strain 536	100	371	CAD66179.1
20		89	23363–23094	Hypothetical protein	No significant homology			
21		173	24692–24171	Hypothetical protein; putative integrase	*E. coli* strain K5	59	100	CAA54707
22		96	25164–24874	Hypothetical protein	*E. coli* strain K5	59	48	CAA54710
23		97	26026–25733	Hypothetical protein	*E. coli*	80	76	AAB40752
24	*iss*	102	26347–26039	Iss (increased serum survival and complement resistance)	*E. coli* strain 102	100	102	AAD41540
25		80	26948–26706	Hypothetical protein	*E. coli* strain EDL933	100	26	AE005326
26		112	26959–27297	Putative transposase of insertion element IS*2*	*E. coli*	98	109	P51026
27		263	27404–28195	Putative integrase of insertion element IS*2*	*E. coli*	97	262	P51026
28		54	28530–28366	Hypothetical protein; homologous to gene *trp1328* on IS*1328*	*Yersinia enterocolitica*	63	50	CAB46575
29		397	29418–30611	Hypothetical protein; putative cobalamin synthesis protein	*E. coli* strain CFT073	65	397	NP_753181
30		103	30750–31061	Hypothetical protein; putative integrase	*Shigella flexneri* 2a strain 301	59	82	NP_709264
31		207	31248–31869	Truncated ORF; homologous to gene HCM1.201 on plasmid pHCM1; putative transposase	*S. enterica* serovar Typhi strain CT18	95	207	NP_569402

[a] Nucleotide positions from the start to stop codons in the pJS332 sequence.

[b] Identity is presented as percentage of amino acid identity between the pJS332 ORF and the best hit as determined with BLAST and FASTA. The range is the number of amino acids (aa) over which this identity exists.

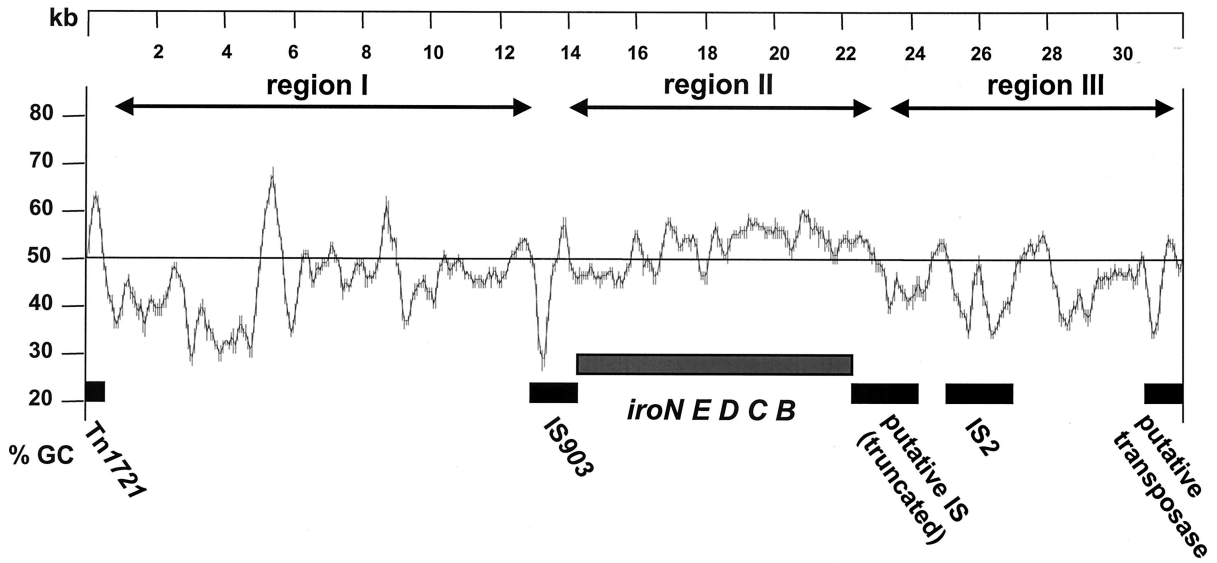

FIG. 2. G+C plot of the DNA sequence of pJS332. Regions I to III are indicated by double-headed arrows; black boxes mark putative transposase genes and IS elements with significantly different G+C content (troughs or peaks). The *iroBCDEN* gene cluster is shown as a dark gray box. The average G+C content of the *E. coli* K-12 chromosome is shown with a black line (50.8%).

coding DNA (65.4%), which is probably due to the accumulation of partially truncated IS sequences. The DNA fragment AA107 used as the initial probe was localized between positions 28807 and 28961 as part of the noncoding DNA of region III. In summary, pJS332 is organized in a modular fashion and consists of three regions that appear to be a composite of DNA fragments derived from plasmids, chromosomal loci (PAI III), and diverse IS elements.

The plasmid-carried *iroBCDEN* cluster is located on a transmissible plasmid related to plasmids of *S. enterica*. The homology of the cloned DNA fragment pJS332 to plasmid R64 of *Salmonella* suggested it for a search for plasmid origins. Plasmid DNA of *E. coli* HE300 was therefore isolated and subjected to Southern hybridization using fragment AA107 as a DNA probe. The results revealed pJS332 to be part of a plasmid named p300, the size of which is about 78.5 kb as determined by restriction analysis using different enzymes (data not shown). After transformation of the entire plasmid DNA of *E. coli* HE300 in *E. coli* S17-1, p300 was mobilized to *E. coli* TH2 by mating. Thus, the pJS332 region carrying the *iroBCDEN* cluster is located on a transmissible plasmid that was probably derived from *Salmonella* plasmid R64, as suggested by the high number of orthologous genes (region I). In addition, both p300 and R64 plasmids confer resistance to trimethoprim, sulfamethoxazole, and tetracycline (15).

RT-PCR experiments detected transcription of the *iroBCDE* gene cluster. The results of the RT-PCR assays confirmed the transcription of *iroN* and *iroBCDE* genes under iron-limited conditions. Interestingly, an amplification product was obtained using primers for the *iroB* gene and ORF 20 upstream of *iroBCDE*, suggesting that both form a transcriptional unit transcribed in an operon fashion (Fig. 3). To investigate the functional expression of the identified *iroBCDEN* gene cluster, the ability to promote the uptake of different catecholate siderophores as iron sources was tested by a plate assay. To do this, the *iroBCDEN* cluster was subcloned (pJS12 and pJS13; Fig. 1), introduced into the *E. coli fepA cir fiu aroB* mutant H5058, and examined in a siderophore cross-feeding assay as described previously (4). The results of the feeding assay revealed that as in *S. enterica*, the *iro* gene cluster of pJS332 promotes the uptake of enterochelin, DHBS, DHBO, and DHBA. Moreover, IroN alone is sufficient to facilitate catecholate siderophore utilization, since plasmids pJS12 (*iroN-iroE*) and pJS15 (*iroN-iroE*::Tp^r), but not plasmid pJS13 (*iroBCDE*) or pJS14 (*iroN*::Tp^r-*iroE*), promoted siderophore uptake by *E. coli* H5058 (Fig. 1 and Table 1).

Phylogenetic analysis of IroN. A phylogenetic tree was constructed from the amino acid sequences of IroN for both *E. coli* and *S. enterica* by the neighbor-joining algorithm (22) and rooted by the sequence of the *E. coli* enterobactin receptor FepA (Fig. 4). It revealed two distinct clusters of *iroN* alleles that grouped by species, with only a little divergence within each cluster. Very recently, Dozois et al. described a plasmid-carried *iro* gene cluster found in the avian pathogenic *E. coli* strain χ7122 (13). The amino acid sequence of this $IroN_{\chi7122}$ is 100% identical to that of $IroN_{HE300}$. In addition, the IroN peptide of the uropathogenic *E. coli* CP9 strain described by Russo et al. (32) is identical to $IroN_{CFT073}$ of *E. coli* CFT073. These results suggest the presence of three phylogenetic lineages of IroN in *E. coli* (Fig. 4).

Distribution of mobilizable *iroBCDEN* gene clusters among uropathogenic *E. coli* strains. To determine the distribution of the plasmid-carried *iroBCDEN* gene cluster and compare it with the distribution of the chromosomal cluster, dot blot hybridizations were performed using probes derived from either the *iroN* gene or region I (*ydfB* gene). In a survey of 50 uropathogenic *E. coli* isolates and 30 *E. coli* isolates from the stools of healthy volunteers, we were able to detect *iroN* in 29 (58%) of the uropathogenic *E. coli* strains, two of which also proved positive for the *ydfB* gene (4%). Six of the isolates were

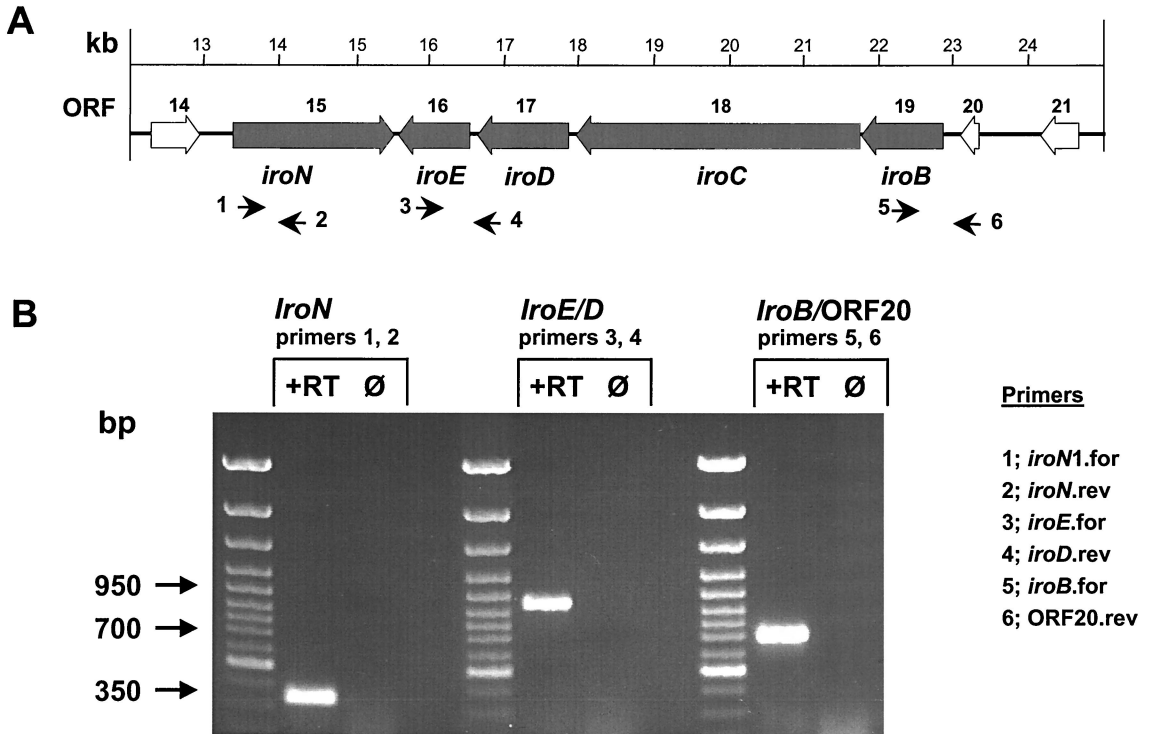

FIG. 3. RT-PCR analysis of the *iroBCDEN* cluster. (A) Genetic organization of the *iroN*, *iroBCDE*, and upstream genes. Numbered arrows below the map indicate the locations of 5′ and 3′ primers used in RT-PCR. Thick arrows indicate the direction of transcription of each gene. (B) Results of RT-PCR amplification assays. Primers used in each reaction are listed above the corresponding bracket. To verify that DNA was not the amplification template, each RT-PCR was run without the addition of RT (lanes Ø). The size of the expected band for each reaction is indicated.

positive for *ydfB* alone. The two isolates positive for both *iroN* and region I (*ydfB*) were compared with *E. coli* strain HE300 with regard to a potential phylogenetic relationship. Interestingly, all three isolates revealed different O antigens, and while the two strains belong to the *E. coli* phylogenetic group B1, the HE300 strain was assigned to group A by means of a PCR-based typing method (10). Thus, these results favor the idea of a horizontal transmission of this particular virulence factor between only distantly related *E. coli* strains. These data further demonstrate that the *iroN* cluster is not linked to plasmid p300 in the vast majority of uropathogenic *E. coli* strains but rather is chromosomally located or at least linked to a different genetic backbone.

DISCUSSION

In the present report we have described the virulence-associated DNA region pJS332, which was isolated from a uropathogenic *E. coli* strain. This region, which is 31,870 bp in size, is located on the transmissible plasmid p300 and reveals a

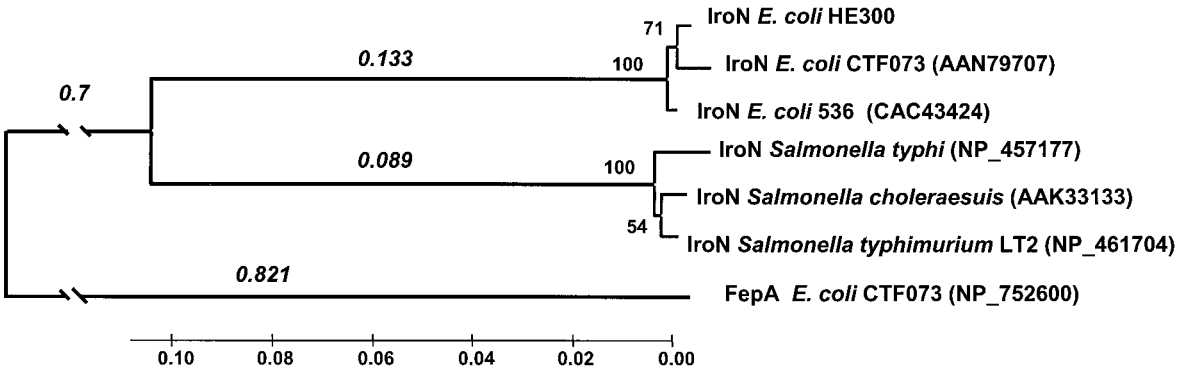

FIG. 4. The phylogenetic tree was constructed using the neighbor-joining algorithm based on the gamma distance with $\alpha = 2$ and rooted by the sequence of the *E. coli* enterochelin receptor FepA. The gamma distance assumes that substitutions follow a gamma distribution with the α parameter specifying the amount of variation across amino acid positions. Branch lengths (in number of amino acid substitutions per site) are given in italics above the major branches. Bootstrap confidence levels are given under the nodes, and accession numbers are shown in parentheses.

composite structure of elements of different origins (chromosomal pathogenicity island, plasmid, and transposable elements) with three distinct DNA regions. Region I resembles a part of plasmid R64 from *S. enterica*, region II is a copy of the *iroBCDEN* cluster found on pathogenicity islands of extraintestinal *E. coli*, and region III comprises different partially truncated insertion sequences. The mosaic nature of p300 is underscored by the observation that the G+C contents of the three DNA regions differ greatly, suggesting that they are of different origins. The *iroBCDEN* gene cluster encodes a catecholate siderophore uptake system, which is induced in uropathogenic *E. coli* isolate CP9 upon incubation in urine (31). The recent work of Russo et al. provided evidence that IroN is a virulence factor of *E. coli* strain CP9 (32). Our study illustrates that the IroN encoded by the plasmid p300 mediates the uptake of different catecholate siderophores such as enterobactin and other linear forms of catecholate siderophores such as DHBA, DHBO, and DHBS. These data broaden the recent results of catecholate siderophore uptake mediated by IroN of *E. coli* (32) by showing the specific uptake of different purified catecholate siderophore compounds for the first time. In a recent study, Dobrindt et al. analyzed the structures of different PAIs of the uropathogenic *E. coli* isolate 536 and found the *iroBCDEN* cluster to be part of PAI III_{536} (12). Upstream of the *iroBCDEN* operon, which is homologous to the *iro* gene cluster of the *Salmonella* species, an *sfa* determinant encoding fimbrial adhesins of the S-adhesin family has been detected (11). Interestingly, it has been shown that the *iroN* gene of strain CP9 is also part of a PAI and is preceded by DNA sequences with homology to the related *prs* or *foc* gene cluster (F1C fimbriae) (31). In addition, the recently published genome sequence of the uropathogenic *E. coli* CFT073 strain reveals a chromosomal *iroBCDEN* gene cluster on a genomic island neighboring an orthologous *sfa*/*foc* gene cluster (38). These data indicate that all *iro* clusters of *E. coli* characterized so far are associated with determinants coding for members of the S-adhesin family. However, this is not true for the *iro* gene cluster of pJS332 described in this study. The homology to the cluster of other *E. coli* is interrupted upstream of the *iroN* gene by insertion of IS*903*, which forms the border with region I. In contrast, the regions downstream of the *iroBCDEN* cluster bear structural similarity in all *E. coli iroBCDEN* gene clusters. Different insertion sequences and other partially deleted mobile elements are scattered in the 3′ vicinity of the clusters, suggesting a hot spot for insertion elements. Further elements contributing to the mosaic nature of pJS332 are the transposable element Tn*1721* and the insertion sequence IS*2*, whose origins remain unknown owing to the versatility and widespread distribution of these elements. The presence of insertion sequences adjacent to gene clusters encoding different iron uptake systems has previously been described, and it has been suggested that the insertion sequences mediate the mobilization of the respective cluster. Thus, the genes encoding the aerobactin siderophore system in *E. coli* are located on the pColV plasmid and flanked by IS*1* elements that may facilitate its transposon-like nature (25). The orthologous aerobactin gene cluster of *Shigella boydii* is part of the genomic island SHI-3 and is framed by IS*600* insertion sequences (29). Therefore, the presence of insertion sequences in the direct neighborhood of the *iroBCDEN* cluster described here is suggestive of a role in its mobilization and transfer. The phylogenetic analysis of different *E. coli* IroN proteins agrees with the results obtained from sequencing the 3′ end of region II of pJS332, which revealed small nucleotide fragments with a high homology to DNA segments downstream from *iroB* in *E. coli* strain CFT073. These data provide circumstantial evidence that the insertion of IS elements followed by site-specific recombination events led to the transfer of the *iro* gene cluster from the chromosomal location to plasmid p300. During the preparation of the present report, Dozois et al. described an *iro* gene cluster carried by plasmid pAPEC-1 of the avian pathogenic *E. coli* strain χ7122 (13). The authors of that study demonstrated that the pAPEC-1 plasmid carries the *iutA* gene for the ferric aerobactin siderophore receptor. As no *iutA* gene was detected in *E. coli* HE300, we suggest a different molecular structure for both pAPEC-1 and p300. Furthermore, the antibiotic resistance (conferred by p300) of *E. coli* strain HE300 to tetracycline and trimethoprim-sulfamethoxazole supports the structural difference. No comparable resistance pattern is detectable in the avian pathogenic *E. coli* strain χ7122. In the present report, we show the coexistence of a virulence factor and antibiotic resistance genes on the same transmissible plasmid. Such colocalizations have previously been described for other *E. coli* pathotypes (34) and suggest that antibiotic-selective pressure also indirectly selects for enhanced virulence.

Pathogenic bacteria have obtained a significant proportion of their genetic diversity through the acquisition of DNA from distantly related organisms (26). The horizontal gene transfers have effectively changed the ecological and pathogenic character of bacterial species, producing extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome. Different genetic elements such as phages, plasmids, transposons, and PAIs are involved in lateral gene transfer (6, 16).

However, it is not known at present whether pathogenicity islands are acquired intact (e.g., by phage transduction) or by a stepwise insertion of genes into the island. It is also possible that pathogenicity islands use diverse mechanisms of horizontal transfer. The data drawn from this study point to a transfer of single virulence determinants, which may at least modify the structure and function of genomic islands. This also supports the view that virulence determinants are often structured in a modular manner and that parts of PAIs can be distributed individually (19). Furthermore, the detection of a plasmid-carried *iro* gene cluster corresponds with previous findings that even virulence factors known to be PAI associated in certain strains commonly exhibit divergent patterns of phylogenetic distribution both among lineages and even within a given lineage (7). This is a strong indication that virulence genes are mobile independently of PAIs and that PAIs are subject to continuous, ongoing remodeling (19). Thus, the presence of the *E. coli iroBCDEN* gene cluster on a transmissible plasmid may explain the particular pattern of phylogenetic distribution described by Johnson et al. (20). The authors of that study found a sporadic appearance of $iroN_{E.\ coli}$ in many different *E. coli* serotypes and suggested that either multiple horizontal acquisition events or multiple scattered deletions of $iroN_{E.\ coli}$ within otherwise $iroN_{E.\ coli}$-positive lineages were responsible. These data are consistent with a model in which PAIs, although perhaps occasionally subject to en bloc horizontal

transfer (8, 16) or total deletion (5, 6, 17), participate to a much greater extent in the horizontal transfer of single virulence factors by providing genomic regions accessible to the insertion, retention, and release of individual virulence genes.

Our future work is to further investigate the mechanisms of transfer and recombination of the plasmid-carried *iroBCDEN* gene cluster as a variable virulence factor module. A better understanding of the prevalence and evolutionary origins of the virulence factors of ExPEC strains would accelerate the development of virulence factor-specific preventive measures that are truly needed against these common and morbid infections.

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