The Pathogenicity Islands (PAIs) of the Uropathogenic Escherichia coli Strain 536: Island Probing of PAI II₅₃₆

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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, FIC), 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 I₅₃₆ and PAI II₅₃₆, which are 70 and 120 kb in size, carry the genes for hemolysin and, in the case of PAI II₅₃₆, P fimbriae. PAI III₅₃₆ carries the S fimbrial gene cluster, and PAI IV₅₃₆ 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In addition, the genes responsible for the capsule synthesis seem also to be located on another PAI (Janke B, et al., unpublished data).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 I₅₃₆ and PAI II₅₃₆, 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 I₅₃₆ and PAI II₅₃₆ 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 II₅₃₆ 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 536recAmutant strain. The recA gene was amplified by polymerase chain reaction (PCR), mutagenized by the introduction of the cat gene, and cloned into plasmid pCDV442, resulting in the 8.8-kb plasmid pPM15. Plasmid pPM15 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 II₅₃₆::sacB strain. A non-coding region downstream of the cryptic integrase gene (Δint) of the bacteriophage P4 at the 5'-end of PAI I₅₃₆ 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'-GGGAAACGCAGATGTGACAAAGGTTAC-3') and 536–3190 (5'-CCC-GGCCCTATCGAGCAACATGAG-3') and subcloned into the vector pGP704. An internal XhoI restriction site of the PAI II₅₃₆-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 pCDV442 by PstI, 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 II₅₃₆::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 II₅₆ specific probes and by PCR experiments.

PCR reactions to analyze the presence or absence of PAI II₅₆. The presence or the absence of PAI II₅₆ 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 mM MgCl₂), 8 μL of 1.25 mM 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 II₅₆ region is shown in figure 1. The nucleotide sequences of the primers were as follows: primer 1: 5'-GCTGCGGACGTGACGACAAAGGGTTAC-3'; primer 2: 5'-GACCTCAGTTGGATTCTATT-3'; primer 3: 5'-AACCGGAATTCTCGCTGA-3'.

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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, Δint = cryptic P4 integrase gene, sacB = gene mediating sucrose sensitivity, hly = hemolysin gene cluster, prf = P-related fimbriae gene cluster, WT = wild-type.

ACAGAAAGTTTTACATGAGCCTTAAGTC-3; primer 4: 5'-AAAACCATCGATGGTGACATACTCAATGATAATAAACCTGTC-3; primer 5: 5'-CAGGACCTGATTTGCTCGG-3; primer 6: 5'-GAAGCTGGACGACGCGACTG-3. After 30 cycles, a 15-µL volume of each PCR sample was analyzed by submersion gel electrophoresis on a 1% (w/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'-GCACACTGCGTCCTGCAAAG-3') and hlydo (5'-GTGCTCCAGATTTGCTGGG-3') and the pap/prf-specific primers papup (5'-TGTCAGATTAACACTCAAAGGGG-3) and papdo (5'-ATGTCCATGCTGGCCTGGT-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% sacrose 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 ~10^-3 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 hlyI determinant is responsible for a much stronger hemolysin production than is the hlyII 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'-GTCAGATTAACACTCAAAGGGG-3') (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 536recA-. In addition, the recA mutant strain was complemented in trans by a cloned recA gene, resulting in strain 536recA+ (pLM10). 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.
To analyze the deletion of PAI II536 independently of the hemolysin detection system, we introduced a sacB cassette into a PAI II536-specific DNA fragment. It was found that ~90% of E. coli strains that could grow on plates with sucrose had lost PAI II536; most of these, however, had retained PAI I536 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 II536-negative variants generated by the sacB system were identical to the PAI II536-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 II536 deletion formation.

**Table 1.** Determination of deletion frequencies of pathogenicity island (PAI) II536 of uropathogenic *Escherichia coli* strain 536 and derivatives by island probing.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Marker</th>
<th>Conditions</th>
<th>Deletion frequencies, × 10⁻⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>536 wild-type</td>
<td>hly⁺</td>
<td>37°C, mid-log phase, LB medium</td>
<td>1.15</td>
</tr>
<tr>
<td>536 recA⁺</td>
<td>hly⁺</td>
<td>37°C, mid-log phase, LB medium</td>
<td>0.92</td>
</tr>
<tr>
<td>536 recA⁺ (pIM10)</td>
<td>hly⁺</td>
<td>37°C, mid-log phase, LB medium</td>
<td>1.08</td>
</tr>
<tr>
<td>536PAI II536::sacB</td>
<td>sacB</td>
<td>37°C, log phase, LB medium</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>37°C, stationary phase, LB medium</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>37°C, log phase, M9 medium</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>37°C, stationary phase, M9 medium</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>42°C, log phase, LB medium</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>42°C, stationary phase, LB medium</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>37°C, log phase, LB + 2% NaCl</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>37°C, stationary phase, LB + 2% NaCl</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>20°C, log phase, LB medium</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td>sacB</td>
<td>20°C, stationary phase, LB medium</td>
<td>10.44</td>
</tr>
</tbody>
</table>

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 I536 and PAI II536.

**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 isolates, 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 II536 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 II536 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 \textit{E. coli} 536 analyzed thus far carry \textit{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.

References