The Pathogenicity Islands (PAIs) of the Uropathogenic *Escherichia coli* Strain 536: Island Probing of PAI II$_{536}$

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Pathogenicity islands (PAIs) represent distinct pieces of DNA that are present in the genomes of pathogenic bacteria but absent from the genomes of related nonpathogenic strains. They carry (often more than one) virulence genes and are linked to transfer RNA (tRNA) loci. In addition, they carry mobility genes and direct repeats at their ends and are often unstable [1]. The first PAIs have been identified in the genomes of uropathogenic *Escherichia coli* (UPEC) [2–4], and PAIs of >20 species of pathogens have been described [5]. UPECs can produce various virulence factors, such as specific adhesins (P, S, type I, F1C), toxins (α-hemolysin, cytotoxic necrotizing factor I), capsules (K1, K5, K12), specific O antigens (O1, O2, O4, O6, O18), iron-uptake systems (aerobactin, yersiniabactin), and factors contributing to serum resistance. Most genes, coding for these factors are located on PAIs [3, 4].

The uropathogenic strain *E. coli* 536, which was isolated from a patient with pyelonephritis, represents a model organism for the analysis of the genetic basis of urovirulence [2, 6]. The strain exhibits the serotype O6:K15:H31 and carries four PAIs in its chromosome. PAI I$_{536}$ and PAI II$_{536}$, which are 70 and 120 kb in size, carry the genes for hemolysin and, in the case of PAI II$_{536}$, P fimbriae. PAI III$_{536}$ carries the S fimbrial gene cluster, and PAI IV$_{536}$ is almost identical to the functional core region of the "high pathogenicity island" (HPI) of pathogenic *Yersinia* species, encoding the yersiniabactin iron-uptake system [4, 7].

In addition, the genes responsible for the capsule synthesis seem also to be located on another PAI (Janke B, et al., unpublished data). As shown recently, PAI I$_{536}$ and PAI II$_{536}$, which are located next to the tRNA genes *selC* and *leuX* at map positions 82 and 97 in the *E. coli* chromosome, respectively, are unstable [2]. With relatively high frequencies, these PAIs can be deleted from the chromosome of strain 536. In previous studies [2], the deletion processes of PAI I$_{536}$ and PAI II$_{536}$ have been analyzed on the basis of the loss of hemolysin production. In this report, however, we use the new method of "island probing," as described by Rajakumar et al. [8], for the first time

to analyze the instability of PAIs of *Shigella flexneri*. Our data indicate, that the deletion of PAI II$_{536}$ is a highly specific, *recA*-independent process, which can be influenced to a certain extent by environmental parameters.

Methods

Bacterial strains. Strain 536 and its derivatives were grown on Luria-Bertani (LB) agar or in LB broth or M9 minimal media.

Construction of the 536recA⁻ mutant strain. The *recA* gene was amplified by polymerase chain reaction (PCR), mutagenized by the introduction of the *cat* gene, and cloned into plasmid pCVD442, resulting in the 8.8-kb plasmid pIM15. Plasmid pIM15 was introduced into strain 536 and double crossover mutants were selected by growth at 30°C on LB agar plates containing sucrose (10%) and chloramphenicol (20 μg/mL).

Construction of the 536PAI II$_{536}$::sacB strain. A non-coding region downstream of the cryptic integrase gene (Δ*int*) of the bacteriophage P4 at the 5′-end of PAI II$_{536}$ was chosen as the integration site for the counter-selectable marker *sacB*. First, a 1613-bp fragment from this area was amplified with the primer pair 536–1597 (5′-GGGAAGCACGATATGCAGCC-3′) and 536–3190 (5′-CCC-GGCTTCATCGACAATGA-3′) and subcloned into the vector pGP704. An internal *Xho*I restriction site of the PAI II$_{536}$–specific fragment was used to make the resulting plasmid pGP1613 linear, and afterward, the protruding ends were blunted. The counter-selectable marker *sacB* and its *cis*-acting regulatory locus, *sacR*, were cut out of the pGP704 derivative pCVD442 by *Pst*I, and the ends of this 2.6-kb fragment were also filled in to enable the blunt-end ligation into pGP1613. The resulting construct, pBMM1, was conjugated into *E. coli* 536, and the double crossover mutant 536PAI II$_{536}$::*sacB* was selected by searching for an ampicillin- and sucrose-sensitive phenotype. The correct integration of the counter-selectable marker was confirmed by Southern blot experiments with PAI II$_{536}$- and *sacB*-specific probes and by PCR experiments.

PCR reactions to analyze the presence or absence of PAI II$_{536}$. The presence or the absence of PAI II$_{536}$ was analyzed by PCR performed in a T3 Thermocycler (Biometra). Amplifications were carried out in a total volume of 50 μL containing 10 μL of 5× buffer (10 m*M* MgCl$_2$), 8 μL of 1.25 m*M* dNTP, 1 μL of each primer (100 pmol/μL), 1 μL of taq polymerase (Qiagen), and 1 μL of bacterial suspension. The location of the primer-specific DNA sequences in the PAI II$_{536}$ region is shown in figure 1. The nucleotide sequences of the primers were as follows: primer 1: 5′-GCCTGG-AGTGTGACAAAGGTTAC-3′; primer 2: 5′-GACCTCAGT-TGAGGTCTATT-3′; primer 3: 5′-AAACGGAATTCCGCTGTA-
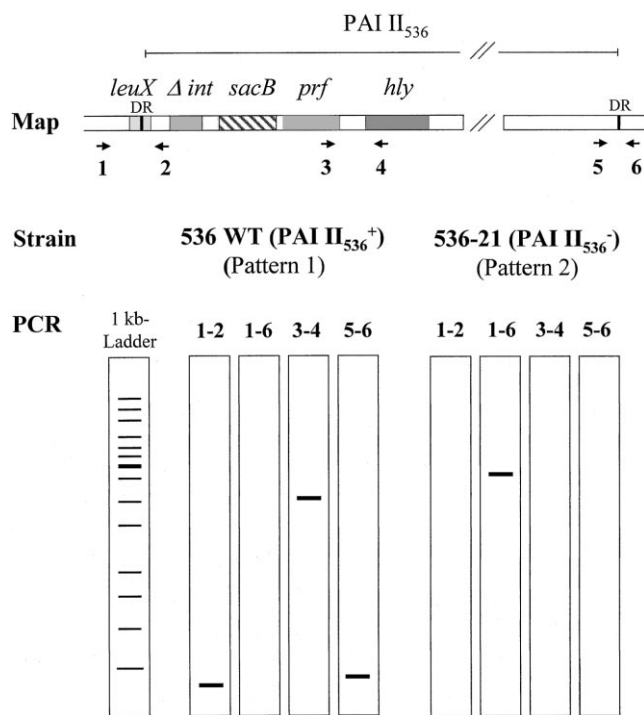
Figure 1. Schematic overview of the polymerase chain reactions (PCR) for the analysis of the deletion of pathogenicity island (PAI) II$_{536}$ in *Escherichia coli* strain 536. The positions of the primers that can be used for the detection of PAI II$_{536}$–positive strains (pattern 1) and of PAI II$_{536}$–deficient mutants (pattern 2) are indicated by arrows. Direct repeats (DR) and putative genes are shown. *leuX* = leucine-specific tRNA, Δ*int* = cryptic P4 integrase gene, *sacB* = gene mediating sucrose sensitivity, *hly* = hemolysin gene cluster, *prf* = P-related fimbriae gene cluster, WT = wild-type.

ACAGAAAGTTTACATGAGCGTTAAGTC-3′; primer 4: 5′-AAACCATCGATGGGTCAGAATACCTCACATGATAATAA-AACCTGTC-3′; primer 5: 5′-CAGGACCTGATTGTCCTGG-3′; primer 6: 5′-GAACTGGACGACGGCACTG-3′. After 30 cycles, a 15-μL volume of each PCR sample was analyzed by submarine gel electrophoresis on a 1% (wt/vol) agarose gel. Strains 536 and 536-21 were used as controls.

Other DNA techniques. Chromosomal and plasmid DNA was isolated as described [2, 6]. DNA sequencing was done on a LICOR MWG sequencer (MWG-Biotech). Sequence analysis was done by use of the BLASTN and BLASTX programs (National Center for Biotechnology Information).

Isolation and characterization of nonhemolytic mutants. Nonhemolytic 536 derivatives were phenotypically identified on LB agar plates supplemented with 5% human blood. The presence of *hly* and *prf* (P-related fimbriae) genes was detected by PCR, using the *hly*-specific primers hlyup (5′-GCACACTGCAGTCTGCAA-AG-3′) and hlydo (5′-GTGTCCACGAGTTGGTTGAT-3′) and the *pap/prf*-specific primers papup (5′-GTGCAGATTAACATCAGG-GG-3′) and papdo (5′-ATGCTCATACTGGCCGTGGT-3′) (data not shown).

Determination of deletion frequencies of PAI II$_{536}$ with the counter-

selectable marker *sacB.* Thirty milliliters of LB or M9 medium were inoculated 1:100 with an overnight culture of 536PAI II$_{536}$::*sacB.* The bacteria were cultivated at 37°C, 42°C, 20°C, and samples were taken during the late-lag and mid-log phases as well as during the early and late stationary phases. Serial dilutions were plated on LB agar and LB agar supplemented with 7% sucrose to determine the colony-forming units (cfu) and the number of PAI II$_{536}$–negative cells. The agar plates had to be incubated at 20°C for at least 48 h to reduce the growth rate and establish the toxic effect of the levansucrase. The deletion frequency of PAI II$_{536}$ was calculated as the quotient of sucrose-resistant cells to cfu. All resulting data are mean values of at least three independent experiments.

Results

Isolation and characterization of PAI II$_{536}$–negative variants on the basis of hemolysin production. As analyzed previously [2], the uropathogenic strain *E. coli* 536 carries two unstable PAIs, PAI I$_{536}$ and PAI II$_{536}$, both of which contain an *hly* gene cluster encoding hemolysin. To determine the frequency and the specificity of PAI deletion, we isolated variants that were nonhemolytic or exhibited a reduced lysis zone following plating on blood agar plates. As determined using the hemolysin indicator system, a deletion frequency of $\sim 10^{-5}$ was estimated for the simultaneous deletion of PAI I$_{536}$ and PAI II$_{536}$ (table 1). While the nonhemolytic variants had lost both PAIs, PAI I$_{536}$ and II$_{536}$ variants exhibiting a reduced hemolysin production had lost PAI I$_{536}$ but retained PAI II$_{536}$. These data are corroborated by the fact that the *hly*I determinant is responsible for a much stronger hemolysin production than is the *hly*II gene cluster (Nagy G, unpublished data). Therefore, it was impossible to determine the deletion rate of PAI II$_{536}$ alone, as all hemolysin-negative variants were PAI I$_{536}$ and PAI II$_{536}$ negative.

To analyze whether the deletion of PAI II$_{536}$ follows a general mechanism, we investigated 8 nonhemolytic mutants in more detail by PCR (figure 1). DNA sequence analysis of the PCR products generated by a PCR reaction with primers 1 and 6 revealed that all variants tested retained one of the two 18 bp–long directly repeated DNA sequences (5′-GTTCGAGTC-CGGCCTTCG-3′) [2] (indicated by pattern 2 in figure 1). These direct repeats are located at both ends of PAI II$_{536}$ in the chromosome, further indicating the generation of the PAI II$_{536}$–negative mutants by a unique mechanism.

Influence of RecA on deletion formation. To analyze whether the RecA protein plays a role in deletion formation of PAI I$_{536}$ and PAI II$_{536}$, we constructed a *recA*-negative mutant of strain 536, which resulted in strain 536*recA*⁻. In addition, the *recA* mutant strain was complemented in trans by a cloned *recA* gene, resulting in strain 536*recA*⁻(pIM10). As indicated in table 1, the presence or absence of *recA* did not influence the deletion rate of strain 536 as determined by use of the hemolysin indicator system.

Analysis of PAI II$_{536}$ deletion formation by island probing.

Table 1. Determination of deletion frequencies of pathogenicity island (PAI) II$_{536}$ of uropathogenic *Escherichia coli* strain 536 and derivatives by island probing.

Strain	Marker used	Conditions	Deletion frequencies, $\times 10^{-5}$
536 wild-type	*hly*[a]	37°C, mid-log phase, LB medium	1.15
536*recA*⁻	*hly*[a]	37°C, mid-log phase, LB medium	0.92
536*recA*⁻ (pIM10)	*hly*[a]	37°C, mid-log phase, LB medium	1.08
536PAI II$_{536}$::*sacB*	*sacB*	37°C, log phase, LB medium	0.54
	sacB	37°C, stationary phase, LB medium	1.5
	sacB	37°C, log phase, M9 medium	1.77
	sacB	37°C, stationary phase, M9 medium	1.6
	sacB	42°C, log phase, LB medium	1.22
	sacB	42°C, stationary phase, LB medium	1.75
	sacB	37°C, log phase, LB + 2% NaCl	2.25
	sacB	37°C, stationary phase, LB + 2% NaCl	2.59
	sacB	20°C, log phase, LB medium	3.44
	sacB	20°C, stationary phase, LB medium	10.44

NOTE.   Evaluation of deletion frequency data by paired *t* test demonstrated that the increased deletion frequency in LB at 20°C is statistically significant ($P < .05$). LB = Luria-Bertani.
  [a] Co-deletion of PAI I$_{536}$ and PAI II$_{536}$.

To analyze the deletion of PAI II$_{536}$ independently of the hemolysin detection system, we introduced a *sacB* cassette into a PAI II$_{536}$–specific DNA fragment. It was found that ~90% of *E. coli* strains that could grow on plates with sucrose had lost PAI II$_{536}$; most of these, however, had retained PAI I$_{536}$ in their chromosomes (data not shown). The deletion frequency of strains grown under standard laboratory conditions was ~0.5–3 × 10^{-5} (table 1). By use of the PCR system indicated in figure 1 (primers 1 and 6) and by DNA sequence analysis of the resulting PCR product, it was shown for 50 randomly selected colonies that the PAI II$_{536}$–negative variants generated by the *sacB* system were identical to the PAI II$_{536}$–negative variants isolated on the basis of their non-hemolytic phenotype. This confirmed that the island probing system, developed on the basis of the counter-selectable marker, *sacB,* can be used to determine the frequency of PAI II$_{536}$ deletion formation.

Influence of different environmental conditions on PAI II$_{536}$ deletion.   To answer the question of whether specific environmental signals influence the deletion of PAI II$_{536}$, we cultivated the mutant strain 536PAI II$_{536}$::*sacB* under different growth conditions and determined the deletion frequency. As shown in table 1, the composition of the medium (LB vs. M9), an increase of temperature from 37°C to 42°C, the growth phase (exponential vs. stationary phase), and osmotic stress (2% NaCl) did not influence the rate of deletion of PAI II$_{536}$. Of interest, growth of strain 536PAI II$_{536}$::*sacB* at lower versus higher temperature (20°C vs. 42°C), especially in the early and late stationary phases, increases the deletion rate by a factor of 10. These data clearly indicate that the rate of deletion of PAI II$_{536}$ is modulated by specific environmental conditions.

Commentary

PAIs of different microorganisms are unstable and mobile. Thus, the *cag*-PAI of *Helicobacter pylori* and the HPI of different *Yersinia* species exhibit a very high deletion rate of up to 10^{-2} and 10^{-1}, respectively [5, 9]. In addition, some PAIs, such as the HPI from *Yersinia,* can jump from one tRNA target site to the other [9]. Other islands, such as the *Staphylococcus aureus* PAI (termed SaPI) or a PAI of *Vibrio cholerae* (termed VPI), can be mobilized by helper phages [5]. The finding that the HPI, which was first described for *Yersinia* species, is found in many other enterobacterial species, including *E. coli* [10], also suggests that various PAIs may represent (former) mobile DNA elements. It has been known for a longer time that the hemolytic phenotype of the PAIs of UPEC are unstable [2]. This observation, which was made on the basis of the hemolysin indicator system, was confirmed and extended by us, using an island probing system for the analysis of PAIs from uropathogenic organisms.

The capacity of pathogens to cause deletions may significantly contribute to their ability to adapt to certain niches. Thus, uropathogens may invade epithelial cells, and a chronic phase of infection may follow. Hemolysin-negative mutants, which do not lyse host cells, may be better adapted than fully virulent microbes to the persistent stage of infection. Similar observations have been made for other pathogens, such as *S. aureus* or pathogenic *Yersinia* species.

Using the island probing system, we can determine the deletion frequencies of PAI II$_{536}$ more precisely (see table 1). Compared with the deletion frequencies found for PAIs of *Yersinia* species or *H. pylori,* the deletion frequencies of 10^{-4} to 10^{-5} determined for the PAI II$_{536}$ are relatively low. It has been shown in this study, however, that environmental conditions can modulate the deletion frequencies, at least to a certain extent. Despite the fact that the increase of deletion formation by growing the strains under low temperature is moderate, our observation opens the possibility to define conditions or even substances that may significantly induce deletions and thus may also generate nonpathogenic variants in vivo. Island probing systems such as the one described here [8] may represent ideal indicator systems to address such questions.

In addition, it is still unclear which kind of enzyme may act on the PAI-specific DNA to induce deletions. As shown at least for the UPEC strain 536, the RecA protein does not play any role in these processes. Many PAIs described thus far, however, seem to represent former integrated bacteriophages, plasmids, or both. Therefore, it is not surprising, that several PAIs carry not only virulence-associated genes but also integrase loci. All four PAIs of *E. coli* 536 analyzed thus far carry *int* genes of the P2/P4 family, which in the case of PAI I$_{536}$ and PAI II$_{536}$, are nonfunctional. The integrase loci of PAI III$_{536}$ and PAI IV$_{536}$, however, represent intact genes, and we cannot exclude the possibility that the PAI III$_{536}$– and/or PAI IV$_{536}$–encoded enzymes may play a role in the deletion of PAI I$_{536}$ and PAI II$_{536}$. Further studies are underway to elucidate this possibility.

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