JOURNAL OF BACTERIOLOGY, May 2004, p. 3086–3096 Vol. 186, No. 10
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.10.3086–3096.2004


# Instability of Pathogenicity Islands in Uropathogenic *Escherichia coli* 536

Barbara Middendorf, Bianca Hochhut, Kristina Leipold, Ulrich Dobrindt, Gabriele Blum-Oehler, and Jörg Hacker*

*Institut für Molekulare Infektionsbiologie, Universität Würzburg, 97070 Würzburg, Germany*

Received 5 December 2003/Accepted 28 January 2004

**The uropathogenic *Escherichia coli* strain 536 carries at least five genetic elements on its chromosome that meet all criteria characteristic of pathogenicity islands (PAIs). One main feature of these distinct DNA regions is their instability. We applied the so-called island-probing approach and individually labeled all five PAIs of *E. coli* 536 with the counterselectable marker *sacB* to evaluate the frequency of PAI-negative colonies under the influence of different environmental conditions. Furthermore, we investigated the boundaries of these PAIs. According to our experiments, PAI $II_{536}$ and PAI $III_{536}$ were the most unstable islands followed by PAI $I_{536}$ and PAI $V_{536}$, whereas PAI $IV_{536}$ was stable. In addition, we found that deletion of PAI $II_{536}$ and PAI $III_{536}$ was induced by several environmental stimuli. Whereas excision of PAI $I_{536}$, PAI $II_{536}$, and PAI $V_{536}$ was based on site-specific recombination between short direct repeat sequences at their boundaries, PAI $III_{536}$ was deleted either by site-specific recombination or by homologous recombination between two IS*100*-specific sequences. In all cases, deletion is thought to lead to the formation of nonreplicative circular intermediates. Such extrachromosomal derivatives of PAI $II_{536}$ and PAI $III_{536}$ were detected by a specific PCR assay. Our data indicate that the genome content of uropathogenic *E. coli* can be modulated by deletion of PAIs.**

Pathogenicity islands (PAIs) represent distinct large chromosomal regions that contribute to the evolution of bacterial pathogens (17). Characteristically, (i) they can be found in pathogenic strains but not or only rarely in nonpathogenic variants, (ii) they are inserted at the 3′ end of tRNA genes and carry (often many) virulence genes, (iii) their G+C content differs from that of the rest of the bacterial chromosome, (iv) they are associated with (sometimes cryptic) fragments of mobile genetic elements such as integrase genes or transposase genes, and in most cases, (v) they are flanked by insertion elements or direct repeats (DRs). Furthermore, some PAIs have the tendency to be deleted from the chromosome.

The uropathogenic *Escherichia coli* (UPEC) strain 536 (O6:K15:H31), which was originally isolated from a patient suffering from a urinary tract infection, is one of the best-characterized model organisms for the study of PAIs. The ongoing sequence project of the genome of this strain has revealed that it carries at least five of these genetic elements (PAI $I_{536}$ to PAI $V_{536}$), which are inserted at different sites of the chromosome and exhibit the main features of PAIs (14, 41; G. Schneider, U. Dobrindt, H. Brüggemann, G. Nagy, B. Janke, G. Blum-Oehler, G. Gottschalk, L. Emody, and J. Hacker, unpublished data). These PAIs carry many of the so far known virulence determinants of *E. coli* 536 (Fig. 1). They encode two α-hemolysin gene clusters (PAI $I_{536}$ and PAI $II_{536}$), P-related fimbriae (PAI $II_{536}$), S-fimbriae (PAI $III_{536}$), and the salmochelin and yersiniabactin siderophore systems (PAI $III_{536}$ and PAI $IV_{536}$, respectively). Interestingly, the genetic structure of PAI $IV_{536}$ is identical to the core element of the so-called high-pathogenicity island (HPI) of *Yersinia* species. PAI $I_{536}$ to PAI $V_{536}$ are associated with the tRNA genes *selC*, *leuX*, *thrW*, *asnT*, and *pheV*, respectively. In general, the above-mentioned tRNA genes can be considered hotspots for the integration of foreign DNA into the prokaryotic chromosome, since they have been described as insertion sites for bacteriophages, conjugative transposons, and several PAIs in other bacterial species (17, 22, 23, 37).

The PAIs of *E. coli* 536 are associated with integrase genes that exhibit the highest homology to the *int* genes of coliphage P4 and the *Shigella flexneri* phage SfX (Fig. 1). Our data indicate that all open reading frames encoding these integrases seem to be functional (B. Hochhut, G. Balling, and J. Hacker, unpublished data). Furthermore, with the exception of PAI $IV_{536}$, the islands are flanked by DRs of different sizes (Fig. 1) (14; Schneider et al., unpublished). These flanking repeat regions correspond to the left and right end junctions (*attL* and *attR*) that result from the integration of phage DNA into the prokaryotic chromosome. Therefore, it is very likely that comparable to the insertion-excision mechanism of bacteriophages, insertion and deletion of PAIs is mediated by the respective PAI-encoded integrase and functions via site-specific recombination between the flanking DRs. The close association of virulence relevant genes and large unstable DNA regions such as PAIs is interesting for two reasons. First, the excision of PAIs and their potential propagation could contribute to genome plasticity and bacterial evolution. Second, deletion of PAIs could play an important role during the transition from an acute to a chronic phase of infection (6, 17).

Instability of PAI $I_{536}$ and PAI $II_{536}$ has been discovered previously on the basis of the formation of nonhemolytic colonies due to the loss of both islands via site-specific recombination between their flanking DRs (6). Furthermore, instability of PAIs is a phenomenon that has been described for other organisms as well, e.g., the *cag* PAI of *Helicobacter pylori*, the

* Corresponding author. Mailing address: Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49 931 312575. Fax: 49 931 312578. E-mail: j.hacker@mail.uni-wuerzburg.de.

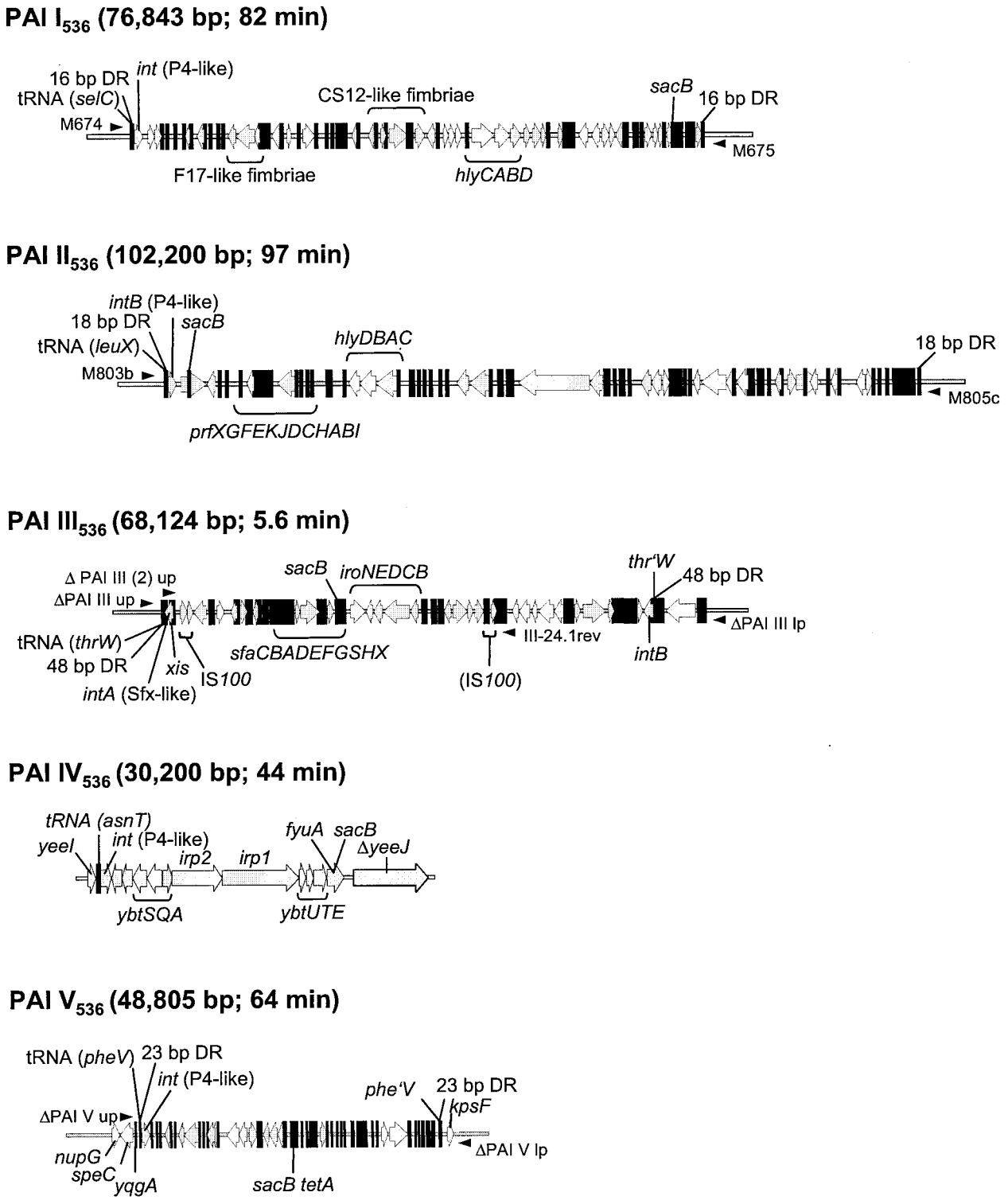


FIG. 1. Organization of PAI $I_{536}$ to PAI $V_{536}$. Localizations of important virulence genes, PAI-associated tRNA genes, integrase genes, DR regions flanking the PAIs, and the insertion site of the counterselectable marker *sacB* are indicated. With the exception of PAI $IV_{536}$, the latter was introduced into noncoding regions of the corresponding PAI. Small black arrows symbolize oligonucleotides used for exclusion PCRs. Sizes of the PAIs and their relative locations in the chromosome (in minutes corresponding to the *E. coli* K-12 chromosome) are also given. Abbreviations: *int*, integrase genes; *hly*, hemolysin determinants; *prf*, P-related fimbrial adhesin-encoding genes; *xis*, excisionase gene; *sfa*, S-fimbriae-encoding genes; *iro*, salmochelin gene cluster; *ybt*, *irp*, and *fyuA*, genes encoding yersiniabactin biosynthesis and uptake; *yeeJ*, *nupG*, *speC*, and *yqgA*, chromosomal genes.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| *E. coli* strains | | |
| 536 | Wild-type UPEC strain (O6:K15:H31) | 4 |
| 536-21 | 536 ΔPAI $I_{536}$ ΔPAI $II_{536}$ | 18 |
| 536 PAI $I_{536}$::*sacB* | *sacB* inserted into PAI $I_{536}$ | This study |
| 536 PAI $II_{536}$::*sacB* | *sacB* inserted into PAI $II_{536}$ | 28 |
| 536 PAI $III_{536}$::*sacB* | *sacB* inserted into PAI $III_{536}$ | This study |
| 536 PAI $IV_{536}$::*sacB* | *sacB* inserted into PAI $IV_{536}$ | This study |
| 536 PAI $V_{536}$::*sacB tetA* | *sacB tetA* inserted into PAI $V_{536}$ | This study |
| 536 $attB_{\lambda}$::*sacB bla* | *sacB* inserted into $attB_{\lambda}$ | This study |
| 536 Δ*recA* PAI $III_{536}$::*sacB* | Δ*recA* | This study |
| 536 Δ*recA* PAI $V_{536}$::*sacB tetA* | Δ*recA* | This study |
| DH5α | $F^-$ Δ*lacU169* (ϕ80 *lacZ*ΔM15) *recA1 hsdR17* | |
| SM10λ*pir* | $F^-$ *recA*::RP4-2-Tc::Mu λ*pir* $Kn^r$ | 29 |
| Plasmids | | |
| pBluescript II KS(−) | *bla*, cloning vector | Stratagene |
| pGEM-T Easy | *bla*, T/A cloning vector | Promega |
| pGP704 | *bla* $ori_{R6K}$ $mob_{RP4}$ | 29 |
| pCVD442 | *bla* $ori_{R6K}$ $mob_{RP4}$ *sacB* | 30 |
| pASK75 | *bla tetR*, expression vector | 42 |
| pBR322 | *bla tetA*, cloning vector | New England Biolabs |
| pBMM3 | pGP704 derivative for insertion of *sacB* into PAI $I_{536}$ | This study |
| pBMM6 | pGP704 derivative for insertion of *sacB* into PAI $III_{536}$ | This study |
| pGP704 *fyuA*::*sacB* | pGP704 derivative for insertion of *sacB* into PAI $IV_{536}$ | Gift from E. Carniel, Institut Pasteur |
| pKS-*sacB tetA* | pBluescript II KS(−) *sacB tetA* | This study |
| pLDR8 | *neo*, *int* expression vector, Ts | 11 |
| pLDR9 | *bla neo*, cloning vector to integrate DNA into $attB_{\lambda}$ | 11 |
| pKD46 | *bla* $repA_{101}$(Ts) *araC* $araB_p$ γ β *exo* | 10 |
| pKD3 | *cat* with flanking FRT sites, template plasmid | 10 |
| pCP20 | *bla*, carries yeast FLP recombinase gene, Ts | 10 |
| pPAI II-CI | *bla*, positive control for detection of PAI $II_{536}$-specific CIs | This study |
| pPAI III-CI (1) | *bla*, positive control for detection of PAI $III_{536}$-specific CIs of deletion type I | This study |
| pPAI III-CI (2) | *bla*, positive control for detection of PAI $III_{536}$-specific CIs of deletion type II | This study |

HPI of *Yersinia pestis* and *Yersinia pseudotuberculosis*, the locus of enterocyte effacement (LEE) of rabbit-specific enteropathogenic *E. coli* (REPEC) strain 84/110-1, and the *Shigella* resistance locus of *S. flexneri* (7, 9, 43, 44). PAI-negative cells appear in the REPEC strain 84/110-1 and in *S. flexneri* 2a with frequencies of around $10^{-6}$ to $10^{-5}$, which are comparable to previously obtained data for PAI $I_{536}$ and PAI $II_{536}$ (43, 44). Other PAIs, like the *Vibrio cholerae* PAI or the *Staphylococcus aureus* PAI (*S. aureus* PAI 1), have not only the tendency to be deleted from the chromosome but both are also transmissible to recipient organisms by transducing phages (25, 32, 39).

In this study, the instability of a complete set of PAIs from one strain was analyzed in detail for the first time. The deletion rate of PAI $I_{536}$ to PAI $V_{536}$ was determined by using the island-probing approach, which is based on insertion of the counterselectable marker *sacB* from *Bacillus subtilis* into the islands (34, 38). Furthermore, the influence of different environmental conditions such as low or elevated temperature, osmotic stress, or depletion of nutrients on the stability of PAIs was investigated.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) Miller broth, M9 minimal medium (40), or artificial urine (AU). AU was prepared according to the instructions of Jackson et al. (24) but with a reduced $CaCl_2$ concentration of 1 mM. For solid medium, agar was added to a final concentration of 1.5% (wt/vol). If necessary, the medium was supplemented with 7% sucrose, 5% sheep blood, 100 μg of ampicillin $ml^{-1}$, 10 μg of tetracycline $ml^{-1}$, or 20 μg of chloramphenicol $ml^{-1}$.

**Oligonucleotides.** A list of oligonucleotides used in this study is available at http://www.uni-wuerzburg.de/infektionsbiologie. Oligonucleotides specific for PAI $III_{536}$, PAI $IV_{536}$, and the capsule region of *E. coli* 536 have been published previously (12, 13, 26). Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany) or Sigma-ARK (Steinheim, Germany).

**Labeling of *E. coli* 536-specific PAIs and $attB_{\lambda}$ with the counterselectable marker *sacB*.** For labeling of PAI $I_{536}$, a noncoding 2.3-kb EcoRI fragment of this island was isolated from a cosmid clone (14) and subcloned into the SmaI site of pBluescript II KS(−). The construct was linearized with SphI, blunted, and ligated with a 2.6-kb PstI fragment of pCVD442 carrying the *sacB* gene and its *cis*-acting regulatory locus *sacR*, which had also been blunted. The complete 4.9-kb insert was finally cut out with XhoI and SacI. The ends were filled in, and the fragment was cloned into the EcoRV restriction site of the suicide vector pGP704, resulting in the vector pBMM3. For labeling of PAI $III_{536}$, a noncoding region of this island was amplified with primer pair PAI III 6.4-1376/PAI III 6.4-3543. The 2.2-kb PCR product was cloned into EcoRV/SmaI-digested pBluescript II KS(−), and the resulting construct was linearized with PstI by cutting only in the center of the PAI $III_{536}$-specific insert. The 2.6-kb PstI fragment of pCVD442 carrying the *sacB* gene was cloned into this restriction site. The entire 4.8-kb insert was cut out with BamHI and XhoI, the ends were blunted, and the fragment was ligated into the EcoRV restriction site of pGP704, resulting in the vector pBMM6. For labeling of PAI $IV_{536}$, we used the vector pGP704 *fyuA*::*sacB*. The plasmids pBMM3, pBMM6, and pGP704 *fyuA*::*sacB* were transferred separately into *E. coli* 536 by conjugation, and double-crossover mutants corresponding to 536 PAI $I_{536}$::*sacB*, 536 PAI $III_{536}$::*sacB*, and 536 PAI $IV_{536}$::*sacB*, respectively, were selected by screening for colonies with an ampicillin- and sucrose-sensitive phenotype. For labeling of PAI $V_{536}$, *tetA* from pBR322 was amplified with primers Tet3 and Tet2, and the *tetA* promoter/operator region from plasmid pASK75 (42) was amplified with oligonucleotides $tet^{p/o}$ and Tet4. Both fragments were assembled in a recombinant PCR (21). The resulting 1.4-kb PCR fragment was cloned into the SmaI restriction site of pBluescript II KS(−). The construct was linearized with XbaI, the ends were blunted and ligated with the *sacB* gene that had been amplified with the primer pair sacB-161/XhoI and sacB-1975 from pCVD442. In the resulting vector pKS-*sacB tetA*, both genes are inserted consecutively in the same orientation. A

subsequent PCR with pKS-*sacB tetA* as a template and the primer pair PAI Vmut 400 and PAI Vmut 499 generated a 3.3-kb PCR product that was used for electroporation of *E. coli* 536/pKD46 by following the protocol of Datsenko and Wanner (10). *E. coli* 536 PAI $V_{536}$::*sacB tetA* was selected by screening for resistance to tetracycline and sensitivity to sucrose. For the insertion of *sacB* into the λ attachment site $attB_\lambda$, the 2.6-kb PstI fragment of pCVD442 encoding the *sacB* gene was cloned into the PstI restriction site of pLDR9 (11). A NotI fragment containing the *sacB* gene, the *attP* site, and the *bla* gene was recircularized and subsequently introduced by electroporation into *E. coli* 536/pLDR8 by following the protocol of Diederich et al. (11). *E. coli* strain 536 $attB_\lambda$::*sacB bla* was isolated by screening for resistance to ampicillin and sensitivity to sucrose.

**Construction of Δ*recA* mutants.** Δ*recA* mutants were constructed with a one-step chromosomal gene inactivation technique (10). The *cat* gene of pKD3 was amplified with the primer pair recA_cat1-recA_cat2, and the resulting PCR product was electroporated into *E. coli* 536/pKD46. Mutants with a replacement deletion of *recA* were selected on agar plates containing chloramphenicol. The *cat* cassette was subsequently removed as described previously (10).

**Preparation and manipulation of DNA.** Plasmid DNA and chromosomal DNA were isolated according to standard protocols (40). Recombinant DNA manipulations were carried out with enzymes supplied by Amersham or New England Biolabs according to the manufacturer's instructions and standard procedures (1). DNA was introduced into *E. coli* K-12 derivatives by transformation with $CaCl_2$ competent cells (40). *E. coli* 536 was transformed by electroporation. Cells were grown to mid-log phase, repeatedly washed with ice-cold water, and resuspended in 10% (vol/vol) glycerol to a cell density of ~$3 \times 10^{10}$ cells $ml^{-1}$. Electroporation was performed with a Bio-Rad gene pulser at 2.5 kV, 25 μF, and 600 Ω in 2-mm-gap electroporation cuvettes.

**PCR analysis and DNA sequencing.** PCR mixes contained 1 μl of cell lysate or 500 ng of chromosomal DNA as a template in a total volume of 50 μl. All PCRs were carried out with *Taq* polymerase from Qiagen (Hilden, Germany) according to the manufacturer's manual in an Eppendorf or Biometra thermocycler. For sequencing reactions, PCR products were cloned into pBluescript II KS(−) or pGEM-T Easy. DNA sequencing was done with the BigDye system (PE Biosystems, Inc.) and ABI-377 automated DNA sequencers (Applied Biosystems, Weiterstadt, Germany). Sequences were analyzed with the BLASTN and BLASTX programs (National Center for Biotechnology Information).

**Determination of deletion rates of *sacB*-labeled PAIs.** Overnight cultures (15 h, 37°C) of *sacB*-labeled derivatives of *E. coli* 536 were diluted 1:100 in 30 ml of LB Miller, AU, or M9 minimal medium. Bacteria were incubated at 37, 42, or 20°C in an orbital shaker at 220 rpm, and samples were taken during the late lag, mid-log, early stationary, and late stationary phases. To determine the rate of spontaneously occurring sucrose resistant ($Suc^r$) colonies, 536 $attB_\lambda$::*sacB bla* was treated accordingly. Serial dilutions were plated on LB agar and LB agar supplemented with 7% sucrose to determine the CFU and the number of $Suc^r$ cells. Agar plates were incubated at 20°C for at least 48 h. The deletion rate of PAIs was calculated as the quotient of $Suc^r$ cells and CFU. All data are mean values of results from at least six independent experiments.

**Postexperimental screening of $Suc^r$ colonies.** To test whether sucrose resistance resulted from excision of the respective PAI, samples of $Suc^r$ colonies were analyzed for the presence or absence of PAI-specific nucleotide sequences. Cells derived from 536 PAI $I_{536}$::*sacB* and 536 PAI $II_{536}$::*sacB* were tested phenotypically for their hemolytic activity on LB agar plates containing 3 to 5% sheep blood. For PAI $III_{536}$ and PAI $IV_{536}$, cells were screened by PAI-specific PCRs. For PAI $V_{536}$, cells were further analyzed on LB plates containing tetracycline. For the control (536 $attB_\lambda$::*sacB bla*), cells were tested on LB plates containing ampicillin.

**PCR assays for detection of deletion of PAI $I_{536}$ to PAI $V_{536}$ (exclusion PCR).** Representative numbers of $Suc^r$ colonies were tested in a PCR assay specific for the chromosomal *att* site after deletion of the respective PAI. This assay is based on the usage of primer pairs binding to chromosomal regions flanking the island (Fig. 1). As the distance between primer binding sites is too large when the PAI is integrated in the chromosome, a PCR product was only amplified after excision of the island. Deletion of PAI $I_{536}$ was detected with the primer pair M674-M675 (6), deletion of PAI $II_{536}$ was detected with M803b-M805c (28), deletion of PAI $III_{536}$ was detected with ΔPAI III up-ΔPAI III lp (type I deletion) and ΔPAI III (2) up–III-24.1rev (type II deletion), and deletion of PAI $V_{536}$ was verified with ΔPAI V up-ΔPAI V lp.

**Detection of CIs by PCR.** For the detection of circular intermediates (CIs), total DNA preparations were adjusted to a concentration of 200 ng $\mu l^{-1}$ and sheared by vortexing and freezing. The PCRs contained 2 μl of 10× reaction buffer, 0.2 μl of 20 mM deoxynucleoside triphosphates, 0.2 μl of each primer (100 pmol $\mu l^{-1}$), 0.2 μl of *Taq* polymerase (Qiagen), and 200 to 500 ng of DNA in a total volume of 20 μl. PAI $II_{536}$-specific CIs were detected with the primer pair leu2 and concat1. Intermediates that resulted from excision of PAI $III_{536}$ were detected with the primer pairs PAI III-ci (1) upnest–PAI III-ci (1) lpnest (type I deletion) and PAI III-ci (2) up–PAI III-ci (2) lp (type II deletion). As negative controls, the ΔPAI $I_{536}$ ΔPAI $II_{536}$ derivative 536-21 and *E. coli* K-12 DH5α were included in the assay. As positive controls, plasmids were constructed that carried, as inserts, the left and right ends of the respective PAI arranged in the orientation corresponding to the CIs. Defined copy numbers of these controls were employed in the assay. To verify that reaction mixes contained equal amounts of DNA, an internal region of the 16S rDNA was also amplified with the primer pair rrsA up-rrsA lp. All PCRs were carried out in an Eppendorf thermocycler (50 cycles of 30 s at 95°C, 30 s at 60°C, and 1 to 2 min at 68°C). Ten-microliter aliquots of the PCR samples were analyzed by submarine gel electrophoresis on a 1% (wt/vol) agarose gel.

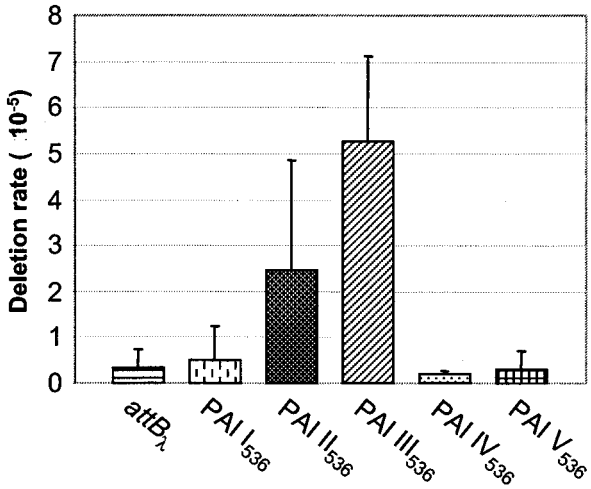


FIG. 2. Deletion rates of PAI $I_{536}$ to PAI $V_{536}$. *E. coli* 536 derivatives with *sacB*-labeled PAI $I_{536}$ to PAI $V_{536}$ and the control (536 $attB_\lambda$::*sacB bla*) were grown overnight in LB medium at 37°C. Deletion rates were calculated as the quotient of $Suc^r$ cells divided by CFU. The data are mean values of the results from at least six independent experiments.

## RESULTS

**PAI $I_{536}$ to PAI $V_{536}$ are deleted with different frequencies.** When the deletion rate of *sacB*-labeled PAIs of *E.coli* 536 was quantified after growth overnight in LB medium at 37°C, it was found that they exhibit different stabilities (Fig. 2). Analysis of strains 536 PAI $II_{536}$::*sacB* and 536 PAI $III_{536}$::*sacB* revealed that these islands are relatively unstable because $Suc^r$ colonies were generated with average frequencies of $2 \times 10^{-5}$ to $3 \times 10^{-5}$ and $5 \times 10^{-5}$, respectively. Postexperimental screening of these colonies demonstrated that the majority of $Suc^r$ colonies resulted from deletion of the corresponding PAI and that the frequency of $Suc^r$ colonies was nearly equivalent to the number of PAI-negative cells in the culture. In contrast, $Suc^r$ colonies of 536 PAI $I_{536}$::*sacB*, 536 PAI $IV_{536}$::*sacB*, and 536 PAI $V_{536}$::*sacB tetA* appeared with frequencies that were about 10-fold lower than the deletion rates of PAI $II_{536}$ and PAI $III_{536}$ and similar or only slightly increased compared to the rate of spontaneous mutations in *sacB* integrated into the λ *att* site (Fig. 2). This rate was determined with frequencies of $3 \times 10^{-6}$ to $4 \times 10^{-6}$ and was similar to previously published observations (5). Additional analyses (see Materials and Methods) revealed that about 50 to 60% of $Suc^r$ colonies derived from 536 PAI $I_{536}$::*sacB* and 30 to 40% of $Suc^r$ colonies derived

from *sacB*-labeled PAI $V_{536}$, respectively, resulted from deletion events of the entire islands or parts of them, whereas in case of PAI $IV_{536}$, sucrose resistance was based on spontaneous mutations in *sacB* rather than on deletion of this PAI.

In summary, PAI $I_{536}$-, PAI $II_{536}$-, PAI $III_{536}$-, and PAI $V_{536}$-negative cells arose with frequencies of $\sim 2 \times 10^{-6}$, $2 \times 10^{-5}$, $5 \times 10^{-5}$, and $\sim 1 \times 10^{-6}$, respectively, while PAI $IV_{536}$ was stable.

**PAI $I_{536}$, PAI $II_{536}$, PAI $III_{536}$, and PAI $V_{536}$, but not PAI $IV_{536}$, are deleted by site-specific recombination.** Analysis of nonhemolytic clones of *E. coli* 536 has previously revealed that PAI $I_{536}$ and PAI $II_{536}$ can be deleted by site-specific recombination between their flanking DR sequences (16 and 18 bp, respectively). Similar to lambdoid phage excision, one copy of the flanking DRs was deleted with the PAI during excision while the other remained in the chromosome (6). To analyze whether Suc$^r$ and PAI-negative colonies of *sacB*-labeled *E. coli* 536 derivatives also resulted from precise excision of the respective islands, exclusion PCR assays were designed. They were based on the amplification of the chromosomal junction region after deletion of a PAI. PCRs with primer pairs binding specifically to the flanking regions of PAI $I_{536}$ and PAI $II_{536}$, respectively, confirmed that these two islands were deleted site specifically from the chromosome of Suc$^r$ and PAI-negative cells.

In contrast to PAI $I_{536}$ or PAI $II_{536}$, PAI $IV_{536}$ is not flanked by DRs. As described previously, *E. coli* 536 only carries a 17-bp sequence at the 3′ end of *asnT* that is identical to the 17-bp DRs flanking the HPI in *Y. pseudotuberculosis*, whereas the repeat at the 3′ end of the island is missing (14, 41). Therefore, it was not too surprising that no precise deletion mutants of PAI $IV_{536}$ were found. Instead, postexperimental screening of Suc$^r$ colonies revealed that deletion of this island occurred very rarely and affected either internal parts of PAI $IV_{536}$ or the entire island with flanking regions of the chromosome. These data confirmed that PAI $IV_{536}$ could be considered a stably integrated island.

For PAI $III_{536}$ and PAI $V_{536}$, it has been postulated that the associated tRNA gene at the 5′ end and a truncated tRNA gene at the 3′ end terminate these islands (Fig. 1). PAI $V_{536}$ is flanked by the intact tRNA gene *pheV* (76 bp) and a partial tRNA gene *phe′V* (22 bp). *phe′V* corresponds to the 3′ end of *pheV* but carries a deletion of 1 bp compared to the sequence of *pheV* (Schneider et al., unpublished). To test whether deletion of PAI $V_{536}$ was mediated by recombination between these two repeats, sucrose-resistant and tetracycline-sensitive (Suc$^r$ Tet$^s$) colonies derived from *E. coli* strain 536 PAI $V_{536}$::*sacB tetA* were analyzed in a PAI $V_{536}$-specific exclusion PCR. A product of the expected size was amplified from circa 45% of the examined colonies. Cloning and sequencing of this product confirmed that site-specific recombination between *pheV* and *phe′V* had caused deletion of the entire PAI $V_{536}$. The regions preceding and following *pheV* in PAI $V_{536}$-negative cells corresponded to the sequences localized immediately upstream and downstream of the PAI in the wild-type strain, indicating that no codeletion of neighboring chromosomal DNA regions had occurred during excision of PAI $V_{536}$. After deletion, the copy of *pheV* remaining in the chromosome carries the 1-bp deletion introduced by *phe′V* during site-specific recombination between the flanking DRs. Since a second gene for a phenylalanine tRNA, *pheU*, is encoded by the *E. coli* chromosome, it remains unclear whether the mutated *pheV* is functional.

TABLE 2. Distribution of PAI $III_{536}$ deletion types

| Strain | Conditions | % Type I[a] | % Type II[b] |
|---|---|---|---|
| 536 PAI $III_{536}$::*sacB* | LB, 37°C | 41 | 59 |
| 536 Δ*recA* PAI $III_{536}$::*sacB* | LB, 37°C | 100 | 0 |
| 536 PAI $III_{536}$::*sacB* | M9, 37°C | 73 | 27 |
| 536 PAI $III_{536}$::*sacB* | LB, 42°C | 67 | 33 |
| 536 PAI $III_{536}$::*sacB* | LB + 2% NaCl, 37°C | 65 | 35 |
| 536 PAI $III_{536}$::*sacB* | LB, 20°C | 71 | 29 |
| 536 PAI $III_{536}$::*sacB* | AU, 37°C | 100 | 0 |

[a] Percentage of Suc$^r$ colonies that were positive in PCR assays with primer pair ΔPAI III up/ΔPAI III lp.
[b] Percentage of Suc$^r$ colonies that were positive in PCR assays with primer pair ΔPAI III (2) up/III-24.1rev.

Suc$^r$ Tet$^s$ colonies that had been negative in the PAI $V_{536}$-specific exclusion PCR contained imprecise deletions of the PAI. This was verified by further PAI $V_{536}$-specific PCRs. In contrast to the above-described site-specific recombination between *pheV* and *phe′V*, the detected deletions seemed unspecific, as they encompassed regions of different sizes surrounding the insertion site of the *sacB tetA* cassette and sometimes even larger areas, including DNA from the core chromosome. However, the frequency of their occurrence was about 10-fold higher than the rate of spontaneous deletions of stable chromosomal regions determined by postexperimental screening of Suc$^r$ colonies derived from *E. coli* strains 536 PAI $IV_{536}$::*sacB* and 536 $attB_\lambda$::*sacB bla* (data not shown). Sequence comparison of the regions located upstream and downstream of the *sacB tetA* insertion site in PAI $V_{536}$ revealed no regions of significant identity that could be responsible for site-specific or homologous recombination leading to the observed deletion types. Hence, they might be caused by the selective pressure for Suc$^r$ colonies, but the underlying mechanism for their occurrence has to be investigated.

Similar to PAI $V_{536}$, PAI $III_{536}$ is flanked by an intact tRNA gene (*thrW*, 76 bp) at the 5′ end and a truncated tRNA gene (*thr′W*) at the 3′ end (14). The DR at the 3′ end encompasses the last 46 bp of *thrW* and the first 2 bp following downstream in the PAI. With 48 bp, the DRs of PAI $III_{536}$ are much larger than those flanking PAI $I_{536}$, PAI $II_{536}$, or PAI $V_{536}$ (Fig. 1), which might at least in part account for the higher deletion rate of this island. To investigate whether deletion of PAI $III_{536}$ is the result of site-specific recombination between *thrW* and *thr′W*, we used Suc$^r$ colonies derived from *E. coli* strain 536 PAI $III_{536}$::*sacB* as templates in an exclusion PCR with the primer pair ΔPAI III up-ΔPAI III lp (Fig. 1). To our surprise, a product of the expected size was only amplified from 41% of the Suc$^r$ cells (Table 2; Fig. 3). Determination of the nucleotide sequence of the PCR product demonstrated that loss of the entire PAI $III_{536}$ with a size of ~68 kb was driven by site-specific recombination between *thrW* at the 5′ end and its truncated copy at the 3′ end. Additional PAI $III_{536}$-specific PCRs that covered the complete PAI with short intervals verified this result. Thus, this deletion process, which will be referred to as a type I deletion, is restricted to the island itself. It is similar to the deletion mechanism of PAI $I_{536}$, PAI $II_{536}$,

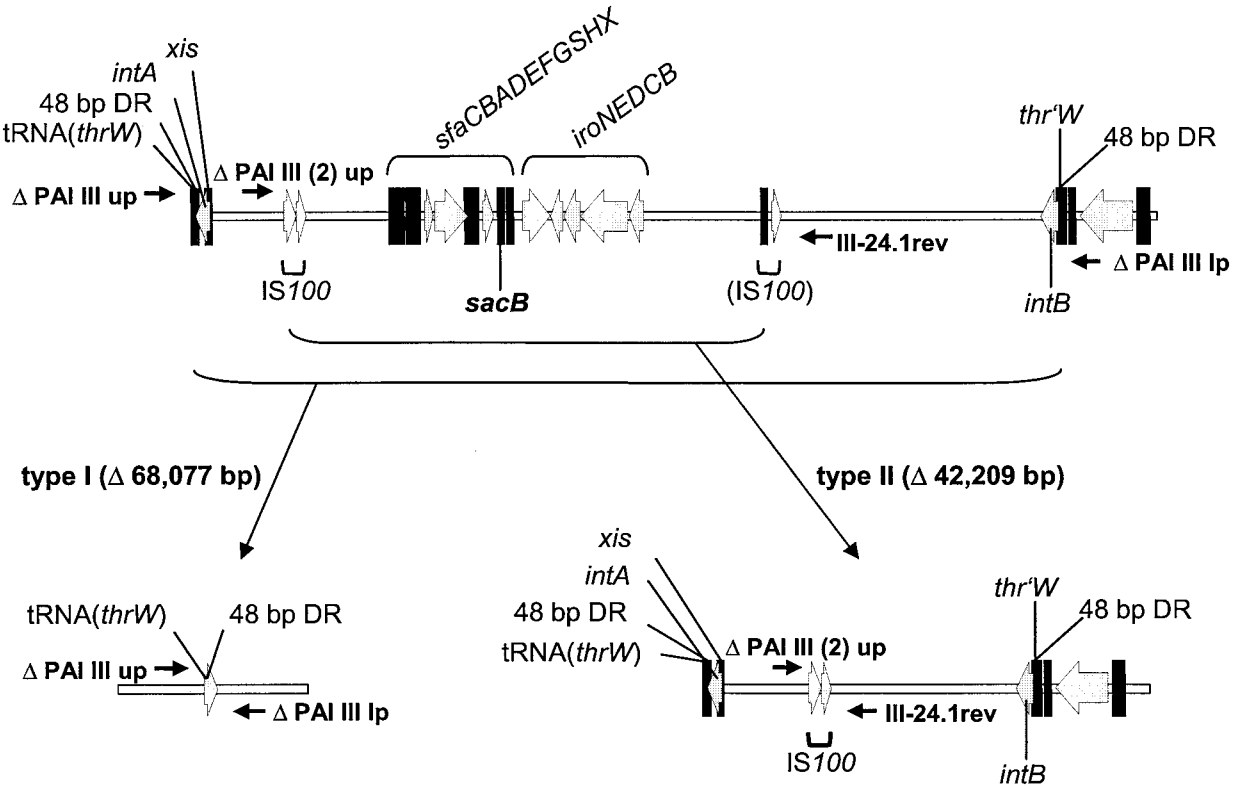


FIG. 3. Deletion types of PAI $III_{536}$. The gene organization in the two types of deletion mutants is shown. Localizations of important virulence genes, PAI-associated integrase genes, direct repeat regions flanking the PAI, IS*100* copies, and the insertion site of the counterselectable marker *sacB* are indicated. Small arrows symbolize primer pairs used for exclusion PCRs. For definitions of abbreviations, see the legend to Fig. 1.

and PAI $V_{536}$ and does not affect neighboring sequences of the core chromosome.

**An internal part of PAI $III_{536}$ can be deleted by homologous recombination.** A screening of the remaining 59% of $Suc^r$ colonies derived from *E. coli* 536 PAI $III_{536}$::*sacB* with PCRs covering the island revealed that in contrast to PAI $V_{536}$ no imprecise deletion had occurred. Instead, all samples tested seemed to have lost the same portion of the island, indicating a second deletion type that covered a shorter part in the center of the PAI including the *sacB* insertion site (type II deletion). Starting from both ends of PAI $III_{536}$, nearly 4 kb of the 5′ end and 23 kb of the 3′ end were still present. Sequence comparison of the supposed boundaries revealed the existence of an IS*100* element (positions 2124 to 4077 of PAI $III_{536}$; GenBank accession no. X16664) and a cryptic IS*100* element (positions 44870 to 46287) (Fig. 3). The intact copy is nearly identical to the IS*100* element of *Y. pestis*, but a mismatch in the 5′ inverted repeat may have rendered it stable. However, a 1,418-bp region of identical nucleotides in both PAI $III_{536}$-specific insertion elements could have been the basis for recombination and loss of the internal part of the PAI. A second exclusion PCR assay with primers that were flanking the two IS*100* copies was designed to confirm this assumption (Fig. 3). PCR products of the expected size could be amplified from all $Suc^r$ colonies that had been negative in the first exclusion PCR assay specific for type I deletion of PAI $III_{536}$. Cloning and sequencing of the PCR product finally demonstrated that a full-length copy of IS*100* remained in the chromosome while the cryptic IS*100* element was deleted together with nearly 42 kb of PAI $III_{536}$.

Whereas the first deletion type of PAI $III_{536}$ was the result of recombination between 48-bp DRs, type II deletion resulted from recombination between larger regions of sequence identity. This suggested that the second deletion type of PAI $III_{536}$ depended on RecA, a protein involved in homologous recombination and DNA repair in *E. coli*. Therefore, a Δ*recA* mutant of *E. coli* strain 536 PAI $III_{536}$::*sacB* was constructed, and $Suc^r$ colonies were isolated. They arose with slightly lower rates than that of the wild-type strain, suggesting that one of the two deletion types required RecA. To determine which mechanism was affected, $Suc^r$ colonies were analyzed in the exclusion PCR assays specific for both deletion types. In contrast to the wild-type strain, where the deletion types appeared with similar frequencies, only type I deletions were detected in the Δ*recA* mutant (Table 2). This indicated that deletion type II of PAI $III_{536}$ was based on homologous recombination between the two IS*100* copies, whereas deletions of type I did not require RecA.

**RecA is not required for deletion of PAI $V_{536}$.** Whereas RecA was required for internal deletions of PAI $III_{536}$, it was not necessary for the site-specific excision of either PAI $I_{536}$, PAI $II_{536}$, or the entire PAI $III_{536}$ (see above) (6, 28). To examine the influence of RecA on the deletion of PAI $V_{536}$, we constructed a Δ*recA* derivative of 536 PAI $V_{536}$::*sacB tetA*. The deletion rate of PAI $V_{536}$ in this strain was comparable to that

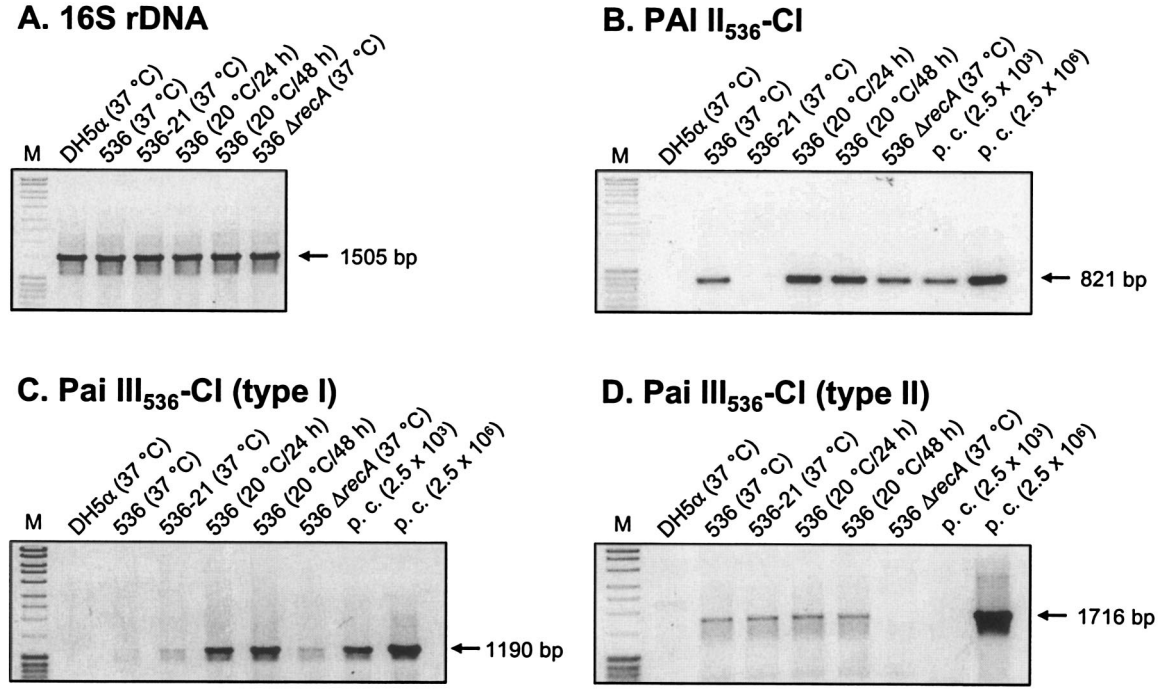


FIG. 4. Detection of CIs. Ethidium bromide-stained 1% agarose gels of PCR products amplified from total DNA preparations were isolated from cultures grown for 15 h at 37°C unless indicated differently. (A) 16S ribosomal DNA (rDNA)-specific control PCR. (B) PCR to detect CIs of PAI $II_{536}$. (C) PCR to detect CIs resulting from excision of PAI $III_{536}$ by site-specific recombination (type I). (D) PCR to detect CIs resulting from excision of PAI $III_{536}$ by homologous recombination (type II). Sizes of the PCR products are indicated. M, 1-kb ladder; p.c., defined copy numbers of plasmid controls.

of the wild-type, but an increase of spontaneous $Suc^r$ colonies was observed, probably due to the accumulation of mutations in a strain that is deficient in DNA repair. PCR analysis of $Suc^r$ $Tet^s$ colonies revealed that both precise and imprecise excision of PAI $V_{536}$ still occurred. However, compared to the wild-type strain, the distribution was altered in the Δ*recA* mutant and a slight increase of site-specific deletions was found (data not shown). Therefore, similar to PAI $I_{536}$ and PAI $II_{536}$, the site-specific excision of PAI $V_{536}$ was RecA independent, whereas the imprecise deletion of this island seemed to be moderately influenced by RecA.

**Detection of CIs of PAI $II_{536}$ and PAI $III_{536}$.** Comparable to the excision mechanism of bacteriophages, PAIs are thought to exist at least transiently as CIs after excision from the chromosome. Since the islands of *E. coli* 536 contain apparently no origin of replication, it has been hypothesized that CIs get lost upon cell division when they fail to reintegrate into the chromosome. In this study, we tried to detect circular intermediates of *E. coli* 536-specific PAIs by a sensitive PCR assay. The primer pairs used for these experiments were oriented towards the right and left PAI-chromosome junctions. A PCR product was only amplified after excision and formation of a CI when the primer binding sites were oriented towards each other. The expected products were amplified with primer pairs specific for CIs resulting from site-specific excision of PAI $II_{536}$ and PAI $III_{536}$, respectively (Fig. 4B and C). Cloning and sequencing of the PCR fragments confirmed that the CIs contained one copy of the PAI-flanking DRs and that this sequence separated the former ends of the island, now oriented towards each other. Formation of the CIs was independent of RecA, as the amount of PCR product derived from a Δ*recA* mutant was comparable to that of the wild type (Fig. 4B and C). CIs corresponding to type II deletion of PAI $III_{536}$ driven by homologous recombination could also be detected in this assay (Fig. 4D) and were verified by nucleotide sequencing. As expected, no specific product was amplified from a Δ*recA* mutant, confirming our previous results.

In contrast to PAI $II_{536}$ and PAI $III_{536}$, CIs of PAI $I_{536}$ or PAI $V_{536}$ could not be detected even if the amount of template DNA or the number of PCR cycles were increased. Therefore, CIs could only be detected for the two islands with a relatively high deletion rate, and the assay was not sensitive enough to detect the probably low numbers of PAI $I_{536}$- and PAI $V_{536}$-specific CIs.

**Deletion of PAI $II_{536}$ and PAI $III_{536}$ is inducible by environmental conditions.** We were interested in whether instability of PAIs in UPEC is influenced by environmental stimuli, such as low or elevated temperature, osmotic stress, nutrient limiting conditions, or growth in AU, which mimics conditions faced by the bacteria in the human host. Therefore, the relative amount of $Suc^r$ colonies was calculated at characteristic time points when cells were grown under different conditions. While an increased amount of $Suc^r$ cells indicated novel deletion events of a particular PAI, stable values during the entire growth curve pointed out that the fraction of PAI-negative cells in the culture was unchanged and that deletion of the respective island was not stimulated under the indicated condition.

Whereas none of the tested stimuli affected deletion rates of PAI $I_{536}$ and PAI $V_{536}$ (Fig. 5A and D), *E. coli* strain 536 PAI $II_{536}$::*sacB* responded to growth in LB medium at 20°C with a

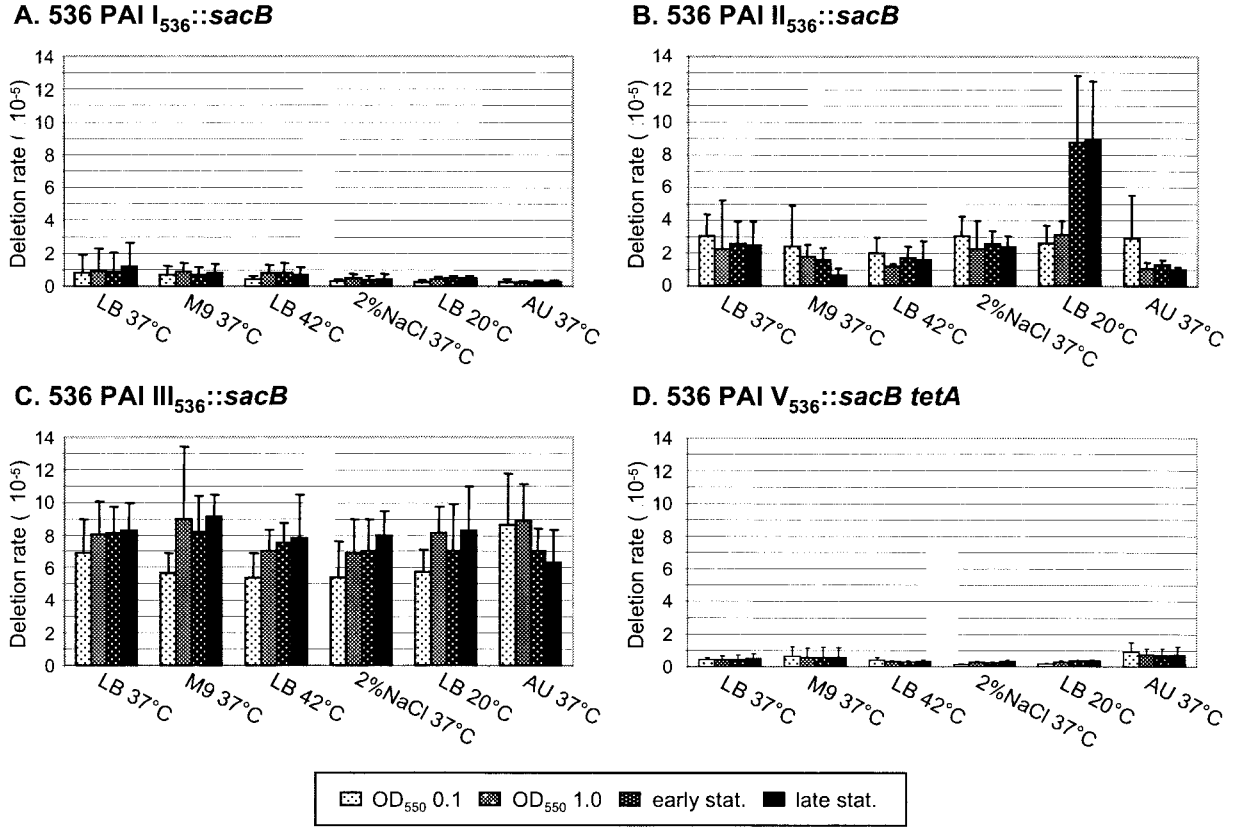


FIG. 5. Influence of environmental factors on deletion of PAI $I_{536}$, PAI $II_{536}$, PAI $III_{536}$, and PAI $V_{536}$. All strains were cultivated under the indicated conditions. Samples were taken during late lag, mid-log, early stationary, and late stationary phase. The deletion rate was calculated as the quotient of $Suc^r$ cells divided by CFU. All data are the results from at least six independent experiments. Evaluation of data by paired *t* test revealed that the increased deletion rate of PAI $II_{536}$ in LB at 20°C is statistically significant ($P < 0.5$).

threefold-higher frequency of $Suc^r$ colonies compared to the deletion rate at 37°C (Fig. 5B) (28). More than 95% of the $Suc^r$ colonies showed reduced hemolytic activity, indicating complete loss of PAI $II_{536}$. A more-detailed analysis of the deletion rate of PAI $II_{536}$ between the mid-log and early stationary phases revealed that excision of this island was induced during the transition from the logarithmic to the stationary phase, i.e., when maximum cell densities (optical density at 550 nm > 2.0) were reached. Furthermore, higher amounts of PAI $II_{536}$-specific CIs were detected in cells grown at low temperatures for 24 and 48 h than in cells grown at 37°C, thereby reflecting an induced excision rate of PAI $II_{536}$ at 20°C (Fig. 4B). However, during stationary-phase growth, no further deletion events seemed to occur. Both stimuli, low temperature and high cell density, were responsible for the increased number of PAI $II_{536}$-negative cells, since a similar effect was not observed when bacteria were grown in LB medium at 37 and 42°C (Fig. 5B) or when the culture reached the stationary phase at an optical density at 550 nm of <2.0 (data not shown). Similarly, high salt concentrations, nutrient limitation, or growth in AU did not induce deletion of PAI $II_{536}$. However, when 536 PAI $II_{536}$::*sacB* was grown in M9 minimal medium, the deletion rate of PAI $II_{536}$ was decreased in the late stationary phase (Fig. 5B). This could point out that PAI $II_{536}$-negative cells are deficient in growth in M9 minimal medium and are outcompeted by the wild type, but the difference between values was not statistically significant. So far, growth differences between PAI-negative and PAI-positive cells have not been observed. Finally, sequence analysis of PAI $II_{536}$ revealed no genes whose deletion would account for growth deficiency.

Similar to PAI $I_{536}$ and PAI $V_{536}$, the deletion rate of PAI $III_{536}$ seemed to be unaffected by the tested environmental conditions. Only slightly increased numbers of $Suc^r$ colonies could be observed during growth in M9 minimal medium, in LB medium at low or high temperatures, or under salt stress (Fig. 5D). However, a closer analysis of the distribution of PAI $III_{536}$-specific deletion types revealed a difference (Table 2). Whereas the basic ratio of type I to type II was unaltered when cells were grown in LB medium at 37°C, deletion type I was increased to more than 60% of the $Suc^r$ cells after incubation at 42°C or in presence of 2% NaCl. In M9 minimal medium or after growth at 20°C, this effect was even more pronounced and the ratio of deletion types changed to circa 70 to 30%. Comparable to PAI $II_{536}$, the increase of type I deletion in cells grown at low temperatures could be confirmed with the PCR assay designed to detect CIs of PAI $III_{536}$ that originate from site-specific excision (Fig. 4C). Finally, when cells were grown in AU, only $Suc^r$ colonies that had lost PAI $III_{536}$ by type I deletion events were isolated (Table 2). In summary, PAI $III_{536}$ was not only the island with the highest deletion rate under all tested conditions but it was also susceptible to all environmental stimuli tested in vitro. The observed shift in the distribution

of PAI $III_{536}$-specific deletion types was the result of either induced type I deletion or decreased type II deletion. Since PAI $III_{536}$ is deleted by site-specific and homologous recombination, an influence of the environmental conditions on *recA* and the PAI $III_{536}$-associated integrase genes has to be evaluated in future experiments.

## DISCUSSION

The capacity of bacteria to modulate their genome structure is an important feature for adapting to changing environmental conditions. Genome flexibility also has an impact on the evolution of new bacterial pathogens. The acquisition of new traits by horizontal gene transfer is one of the driving forces in the emergence of new bacterial variants, but point mutations, gene rearrangements, or loss of genetic information also play crucial roles (15, 27, 31). In this context, it has been interesting to find that instability seems to be a characteristic of PAIs in particular pathogens, including UPEC (6, 16, 33). Furthermore, other PAIs have been identified that share the tendency to be deleted from the bacterial chromosome (2, 7, 9, 34, 43, 44). In this study, we applied the island-probing approach to analyze for the first time the (in)stability of all so far identified PAIs of one pathogenic strain in detail.

The deletion rates of $10^{-6}$ to $10^{-5}$ that were obtained for PAI $I_{536}$ to PAI $V_{536}$ were similar to the rates previously reported for other PAIs (34, 43, 44). With the exception of PAI $III_{536}$, where a second deletion mechanism based on homologous recombination between a complete and a truncated IS*100* copy was detected, excision and subsequent deletion of PAIs in *E. coli* 536 is mediated by site-specific recombination between short flanking DR sequences. It can be speculated whether the length of the flanking DRs is one reason for the observed differences in the stability of *E. coli* 536-specific PAIs. However, a direct connection between the size of flanking repeats and excision frequency can be ruled out by comparing PAI $I_{536}$ and PAI $II_{536}$, which are both flanked by DRs of similar length (16 and 18 bp, respectively) but are deleted with rates differing nearly 10-fold. In case of PAI $V_{536}$, the mismatch in the DR located at the 3′ end of the island (*phe′V*) may decrease the efficiency of site-specific recombination, resulting in a relatively low deletion rate. Interestingly, the identified mismatch is also present in the 3′ DR of the LEE in REPEC strain 84/110-1 (43). Similar to PAI $V_{536}$, the relatively low deletion rate of this island may be ascribed to the imperfect DR. As it is unlikely that the same mismatch developed independently and spontaneously by point mutation in the 3′ DR of both islands, the REPEC LEE and PAI $V_{536}$, it can be speculated that it has been already present in a precursor of the two islands. Finally, PAI $IV_{536}$ is stably integrated into the chromosome of strain 536, apparently due to deletion of one of the flanking DR sequences, as it has also been reported for *Y. enterocolitica* Ye8081 (2, 41). In contrast, HPIs of *Y. pestis* and *Y. pseudotuberculosis* are relatively unstable. In *Y. pestis*, the HPI core element is deleted together with the flanking pigmentation segment by homologous recombination between two IS*100* elements comparable to type II deletion of PAI $III_{536}$, and in *Y. pseudotuberculosis*, deletion of the HPI core region is due to site-specific recombination between 17-bp DRs (7, 8).

Besides the length and integrity of the flanking DRs, the levels and activities of PAI-associated integrases probably contribute to the frequency of excision of PAIs in *E. coli* 536. It has been shown that P4-like integrases of the REPEC LEE or the HPI of *Y. pestis* mediate site-specific integration of small artificial DNA fragments corresponding to an *attP* site into all *asn* tRNA loci or into *pheU*, respectively (35, 43). Preliminary data indicate that integrases of PAIs in *E. coli* 536 also trigger deletion of their islands. Therefore, it will be interesting to investigate their activity in more detail in future experiments.

Our data support the hypothesis that stabilization of PAI $I_{536}$ and PAI $V_{536}$ is an ongoing process, since both islands have decreased deletion rates compared to PAI $II_{536}$ and PAI $III_{536}$. It has been hypothesized that PAIs have been acquired by horizontal gene transfer followed by integration into the bacterial genome (15, 17). If their genetic features turn out to be advantageous for the host organism, they are subjected to selective pressure, thereby favoring mutations that render the islands stable. Examples are the HPI of *Y. enterocolitica* or some islands of *Salmonella enterica* that seem to have lost all former traits of mobility (2, 17, 36).

It has been speculated that the loss of virulence determinants may play a crucial role during the transition from an acute state of disease to chronic infection (6, 17). Therefore, in addition to the mechanisms underlying the deletion of PAIs in *E. coli* 536, we were particularly interested in examining the influence of environmental conditions on their stability. We confirmed previous results that deletion of PAI $II_{536}$ is significantly induced at 20°C (28) and found that both low temperature and high cell density contribute to this effect. In contrast, other stimuli tested, such as growth at high temperature, salt stress, depletion of nutrients, or growth in AU did not change the deletion rate of PAI $II_{536}$. One habitat, where bacteria are exposed to low temperature as well as a high density of cells, is the complex community of naturally occurring biofilms. Therefore it can be speculated that such growth conditions facilitate excision and mobilization of PAIs, e.g., by transducing phages that may be induced within biofilms. Interestingly, the mobile islands *V. cholerae* PAI and *S. aureus* PAI 1 are both transmissible to recipient organisms via phage transduction, and it would be interesting to analyze whether their transfer rates are also modulated by environmental parameters. In the case of PAI $III_{536}$, the deletion rate was not significantly altered by environmental stimuli, but comparable to PAI $II_{536}$, growth at low temperatures affected the excision of this island and resulted in a shift of PAI $III_{536}$-specific deletion types to site-specific recombination (deletion type I). The same effect could be observed when cells were grown in M9 minimal medium, at high temperature, or under salt stress, and it was most pronounced in AU, indicating that modulation of PAI stability could be important in the environment and in the host during infections.

It is believed that loss of PAIs is preceded by excision and circularization of the respective islands (6). Detection of CIs of PAI $II_{536}$ and PAI $III_{536}$ with a specific PCR assay confirmed this assumption. Even though a precise quantification of CIs was not possible with this assay, the results corroborated the findings that environmental stimuli have an impact on the deletion rate of at least two PAIs of *E. coli* 536. Excision of

PAIs generates less-virulent variants, and it remains to be studied in more detail which role deletion of PAIs plays in natural habitats of UPEC strains. In contrast to PAI $II_{536}$ and PAI $III_{536}$, no modulation of the deletion rate and no CIs were found in the case of PAI $I_{536}$ and PAI $V_{536}$, again suggesting that these islands are relatively stably inserted into the chromosome.

The basis for enhanced deletion of PAI $II_{536}$ and PAI $III_{536}$ is yet unknown. It can be speculated that island-associated integrase genes are differentially regulated in response to environmental stimuli, thereby altering the frequency of excision. As the elevated deletion rate of both islands is closely connected to high cell density, quorum sensing may play a role for this effect. It has recently been shown that the time when autoinducer 2 of the quorum-sensing circuit in *E. coli* is produced overlaps with the time point when deletion of PAI $II_{536}$ and PAI $III_{536}$ is induced (3, 19). Additionally, the alternative sigma factor of the stationary growth phase ($\sigma^s$) is another candidate that may play a role in the increased deletion of PAIs in *E. coli* 536 (20).

Future experiments will focus on the role of integrases and on the impact of environmental stimuli during deletion of PAIs. Finally, it remains to be studied in more detail which role deletion of PAIs plays in natural habitats of UPEC strains.

## ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB479), the Fonds der Chemischen Industrie, and the Bayerische Forschungsstiftung.

We thank E. Carniel for kindly providing plasmid pGP704 *fyuA*::*sacB*, G. Schneider for the nucleotide sequence of PAI $V_{536}$, and A. Siegl for technical assistance.

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