

## Analysis of Genome Plasticity in Pathogenic and Commensal *Escherichia coli* Isolates by Use of DNA Arrays

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**Genomes of prokaryotes differ significantly in size and DNA composition. *Escherichia coli* is considered a model organism to analyze the processes involved in bacterial genome evolution, as the species comprises numerous pathogenic and commensal variants. Pathogenic and nonpathogenic *E. coli* strains differ in the presence and absence of additional DNA elements contributing to specific virulence traits and also in the presence and absence of additional genetic information. To analyze the genetic diversity of pathogenic and commensal *E. coli* isolates, a whole-genome approach was applied. Using DNA arrays, the presence of all translatable open reading frames (ORFs) of nonpathogenic *E. coli* K-12 strain MG1655 was investigated in 26 *E. coli* isolates, including various extraintestinal and intestinal pathogenic *E. coli* isolates, 3 pathogenicity island deletion mutants, and commensal and laboratory strains. Additionally, the presence of virulence-associated genes of *E. coli* was determined using a DNA “pathoarray” developed in our laboratory. The frequency and distributional pattern of genomic variations vary widely in different *E. coli* strains. Up to 10% of the *E. coli* K-12-specific ORFs were not detectable in the genomes of the different strains. DNA sequences described for extraintestinal or intestinal pathogenic *E. coli* are more frequently detectable in isolates of the same origin than in other pathotypes. Several genes coding for virulence or fitness factors are also present in commensal *E. coli* isolates. Based on these results, the conserved *E. coli* core genome is estimated to consist of at least 3,100 translatable ORFs. The absence of K-12-specific ORFs was detectable in all chromosomal regions. These data demonstrate the great genome heterogeneity and genetic diversity among *E. coli* strains and underline the fact that both the acquisition and deletion of DNA elements are important processes involved in the evolution of prokaryotes.**

Horizontal gene transfer and gene reduction represent two mechanisms contributing to the evolution of prokaryotic genomes “in quantum leaps” (20). Thus, the acquisition of plasmids and phages, as well as large DNA regions called “genomic islands,” plays an important role in the development of new species, subspecies, and pathotypes. Among others, the species *Escherichia coli* represents an excellent model to study evolution of prokaryotic genomes in detail (36). *E. coli* is an ideal example for these studies, as numerous ecotypes, adapted to the intestines of humans and various animals, exist. In addition, the species *E. coli* comprises various pathotypes, which act as causative agents in human as well as veterinary medicine. They can be grouped as extraintestinal pathogenic *E. coli* (ExPEC), causing urinary tract infections, newborn meningitis, or sepsis, and as intestinal pathogenic *E. coli* (IPEC), causing enteric and diarrheal diseases. The broad spectrum of pathogenic features and of different clinical symptoms caused by *E. coli* pathotypes mirrors the presence of different subsets of

virulence-associated genes in certain pathotypes which are absent in commensal isolates (42, 45). The localization of many virulence-associated genes on mobile genetic elements, such as bacteriophages, plasmids, and pathogenicity islands (PAIs), indicates that horizontal gene transfer plays a major role in the evolution of different bacterial pathotypes (14). The genome sizes of natural *E. coli* isolates have been shown to be very heterogenous and may differ by as much as 1 Mb (5). This heterogeneity is thought to be the result of the deletion, as well as the acquisition, of genetic elements. The estimation that ~18% of all open reading frames (ORFs) of the *E. coli* strain MG1655 were horizontally acquired and that, for the majority of them, this occurred relatively recently (36) underlines the high variability of the gene content within this species.

To characterize the genetic diversity and genome structures among different pathogenic and commensal variants of *E. coli*, we applied a whole-genome approach. Using DNA arrays, the genome contents of seven ExPEC (including a fecal O18:K1 strain) and eight IPEC isolates, as well as of eight commensal strains from healthy volunteers (including the laboratory strains MG1655 and B), were determined by investigation of the presence of all 4,290 translatable ORFs of the sequenced nonpathogenic *E. coli* K-12 strain MG1655 (6). In addition, a

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TABLE 1. Strains used in this study

Pathotype	Strain <sup>a</sup>	Serotype	Chromosomal size (Mb)	ECOR group <sup>b</sup>	Known virulence-associated genes on horizontally transferred DNA elements <sup>c</sup>	Known horizontally transferred DNA elements
UPEC	536	O6:K15:H31	4.92	B2	<i>sfaI</i> , <i>prf</i> ( <i>papG</i> allele III), <i>hly</i> (PAI I <sub>536</sub> + PAI II <sub>536</sub> ), <i>kps</i> (group II), <i>fyuA</i> , <i>iro</i> , <i>malX</i>	PAIs
UPEC	536-21*	O6:K15:H31	4.69	B2	<i>sfaI</i> , <i>kps</i> (group II), <i>fyuA</i> , <i>iro</i> , <i>malX</i>	PAIs
UPEC	J96	O4:K:H5	5.11	B2	<i>foc</i> , <i>pap</i> ( <i>papG</i> allele I), <i>prs</i> ( <i>papG</i> allele III), <i>hly</i> (PAI I <sub>J96</sub> + PAI II <sub>J96</sub> ), <i>kps</i> (group III), <i>fyuA</i> , <i>iro</i> , <i>cnf1</i> , <i>malX</i>	PAIs
UPEC	J96-M1*	O4:K:H5	5.06	B2	<i>foc</i> , <i>pap</i> ( <i>papG</i> allele I), <i>hly</i> (PAI I <sub>J96</sub> ), <i>kps</i> (group III), <i>fyuA</i> , <i>iro</i> , <i>malX</i>	PAIs
UPEC	764	O18:K5:H5/11	5.07	B2	<i>foc</i> , <i>hly</i> , <i>fyuA</i> , <i>iutA</i> , <i>kps</i> (group II), <i>malX</i>	PAIs
UPEC	764-2*	O18:K5:H5/11	5.02	B2	<i>foc</i> , <i>fyuA</i> , <i>iutA</i> , <i>kps</i> (group II), <i>malX</i>	PAIs
UPEC	P42	O1:K1:H7	5.06	B2	<i>foc</i> , <i>pap</i> ( <i>papG</i> allele II), <i>fyuA</i> , <i>kps</i> (group II), <i>malX</i>	PAIs
UPEC (ABU) <sup>d</sup>	83972	OR:K5:H <sup>-</sup>	4.88	B2	<i>kps</i> (group II), <i>iutA</i> , <i>fyuA</i> , <i>malX</i>	PAIs
MNEC	IHE3034	O18:K1:H7/9	4.84	B2	<i>sfaII</i> , <i>ibeA</i> , <i>iro</i> , <i>kps</i> (group II), <i>fyuA</i> , <i>malX</i>	PAIs
Fecal isolate	F54	O18:K1	5.02	B1	<i>traT</i> , <i>fyuA</i> , <i>kps</i> (group II), <i>malX</i>	ND
EPEC	C9221a	O6:K15:H16	4.79	A	<i>est</i> , <i>elt</i> , <i>fyuA</i> , <i>kps</i> (group II)	PAIs, plasmid, phage
EPEC	E2348/69	O127:H6	4.7	B2	<i>eae</i> , <i>esp</i> , <i>astA</i> , <i>bfp</i> , <i>per</i>	PAIs, plasmid
EIEC	EDL1284	O124:H <sup>-</sup>	4.68	A	<i>ipaBCD</i> , <i>mxiAB</i> , <i>icsA</i> , <i>iuc</i> , <i>fyuA</i>	PAIs, plasmid
EHEC	4797/97	O91:H <sup>-</sup>	5.25	B1	<i>stx2d</i>	PAI, phage
EHEC	5714/96	O103:H2	4.89	D	<i>stx1</i> , <i>eae</i> (ε), <i>hlyA</i> , <i>etp</i> , <i>sfp</i>	PAI, plasmid, phage
EHEC	1639/77	O111:H <sup>-</sup>	4.85	B1	<i>stx1</i> , <i>eae</i> (γ)	PAI, plasmid, phage
EHEC	SF493/89	O157:H <sup>-</sup>	4.87	D	<i>stx2</i> , <i>eae</i> (γ), <i>hlyA</i> , <i>etp</i> , <i>sfp</i>	PAI, plasmid, phage
EAEC	DPA065	O119	4.69	A	<i>astA</i> , <i>fyuA</i> , <i>kps</i> (group II)	PAI, plasmid
Commensal isolates	MGS 6	ND <sup>e</sup>	4.71	A		ND
	MGS 32	ND	4.95	B1		ND
	MGS 73	ND	4.99	A		ND
	MGS 89	ND	5.03	A		ND
	MGS 104	ND	5.03	A		ND
	MGS 124	ND	5.07	A	<i>traT</i> , <i>papEF</i> , <i>fyuA</i> , <i>iutA</i> , <i>kps</i> (group II)	PAIs
Laboratory strains	B	ND	4.7	A		Phages
	MG1655	K-12	4.64	A		Phages

<sup>a</sup> PAI deletion mutants of the UPEC strains 536, J96, and 764 are marked by asterisks.

<sup>b</sup> Strains were grouped based on the triplex PCR as recently described (13).

<sup>c</sup> The presence of virulence-associated genes of ExPEC and of the fecal nonpathogenic *E. coli* isolates was determined using the multiplex PCR described by Johnson and Stell (28).

<sup>d</sup> ABU, asymptomatic bacteriuria.

<sup>e</sup> ND, not determined.

newly developed “*E. coli* pathoarray,” which currently consists of 456 probes specific for typical virulence-associated genes of ExPEC, IPEC, and *Shigella*, was used to assess the distribution of these genes or their homologues among the pathogenic and commensal strains used in this study. Furthermore, the genome contents of three uropathogenic *E. coli* (UPEC) isolates were compared with those of their derivatives which had lost large chromosomal regions called PAIs. With these approaches, we address several issues concerning the large genetic diversity and the mechanisms involved in genome optimization in *E. coli*. Our results underline the fact that *E. coli* can be used as a paradigm to analyze the evolution of bacteria by whole-genome approaches.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Twenty-six *E. coli* strains which belong to the Institut für Molekulare Infektionsbiologie (Würzburg, Germany) strain collection were used in this study. These strains exhibit different genome sizes and contain different additional mobile genetic elements (Table 1). The

UPEC strains 536, J96, and 764; the asymptomatic bacteriuria strain 83972; the newborn meningitis-causing *E. coli* (MNEC) strain IHE3034, and the enterohemorrhagic *E. coli* (EHEC) strains 4797/97, 5714/96, 1639/77, and SF493/89, as well as E2348/69 (enteropathogenic *E. coli* [EPEC]), have been described elsewhere (3, 7, 25, 31, 32, 35, 37, 46, 52). The deletion mutants 536-21, J96-M1, and 764-2 have been described before (7, 8, 34). The enteroaggregative *E. coli* (EAEC) strain DPA065 was provided by A. Giammanco (University of Palermo, Palermo, Italy). The enterotoxigenic *E. coli* (ETEC) strain C9221a and the enteroinvasive *E. coli* (EIEC) strain EDL1284 belong to the strain collection of the Institut für Molekulare Infektionsbiologie. The UPEC strain P42 and the fecal O18:K1 isolate F54 belong to the strain collection of the Department of Microbiology, Immunology and Glycobiology, Lund University, Lund, Sweden. The *E. coli* K-12 strain MG1655 and *E. coli* strain B, as well as the six commensal fecal isolates, have been described before (6, 41, 56). All of the strains were grown in Luria-Bertani medium (51).

**Genome comparison using *E. coli* gene arrays.** Total genomic DNAs from the different *E. coli* strains were used to probe Panorama *E. coli* gene arrays (Sigma-Genosys, Cambridge, United Kingdom). Two micrograms of total genomic DNA of each of the different strains was used as a template for direct incorporation of [<sup>33</sup>P]dATP (Amersham Pharmacia, Freiburg, Germany) by a randomly primed polymerization reaction using 0.75 μg of random hexamer primers (New England Biolabs, Frankfurt [Main], Germany) and 10 U of Klenow fragment of

DNA polymerase I (New England Biolabs) according to the manufacturers' recommendations. Unincorporated nucleotides were removed with Microspin S 200 HR spin columns (Amersham Pharmacia). Prior to hybridization, the DNA macroarrays were rinsed in  $2\times$  SSPE ( $1\times$  SSPE is 0.18 M NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , and 1 mM EDTA [pH 7.7]) solution and subsequently prehybridized for 3 h at  $65^\circ\text{C}$  in 5 ml of hybridization solution ( $5\times$  SSPE, 2% sodium dodecyl sulfate,  $1\times$  Denhardt's solution, 100  $\mu\text{g}$  of sheared denatured herring sperm DNA/ml). After the addition of the probe denatured in 3 ml of hybridization solution, the arrays were incubated for 12 to 18 h at  $65^\circ\text{C}$ . After hybridization, the blots were washed according to the manufacturer's guidelines. The washed filters were air dried and exposed overnight to a PhosphorImager screen (type, super resolution) prior to being scanned on a Typhoon 8600 variable mode imager (Molecular Dynamics). Before rehybridization, the *E. coli* gene arrays were stripped following the manufacturer's recommendations. Complete removal of radioactivity was confirmed by phosphorimaging after overnight exposure to a PhosphorImager screen.

For every *E. coli* strain investigated in this study, the *E. coli* gene arrays were hybridized in four different experiments using independently labeled DNA probes. The scanned *E. coli* gene arrays were analyzed with ArrayVision software (Imaging Research, St. Catharines, Canada), followed by visual inspection. Calculation of normalized intensity values of the individual spots was performed using the overall spot normalization function of ArrayVision. Background values were measured in the surrounding region of every secondary grid, which contains probes for four ORFs arrayed in duplicate. The mean of the normalized intensity values of the duplicate spots of each gene was used for further analysis. To avoid extreme intensity ratios for genes close to or below the detection limit, signal intensity values corresponding to a signal-to-noise (S/N) ratio of  $<1.0$  were scaled up to a value corresponding to an S/N ratio of 1.0. ORFs were recorded as lacking-undetectable if the S/N ratio was  $<1.0$  in at least three of the four hybridization experiments. In addition, *E. coli* K-12 strain MG1655-specific ORFs were recorded as lacking-undetectable if the ratio of the individual S/N ratios of the analyzed strain and that of the reference strain MG1655 was  $<0.3$  in at least three of four experiments. The missing-undetectable ORFs were then aligned with their chromosomal locations to determine the number and the sizes of chromosomal regions absent in the different *E. coli* strains. In addition, the fact that the ORFs are arranged on the DNA macroarrays without regard to their chromosomal localization minimizes the record of false-negative spots, at least with respect to regions consisting of more than one gene, because the probability that two adjacent ORFs would be recorded as absent due to hybridization artifacts is very low. The *E. coli* gene arrays were hybridized with labeled genomic DNA of the *E. coli* strain MG1655 as a positive control and that of the *Staphylococcus aureus* strain Wood 46 as a negative control. Generally, the quotients of the individual S/N ratios obtained from hybridization with genomic DNA of *S. aureus* and those of *E. coli* strain MG1655 were  $<0.3$ .

**Development of the *E. coli* pathoarray.** To facilitate detection of virulence-associated genes of ExPEC or diarrheagenic *E. coli*, a DNA array was developed which contains probes ( $n = 212$ ) specific for the majority of ORFs located on five PAIs of UPEC strain 536, as well as for all other ExPEC-specific virulence genes ( $n = 100$ ) described so far. The *E. coli* pathoarray also contains probes specific for several typical virulence-associated genes of IPEC and *Shigella* ( $n = 95$ ) (Fig. 1). PCR products of 300 to 500 bp which were specific for the individual genes of interest were generated. The quality and concentration of the PCR products were checked prior to spotting them as double spots onto nylon membranes. These probes enable the detection of genes encoding typical toxins, siderophores, and fimbrial and nonfimbrial adhesins, as well as of other genes which have been described as being involved in the virulence of ExPEC and/or IPEC or which are present on PAIs and other mobile genetic elements frequently present in pathogenic *E. coli* variants. Primers were generated on the basis of available DNA sequences of PAI I<sub>536</sub> to PAI V<sub>536</sub> of UPEC strain 536, as well as on the basis of publicly available DNA sequences, including those of PAIs of other ExPEC and ETEC isolates (J96, CFT073, and 10407), complete genomes (O157:H7 strains EDL933 and Sakai), and single virulence genes of interest. Genomic DNAs of the strains from which the DNA sequences of the different virulence-associated genes had been initially determined were used as templates for probe generation. Supplementary information on primer sequences and genes included in the *E. coli* pathoarray are available (<http://www.uni-wuerzburg.de/infektionsbiologie>).

**PCR.** 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). The grouping into the main phylogenetic lineages of the *E. coli* Collection of Reference Strains (ECOR) was done by a triplex PCR described previously (13). Virulence assess-

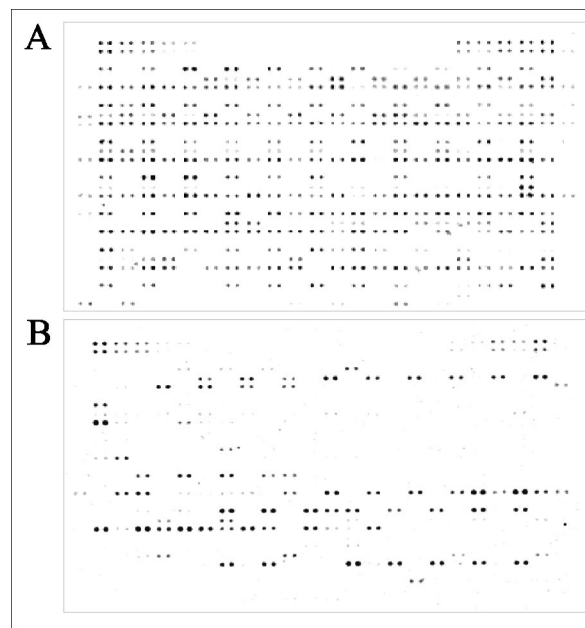


FIG. 1. Analysis of the presence of virulence-associated genes among pathogenic, commensal, and laboratory *E. coli* strains using the *E. coli* pathoarray. (A) Signal intensities after DNA-DNA hybridization with labeled genomic DNA of the UPEC strain 536. (B) Signal intensities after DNA-DNA hybridization with labeled genomic DNA of the commensal *E. coli* isolate MGS 73 isolated from a healthy volunteer.

ment of the ExPEC and nonpathogenic *E. coli* strains included a multiplex PCR specific for a set of typical virulence-associated genes of ExPEC (28).

**PFGE.** The chromosomal sizes of the different *E. coli* isolates were determined by pulsed-field gel electrophoresis (PFGE). Genomic DNA for the analysis by PFGE was prepared in agarose plugs and cleaved with the restriction enzyme *I-CeuI*. PFGE was performed with the CHEF-Dr III system (Bio-Rad, Munich, Germany) in  $0.5\times$  Tris-borate-EDTA buffer at  $6.5\text{ kV/cm}^2$  and  $12^\circ\text{C}$ . Electrophoresis was carried out with pulse times increasing from 5 to 50 s over a period of 22 h.

**DNA sequence analysis.** The genomic region which replaces the Rac prophage, present in *E. coli* strain MG1655, in UPEC strain 536 was sequenced by primer walking (using an ABI-310 sequencer) starting from the DNA fragment A2 obtained by subtractive hybridization analysis of genomic DNA of strain 536 with that of the nonpathogenic *E. coli* strain MG1655 (27). Primer walking was continued until at least 2 kb of the *E. coli* MG1655-specific chromosomal backbone had been sequenced at both ends of this DNA region. In addition, primer walking was also started from the conserved chromosomal backbone of *E. coli* to confirm that the up- and downstream regions of the ORFs b1367 and b1344, respectively, overlap with the so-far-unknown DNA sequence found on subtractive hybridization analysis fragment A2.

**Nucleotide sequence accession number.** The nucleotide sequence of the genomic region replacing the Rac prophage in *E. coli* strain 536 and the flanking DNA stretches was submitted to the EMBL database (accession number AJ496193).

## RESULTS

**Overall analysis of genetic diversity in *E. coli* using the *E. coli* K-12-specific DNA arrays.** In our study, we analyzed the genome structures of 23 *E. coli* isolates representing different phenotypes of *E. coli*, as well as nonpathogenic commensal isolates, including the laboratory strain *E. coli* B. The group of ExPEC isolates consisted of five UPEC strains and one MNEC strain. In addition to MNEC strain IHE3034, the genome contents of another O18:K1 strain, i.e., fecal isolate F54, was

investigated, as well as those of deletion mutants of the UPEC strains 536, J96, and 764, which had lost PAIs. The genetic diversity of four EHEC isolates of serotypes O157:H<sup>-</sup>, O111, O103, and O91, as well as those of four additional intestinal pathogens representing the ETEC, EPEC, EIEC, and EAEC pathotypes, were also characterized. Six nonpathogenic commensal isolates from feces of healthy volunteers, as well as the laboratory strains *E. coli* B and MG1655, were included in this study to assess whether genetic diversity in pathogenic isolates differs from that in nonpathogenic strains. According to a rapid method to group *E. coli* strains into the different main phylogenetic groups of the ECOR (13), most of the pathogenic strains tested in this study belong to the ECOR groups B2 and D, to which virulent *E. coli* isolates mainly belong, whereas the nonpathogenic strains are members of ECOR groups A and B1. The different isolates differ with respect to their chromosomal sizes (between ~4.67 and 5.25 Mb, as analyzed by PFGE) and to the presence of already known virulence-associated genes located on different types of mobile genetic elements, such as bacteriophages, plasmids, and PAIs (Table 1).

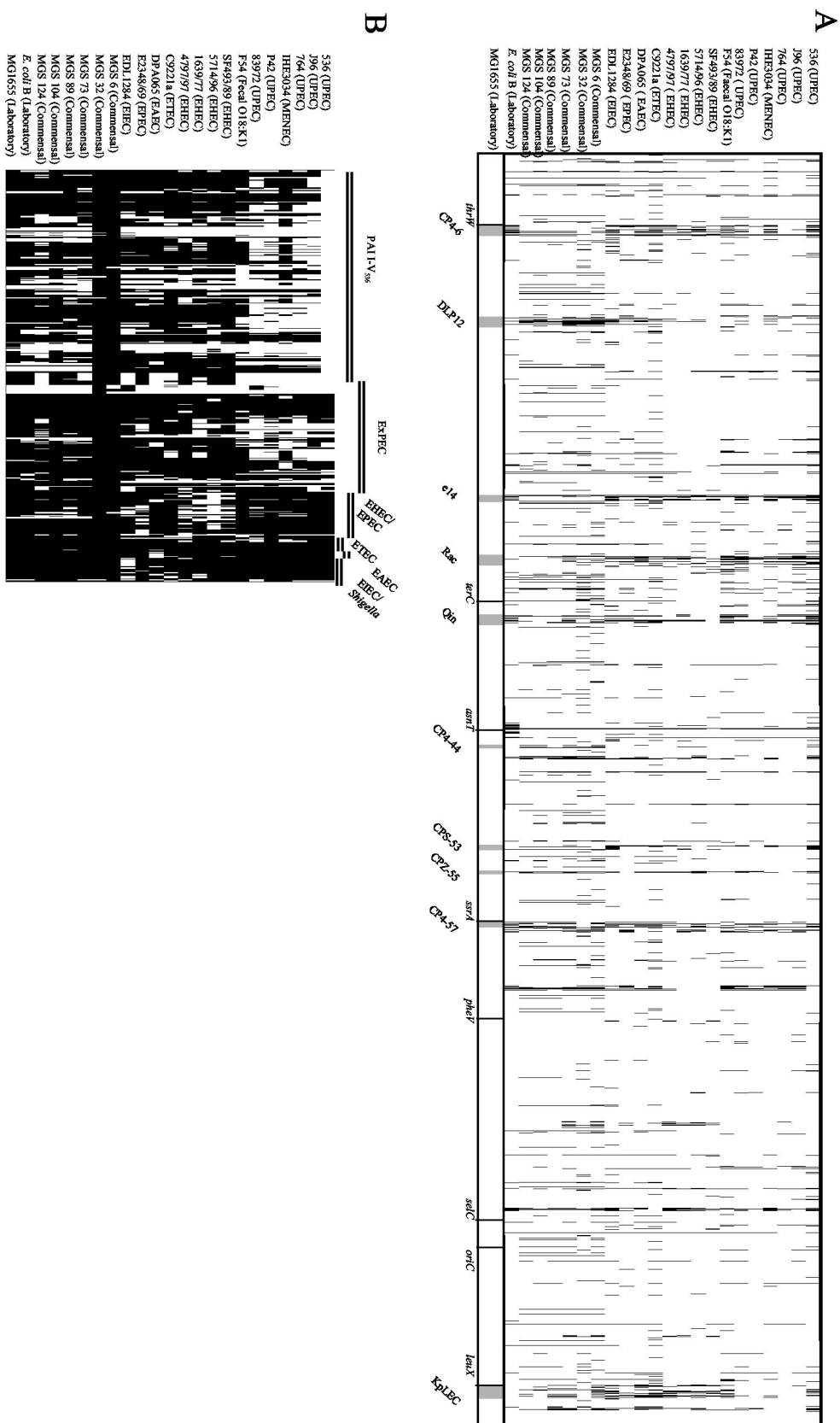
The genome contents of the 22 pathogenic and commensal *E. coli* isolates were compared to that of the nonpathogenic *E. coli* K-12 strain MG1655 by DNA-DNA hybridization using an *E. coli* strain MG1655-specific DNA array which has been designed on the basis of the previously published annotated genome sequence (6). Generally, the results of the DNA-DNA hybridizations of genomic DNA isolated from these *E. coli* strains with *E. coli* K-12 gene arrays demonstrated that the genome contents of the different *E. coli* isolates differ markedly from that of *E. coli* strain MG1655 (Fig. 2A). On average, 5.8% of the translatable ORFs present in the nonpathogenic reference strain (6) were absent in the individual isolates, in which between 3 and 10% of the translatable ORFs of *E. coli* strain MG1655 were not detectable (Tables 2 and 3). Based on the functional GenProtEC database classification of the chromosomally encoded genes and proteins of *E. coli* K-12 (<http://genprotec.mbl.edu>), the majority of these missing ORFs in every strain can be functionally grouped as coding for hypothetical, unclassified, or unknown gene products. The 22 *E. coli* isolates also exhibited a great diversity in ORFs which represent mobile genetic elements or which code for structural components of the cell. The alterations were found to be scattered over the entire *E. coli* MG1655 chromosome. However, prophages of strain MG1655 represent chromosomal variation "hot spots." Generally, the presence of 10 prophages described in *E. coli* strain MG1655 is variable in the 22 strains tested (Fig. 2A). In most of the *E. coli* isolates used in this study, several DNA segments covering the genomic regions described as prophages CP4-6, e14, Qin, and CP4-57 showed a very high density of undetectable clusters of ORFs in comparison to the MG1655 chromosome. The prophages CP4-6 and CP4-57 are located downstream of the tRNA or tRNA-like loci *thrW* and *ssrA*, respectively. Whereas the prophage DLP12 was predominantly absent in nonpathogenic isolates, pathogenic strains frequently lacked the Rac prophage. The chromosomal contexts of several other tRNA-encoding genes (e.g., *serX*, *argW*, *ileY*, *pheV*, and *leuX*) were also found to contain alterations in some of the pathogenic and commensal isolates in comparison to the corresponding sequences in *E. coli* MG1655. Several variable chromosomal regions among the studied isolates contain

ORFs with homology to ORFs of other accessory genetic elements, e.g., insertion sequence elements.

According to the results of the DNA-DNA hybridization, the presence of 1,165 translatable ORFs (27.2% of all translatable ORFs) of the *E. coli* K-12 strain was variable among the pathogenic and commensal strains tested. The vast majority of these variable chromosomal regions represent hypothetical and so far uncharacterized ORFs and prophages of *E. coli* strain MG1655, as well as ORFs involved in lipopolysaccharide biosynthesis, which belong to the functional ORF category "cell structure." The conserved *E. coli*-specific genetic backbone, or "core genome," is therefore estimated to contain at least 3,100 translatable ORFs of strain MG1655. This number of translatable ORFs includes all 232 supposedly essential genes of *E. coli* as they are compiled in the Profiling of *Escherichia coli* chromosome database (<http://www.shigen.nig.ac.jp/ecoli/pec/About.html>).

***E. coli* K-12 genes absent in pathogenic, commensal, and laboratory *E. coli* strains.** Certain genes present in *E. coli* K-12 were not detectable in some of the pathogenic and nonpathogenic strains investigated in our study. The *fec* operon encoding a ferric citrate uptake system was not detectable in UPEC strain 536, all four EHEC strains tested, strain EDL1284 (EIEC), or fecal isolate MGS 6. Strain EDL1284 also lacks the *fim* gene cluster, which codes for type 1 fimbriae. The genome of the EAEC strain DPA065 does not contain the *hsd*-encoded type I restriction modification system. Some of the *hsd* genes were also absent in other strains tested. Whereas seven of the investigated strains showed no hybridization signal with the *hipB*-specific probe, the entire *hip* operon, which is involved in the resistance to the lethal consequences of inhibition of peptidoglycan or DNA synthesis, was not detectable in ETEC strain C9221a. With the exception of UPEC strain P42 and the six fecal isolates from healthy volunteers, the *relEB* genes coding for a cytotoxin-antitoxin system could not be detected in any strains. Similarly, the complete *mcr* operon involved in methylation restriction was present only in UPEC strains J96 and 764, in EHEC strain 1639/77, and in the nonpathogenic isolates MGS 32, MGS 104, and MGS 124, as well as in *E. coli* strain B. Several *csp* genes whose products are involved in cold shock adaptation were not present in some of the tested pathogenic *E. coli* strains. The DNA-DNA hybridizations demonstrated that the different *E. coli* isolates differed markedly in the composition of the *waa* and *wbb* operons (formerly known as *rfa* and *rfb*, respectively), which are required for lipopolysaccharide biosynthesis. Several of the above-mentioned determinants are supposed to have been acquired via horizontal gene transfer. This is also supported by our results, which indicate that the presence of these genes is variable among the different *E. coli* strains tested, underlining the importance of horizontal gene transfer for the evolution of different *E. coli* variants.

**Detection of virulence-associated genes in pathogenic, commensal, and laboratory *E. coli* strains.** The 23 pathogenic and commensal isolates, including the laboratory strains B and MG1655, were screened for the presence of PAI-specific sequences of the UPEC strain 536, as well as for typical virulence-associated genes of ExPEC, IPEC, and *Shigella* using the *E. coli* pathoarray. Using sequences of the known PAIs of UPEC strain 536 and of virulence-associated genes of other



**FIG. 2.** Detection of genomic alterations among pathogenic, commensal, and laboratory *E. coli* strains using DNA arrays. (A) Comparison of genomes of different pathogenic, commensal, and laboratory *E. coli* isolates and the *E. coli* K-12 strain MG1655 using Panorama *E. coli* gene arrays. The individual chromosomes are displayed linearly and in equal lengths. Missing-undetectable ORFs are marked by vertical black lines in the individual chromosomes. The positions of the undetectable ORFs refer to the *E. coli* MG1655 chromosome. The positions of tRNA genes frequently used as chromosomal insertion sites of horizontally acquired DNA elements and those of 10 prophages of strain MG1655, as well as the chromosomal origin and terminus of replication, are marked within the map of *E. coli* strain MG1655. (B) Detection of virulence-associated genes of *E. coli* and *Shigella* among pathogenic and commensal *E. coli* isolates using the *E. coli* pathoarray. Virulence-associated genes are grouped with regard to typical *E. coli* pathotypes. Missing-undetectable ORFs are marked by vertical black lines.

TABLE 2. Number of *E. coli* K-12-specific ORFs absent in different pathogenic, commensal, and laboratory *E. coli* strains

Strain	No. of absent ORFs in functional category:				Total
	Hypothetical unclassified, unknown	Phage, transposon, or plasmid	Cell structure	Other	
536	206	40	21	116	383
536-21	206	29	21	116	372
J96	132	8	11	46	197
J96-M1	132	8	11	46	197
764	84	8	11	22	125
764-2	84	8	11	22	125
P42	78	9	2	23	112
83972	148	19	15	52	234
IHE3034	114	9	18	61	202
F54	184	9	11	90	294
C9221a	196	14	14	185	409
E2348/69	119	10	15	65	209
EDL1284	184	11	22	129	346
4797/97	125	9	14	64	212
5714/96	84	25	10	59	178
1639/77	105	7	10	37	159
SF493/89	75	9	11	37	132
DPA065	145	9	11	107	272
MG5 6	208	23	15	181	427
MG5 32	230	9	16	124	379
MG5 73	202	20	12	116	350
MG5 89	136	12	12	120	280
MG5 104	152	20	13	127	312
MG5 124	142	14	13	107	276
<i>E. coli</i> B	140	9	30	48	227
<i>E. coli</i> MG1655	0	0	0	0	0

pathogenic *E. coli* strains, this array was recently designed in our laboratory. Generally, sequences specific for PAI I<sub>536</sub> to PAI V<sub>536</sub>, as well as typical virulence-associated genes of ExPEC, were more frequently detectable in ExPEC strains (61.3 and 42% of the probes specific for PAI I<sub>536</sub> to PAI V<sub>536</sub> ORFs and other ExPEC virulence genes spotted on the pathoarray) than in IPEC (27.4 and 24%, respectively). Accordingly, IPEC isolates harbored more genes which are typical for these pathotypes (24.2% of the probes specific for virulence genes of IPEC) than ExPEC (4.8%). However, even in commensal strains, a considerable number of "virulence-associated" genes or their homologues present on PAI I<sub>536</sub> to PAI V<sub>536</sub> ORFs (22.6%), in ExPEC (14%), and in IPEC (3.9%) were detectable (Fig. 1). The "pathogene" content varies considerably among the different pathogenic and commensal isolates (Fig. 2B and Table 3). The results of the *E. coli* pathoarray hybrid-

ization were partially checked by PCR using the primers designed for probe generation. The vast majority of strong hybridization signals on *E. coli* pathoarrays could be confirmed or resulted in strong PCR products, suggesting the presence of these genes or their homologues in the different genomes.

Comparison of the genomes of two O18:K1 isolates (*E. coli* strains IHE3034 and F54) indicated that the overall hybridization patterns of both strains resemble each other and differ from those of the other ExPEC strains tested in the absence of PAI II<sub>536</sub>-specific sequences (Fig. 2B). Although their virulence gene contents are not identical, they contain typical virulence-associated genes of *E. coli* O18:K1 strains which frequently cause newborn meningitis: the K1 capsule-encoding determinant, as well as the *ibeA* gene, which has been shown to be required for the ability to cross the blood-brain barrier (24), were detectable. In addition, both strains contain the type 1 fimbrial gene cluster (*fim*) and genes whose products are involved in iron acquisition, i.e., genes coding for the hemin receptor ChuA, as well as for the irobactin and yersiniabactin siderophore systems. The MNEC isolate IHE3034 also carries the *cdt* gene cluster coding for the cytolethal distending toxin. According to these results, the strains of the same serotype exhibit very similar but not identical sets of virulence genes.

Comparison of the virulence gene contents of four EHEC isolates of different serotypes showed that these strains differ with respect to their virulence gene repertoires, but several genes which have been previously described for EHEC O157:H7 strain EDL933 are also present in these strains, i.e., the type 1 fimbrial gene cluster, the *stx1* or *stx2* determinant coding for shigatoxin, the *eae* and *tir* genes involved in the attaching-and-effacing phenotype, and the O-island ORFs Z0250, Z1542, Z4326, and Z4852 (47). Other EHEC PAI genes, e.g., the tellurite resistance gene cluster *trABCD* (54), were detectable only in the O157:H<sup>-</sup> and the O111 strains. Interestingly, the O157:H<sup>-</sup> strain also carries the *cdtIII* gene cluster coding for cytolethal distending toxin III. Probes specific for the P-fimbrial gene cluster usually found in ExPEC gave strong hybridization signals upon hybridization with genomic DNA of the O111 isolate. Virulence genes which are located on pO157, e.g., genes coding for the EHEC hemolysin or the plasmid-encoded catalase, have been detected only upon DNA-DNA hybridization using genomic DNAs of the O157:H<sup>-</sup> and O103 EHEC strains. These results underline the fact that non-O157:H7 EHEC isolates differ with regard to their virulence gene pools from O157:H7 strains, such as strain EDL933 or Sakai (23, 47).

TABLE 3. Presence of *E. coli* MG1655-specific genes and virulence-associated genes of *E. coli* in different pathogenic, commensal, and laboratory *E. coli* isolates

Strains	% of genes detected			
	<i>E. coli</i> MG1655-specific genes ( <i>n</i> = 4,290)	Genes present on PAI I <sub>536</sub> -PAI V <sub>536</sub> ( <i>n</i> = 212)	Virulence-associated genes of ExPEC ( <i>n</i> = 100)	Virulence-associated genes of IPEC ( <i>n</i> = 95)
ExPEC ( <i>n</i> = 7)	94.8	61.3	42	4.8
IPEC ( <i>n</i> = 8)	94.4	27.4	24	24.2
Nonpathogenic <i>E. coli</i> ( <i>n</i> = 8) <sup>a</sup>	93.4	22.6	14	3.9
All strains ( <i>n</i> = 23)	94.2	37.1	26.7	11.3

<sup>a</sup> Including commensal isolates and *E. coli* laboratory strains B and MG1655.

TABLE 4. Presence of *E. coli* MG1655-specific genes and virulence-associated genes of *E. coli* in different UPEC isolates and their PAI deletion mutants

Strain	% of genes detected			
	<i>E. coli</i> MG1655-specific genes ( $n = 4,290$ )	Genes present on PAI <sub>I536</sub> -PAI V <sub>536</sub> ( $n = 212$ )	Virulence-associated genes of ExPEC ( $n = 100$ )	Virulence-associated genes of IPEC ( $n = 95$ )
536	91.1	100	42	2.1
536-21	91.2	63.7	39	2.1
J96	95.4	71.2	40	2.1
J96-M1	95.4	51.9	25	2.1
764	97.1	62.7	53	6.3
764-2	97.1	57.1	51	6.3

**Comparison of genome contents of the wild-type UPEC strains and their PAI deletion mutants.** It is well known that pathogenic bacteria have a tendency to delete large chromosomal regions (PAIs) comprising virulence-associated genes (7, 8, 17). In order to analyze the mechanisms resulting in the deletion of PAIs, the genome contents of three UPEC isolates and one corresponding PAI deletion mutant were compared. Comparison of the genomes of the uropathogenic wild-type isolates 536, J96, and 764 with those of their corresponding PAI deletion mutants 536-21, J96-M1, and 764-2, respectively, demonstrated that only strain-specific genetic information was deleted but no chromosomal regions which belong to the K-12-specific genetic backbone (Table 4). These results underline the fact that the deletion of PAIs is a specific process which is independent of general genome plasticity in these organisms. The hybridization patterns of the *E. coli* pathoarray indicate that fewer *E. coli* pathogens were detectable in PAI deletion mutants than in the corresponding wild-type strains. The observation that PAIs of different ExPEC isolates are not completely identical with regard to genetic organization and gene content, although they share a great number of identical and homologous genes (15, 29), is also supported by the comparison of the *E. coli* pathoarray hybridization patterns of the ExPEC strains 536, J96, and 764 with those of their PAI deletion mutants 536-21, J96-M1, and 764-2. Although the deleted chromosomal regions are not identical in these deletion mutants, the proportions of undetectable sequences specific for PAI I<sub>536</sub> to PAI V<sub>536</sub> compared to other undetectable virulence-associated genes of ExPEC or IPEC are similar in the three UPEC isolates and their PAI deletion mutants (Table 4).

## DISCUSSION

Variants of the species *E. coli* are adapted to various host organisms, e.g., humans, monkeys, horses, and birds, in which they belong to the normal intestinal flora. In addition, pathogenic strains have the capacity to cause sepsis or local infections of the intestines, as well as of the kidney, bladder, and brain, in different hosts. The ecological and pathogenic diversity of *E. coli* strains is a prerequisite for the use of *E. coli* as a model to study processes of bacterial genome evolution. These mechanisms are reflected in the fact that the genomes of different strains show remarkable variations. The genomes of *E. coli* K-12 and of strains of the ECOR vary in size from 4.5 to 5.5 Mb (5, 44). The high variability in gene content among different *E. coli* strains is mainly due to the acquisition of

foreign DNA and to deletion of genetic information. As indicated in Table 1, the strains investigated in this study also exhibit marked differences in their chromosomal sizes. The affiliation of the majority of the pathogenic strains with the major phylogenetic ECOR groups B2 and D, as well as that of the nonpathogenic and commensal isolates with ECOR groups A and B1, respectively, was determined by PCR and confirms the general view of the distribution of pathogenic and nonpathogenic isolates in the ECOR groups (10). All ExPEC and IPEC strains, as well as the commensal strain MGS 124, carry genomic islands in their genomes whose products contribute to their individual traits. In addition, EHEC isolates were able to pick up bacteriophages encoding Shiga toxins. Furthermore, the genomes of IPEC strains comprise large plasmids encoding adhesins, toxins, and other virulence factors.

In order to systematically assess the genetic variability of bacteria, several genome comparison techniques, e.g., macrorestriction analysis, PFGE, and genomic subtraction, have been used (9, 39, 49, 58). DNA array technology is a powerful new tool for comparative genomics which has recently been used for the analysis of genome variability among bacterial species or closely related bacteria (2, 4, 18, 19, 43, 50, 53), as well as for detection of specific groups of trait-conferring genes (12, 21). DNA array technology has been utilized in this study to characterize the amount of genome variation among pathogenic and commensal *E. coli* isolates in general and between ExPEC and IPEC strains in particular. The use of PCR product-based DNA arrays to determine the presence or absence of DNA regions does not allow explicit answers to the question of whether specific genes are present, as hybridization signals can also result from cross hybridization of homologues or of genes with conserved domains. Additionally, no statement can be made as to whether the detected genes are functional, as non-functional genes and even gene fragments which are frequently present, especially on PAIs, will result in a hybridization signal. However, we think that, based on the criteria for signal intensities which we described in Materials and Methods, the use of DNA array technology allows us to sufficiently assess the genetic diversity and genome content among different isolates of one species, as discussed below.

The genome contents of different pathogenic, commensal, and laboratory *E. coli* isolates, as mirrored by the hybridization signals on the *E. coli* gene arrays, is very heterogeneous (Fig. 2A). Depending on the individual isolate, the number of ORFs absent in comparison to strain MG1655 varies between 112

(UPEC strain P42) and 427 (fecal isolate MGS 6). Generally, these alterations show no preferential chromosomal localization and can be scattered all over the chromosome (Fig. 2A). However, we found recently that the *E. coli* MG1655-specific chromosomal region downstream of gene *thrW*, which represents the prophage CP4-6, shows a high density of chromosomal variation in all other *E. coli* strains tested so far (16). Genome comparison shows that this is true for several other prophage sequences present in strain MG1655. Therefore, these regions can be regarded as chromosomal variation hot spots and seem to be *E. coli* K-12 specific. It has been speculated that the 40-kb chromosomal segment downstream of the tRNA-encoding gene *thrW* (b0245 to b0286) has been acquired by horizontal gene transfer (55, 57). The fact that the prophages which have been identified in *E. coli* strain MG1655 (36) are considered to have been recently acquired by *E. coli* laboratory strains, as they are missing in some natural *E. coli* isolates, has been mentioned before (43). As a result of our study, a 20-kb DNA stretch between ORFs b1345 and b1375 was characterized by the frequent occurrence of clusters of undetectable ORFs in almost all pathogenic *E. coli* isolates studied. This chromosomal region is covered in *E. coli* strain MG1655 by prophage Rac. Sequence analysis of the chromosomal region of the UPEC strain 536 replacing prophage Rac (data not shown; available under accession number AJ496193) indicates that this strain originally possessed Rac-specific sequences which were later deleted. Instead, a 2-kb genomic region is located within the K-12 chromosomal backbone between the putative ORFs b1344 and b1376. This fragment has a G+C content of 35% and exhibits no significant homology on the nucleotide level. A small putative ORF coding for a protein with 72% identity to amino acids 18 to 104 of the SitD protein of *Salmonella enterica* serovar Typhimurium (AF128999) is located within this region. The fact that a fragment of *intR* (b1345), which encodes the putative transposase-integrase of prophage Rac, is still present in the chromosome of strain 536 implies that this mobile genetic element was lost upon integration of the DNA region currently present in *E. coli* strain 536.

Genetic mechanisms of bacterial-genome evolution are often associated with tRNA genes. Several lysogenic bacteriophages preferentially use the 3' ends of tRNA genes as chromosomal insertion sites, implying that they may have served as vehicles for the integrated foreign DNA (11, 48). Other genes coding for tRNAs or tRNA-like molecules are also associated with bacteriophage integrase genes in many species. Additionally, in many prokaryotes, including *E. coli*, tRNA genes are frequently associated with genetic elements designated genomic islands. The insertion of genomic islands or PAIs and plasmids into tRNA loci has been described in many pathogenic and nonpathogenic bacteria (22, 26, 30, 33, 40, 44). The sequence contexts of several tRNA-encoding genes of different *E. coli* isolates (e.g., *thrW*, *ssrA*, *serX*, *argW*, *ileY*, *pheV*, and *leuX*) differ from that of strain MG1655. Also among these tRNA loci were some of those which are known to be frequently associated with PAIs in several pathogenic bacteria. These results demonstrate that the chromosomal contexts of many tRNA loci represent variable regions of the *E. coli* chromosome.

Observed sequence alterations in the vicinity of tRNA genes may be due to the integration of foreign DNA fragments.

However, they may also be the result of gene reduction. The latter process, from our point of view, is underestimated as a fundamental mechanism of genome evolution, at least in prokaryotes. It is known that the genome of *Shigella flexneri*, in contrast to the very similar genome of *E. coli*, lacks the *cadA* fragment, encoding the enzyme lysine decarboxylase. This "hole" seems to be important for the evolution of *Shigella* as a pathogen (38). The same is true for the recently evolved human pathogen *Yersinia pestis*, which has lost many functions compared to its ancestor *Yersinia pseudotuberculosis* (1). The determination of *E. coli* K-12-specific genomic regions, which are absent from *E. coli* pathogens, may lead to the identification of other genes whose loss could be a benefit for *E. coli* virulence or adaptation under the corresponding growth conditions. A group of genes which was not detectable in many IPEC isolates but which was present in all extraintestinal and commensal strains tested comprises several genes (*hofG*, *hofH*, *pshM*, *bfr*, and *yjhN*) which can be functionally grouped as coding for transport and binding proteins. The *fec* operon, which encodes a siderophore system, was not detectable in any EHEC strains tested and is also absent in the two sequenced O157:H7 strains. Whether the frequent lack of these genes represents a putative characteristic of EHEC or IPEC strains will have to be further analyzed. The eight K-12-specific ORFs missing in all other tested strains seem to represent *E. coli* strain MG1655-specific genetic information. In contrast, 44 ORFs present in strain MG1655 were absent in fecal commensal isolates of healthy volunteers but present in pathogenic isolates. Genome comparison and determination of the chromosomal sizes of three UPEC strains and their PAI deletion mutants demonstrated that no *E. coli* MG1655-specific genetic information has been deleted together with PAIs. The chromosomal-size differences of strains 536 and 536-21, together with the results of the different whole-genome approaches, confirm earlier findings that the mutant strain 536-21 lost only PAI I<sub>536</sub> and PAI II<sub>536</sub>, which represent chromosomal regions of ~75 and 102 kb, respectively (7, 15), and demonstrate the specific character of PAI deletion. This was further substantiated by comparison of two other UPEC isolates and their corresponding PAI deletion mutants. Deletion of PAIs is thought to be mediated by specific enzymes, such as bacteriophage integrases (22). The genetic structures of the deleted PAIs of the UPEC strains J96 and 764 resemble that of strain 536, as the deletion mutants J96-M1 and 764-2 lost more sequences specific for PAI I<sub>536</sub> to PAI V<sub>536</sub> from their homologues than other ExPEC or IPEC-specific DNA stretches.

Analysis of the presence of virulence-associated genes of *E. coli* and *Shigella* among different ExPEC and IPEC pathotypes, as well as among commensal isolates, indicated that sequences specific for PAI I<sub>536</sub> to PAI V<sub>536</sub> are widespread in pathogenic and many commensal isolates. Our results also demonstrate that the presence of DNA regions described for ExPEC or IPEC is markedly higher in strains belonging to the corresponding pathotype. However, virulence-associated sequences, or at least their homologues, have also been detected to a lesser extent in commensal isolates or strains of other pathotypes. The relatively frequent occurrence of sequences specific for PAI I<sub>536</sub> to PAI V<sub>536</sub>, not only among ExPEC isolates, results from the fact that many putative ORFs present on PAIs of the UPEC strain 536 are similar to fragments of genes of



strain MG1655 or belong to different accessory DNA elements, such as transposons, insertion sequence elements, plasmids, and bacteriophages, whose presence is not restricted to PAIs and which can also be found frequently on chromosomal or extrachromosomal DNAs of other bacteria. Therefore, the high frequency with which PAI-specific sequences of strain 536 have been detected in other, even commensal, isolates can be due to similarity or identity to genes or gene fragments which are present on PAIs but which are not important for virulence. This is also true of many ORFs located on the locus of enterocyte effacement-PAI of EHEC and EPEC. The great number of ExPEC-specific DNA sequences even among intestinal pathogenic and nonpathogenic isolates can also be partially explained by the fact that several determinants we included in this group of genes encode gene products which are fitness rather than virulence factors, e.g., aerobactin or type 1 fimbriae, and which are known to be frequently present in pathogenic and commensal *E. coli* variants. The results obtained with the *E. coli* pathoarray demonstrate on one hand that the accumulation of virulence-associated sequences determines the pathotypes and pathogenicity of *E. coli* strains and on the other the great heterogeneity of the gene contents of different *E. coli* variants, even among members of the same pathotype or serotype.

Taken together, the results of this study clearly indicate that the genetic diversity among pathogenic and commensal *E. coli* isolates is very high with respect to genomic alterations. This may be indicative of frequent acquisition of foreign DNA by horizontal gene transfer, as well as of a high frequency of deletions during the evolution of bacterial genomes.

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