

## Region-Specific Insertion of Transposons in Combination with Selection for High Plasmid Transferability and Stability Accounts for the Structural Similarity of IncP-1 Plasmids<sup>∇</sup>

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**The overall architecture of IncP-1 plasmids is very conserved in that the accessory genes are typically located in one or two specific regions: between *oriV* and *trfA* and between the *tra* and *trb* operons. Various hypotheses have been formulated to explain this, but none have been tested experimentally. We investigated whether this structural similarity is due to region-specific transposition alone or also is reliant on selection for plasmids with insertions limited to these two regions. We first examined the transposition of Tn21Km into IncP-1β plasmid pBP136 and found that most Tn21Km insertions (67%) were located around *oriV*. A similar experiment using the *oriV* region of IncP-1β plasmid pUO1 confirmed these results. We then tested the transferability, stability, and fitness cost of different pBP136 derivatives to determine if impairment of these key plasmid characters explained the conserved plasmid architecture. Most of the pBP136 derivatives with insertions in transfer genes were no longer transferable. The plasmids with insertions in the *oriV-trfA* and *tra-trb* regions were more stable than other plasmid variants, and one of these also showed a significantly lower fitness cost. In addition, our detailed sequence analysis of IncP-1 plasmids showed that Tn402/5053-like transposons are situated predominantly between the *tra* and *trb* operons and close to the putative resolution site for the ParA resolvase, a potential hot spot for those transposons. Our study presents the first empirical evidence that region-specific insertion of transposons in combination with selection for transferable and stable plasmids explains the structural similarity of IncP-1 plasmids.**

Horizontal gene transfer is now well recognized as one of the key mechanisms of adaptive evolution of bacteria (20, 50, 53). Mobile genetic elements such as plasmids, transposons, and integrative and conjugative elements are known to promote horizontal gene transfer events and DNA rearrangements (18, 53, 57). Many of these elements carry so-called “accessory genes” that encode various functions such as resistance to antibiotics, degradation of xenobiotics, virulence, and symbiosis (18, 58). Conjugative plasmids are genetic elements that can efficiently transfer from cell to cell (50, 62). In addition to their so-called “backbone” genes, which encode the machineries for plasmid replication, regulation, stable inheritance, and transfer, they also serve as vectors for other mobile genetic elements such as transposons and integrons that carry the accessory genes (49). Comparative studies of conjugative plasmids have shown that the DNA sequence of plasmid backbones is conserved, which is in contrast to the broad genetic diversity of the accessory genes (2, 14, 35, 45, 59).

The incompatibility group P-1 (IncP-1) plasmids are among the best-studied plasmids of gram-negative bacteria and are of particular interest because of their broad host range, high transfer frequencies, and the wide variety of accessory genes they carry (2, 51). In contrast to their phenotypic diversity,

their basic architecture is very conserved, in that the accessory fragments are usually located in one or both of two specific regions, i.e., between *oriV* and *trfA* and between the *tra* and *trb* operons (2, 51). To date, at least four subgroups of IncP-1 plasmids ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) have been defined based on the phylogenetic relatedness of the *trfA* genes, which encode the plasmid replication initiation protein, and a fifth subgroup has been proposed (4). Strikingly, all these IncP-1 plasmids show the same typical architecture.

There are at least three hypotheses to explain the structural similarity of IncP-1 plasmids: (i) transposons specifically transpose into these regions of the IncP-1 backbone; (ii) transposition is completely random, but plasmids with insertions in the two specific regions are most stable or transferable, or least costly to their host, and therefore persist longer over evolutionary time than cognate plasmids with insertions in other sites (12, 41); or (iii) a combination of region-specific insertion and selection explains the common plasmid structure. Consistent with the first hypothesis, it has been proposed previously that 20-bp inverted repeats (IRs) with a consensus sequence of CATGCCANNTCYGRGATG found in the *oriV-trfA* and *tra-trb* regions of IncP-1β plasmids might be involved in the acquisition of transposons (24, 52). However, this hypothesis has not been tested experimentally, in part because there were no IncP-1 plasmids available that lacked insertions in both of the regions. Conversely, inconsistent with this first hypothesis is the observed transposon (Tn7) insertions in various backbone genes of IncP-1α plasmids, which had major effects on the plasmid phenotype (e.g., host range) (5,

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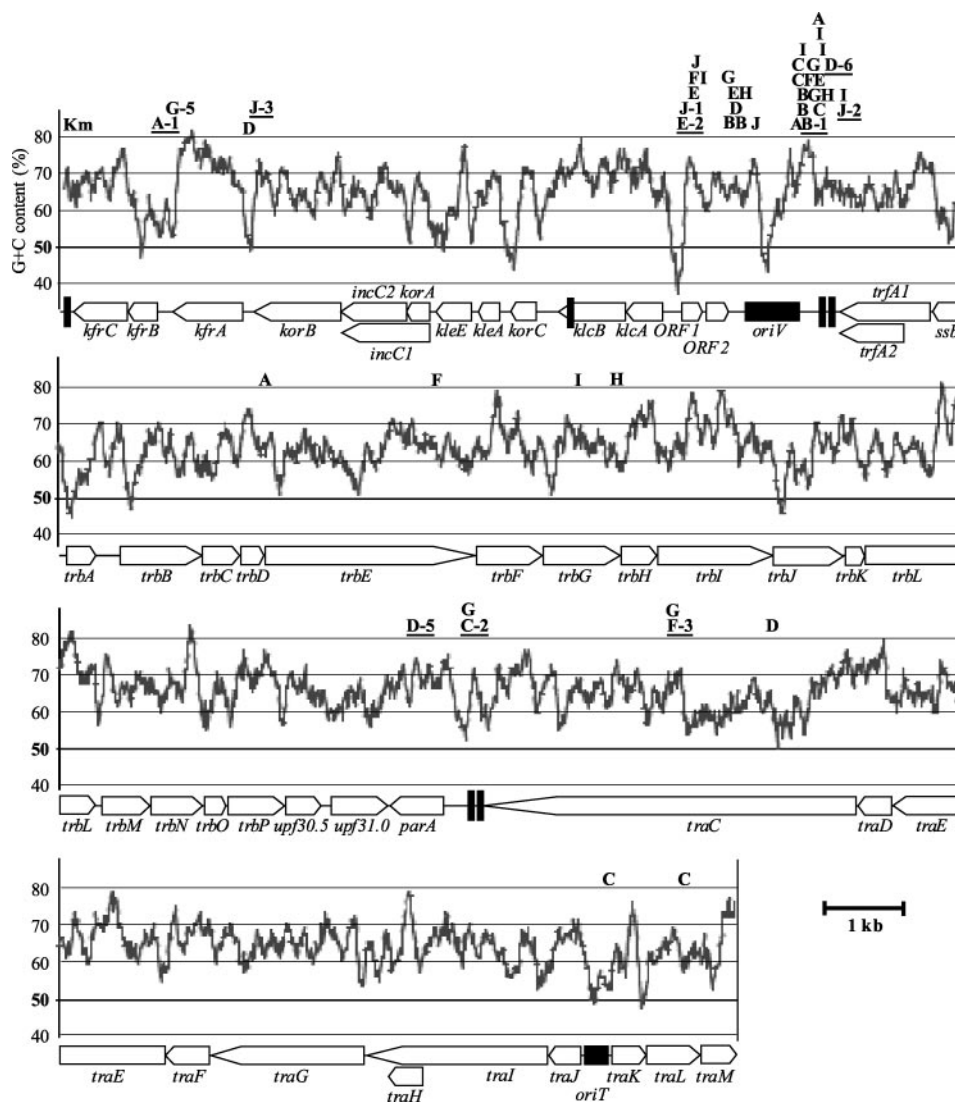


FIG. 1. Physical map and G+C content of pBP136, showing the insertion sites of Tn21/Km. The letters A to J indicate insertion sites in plasmids obtained from 10 independent mating experiments. Plasmids with insertions at the sites shown by underlined letters were used to investigate the plasmid stability and cost. Km indicates the location of the *Km<sup>r</sup>* gene in pBP136Km. The black vertical bars represent the positions of the 20-bp IRs. The G+C content of pBP136 was calculated in an average calculation span of 100 bp. See reference 26 for detailed information on the genetic structure and function of the plasmid.

36). However, these studies did not test the second and third hypotheses.

Recently, the IncP-1 $\beta$  plasmid pBP136, isolated from the human pathogen *Bordetella pertussis*, was found not to carry any transposons or their footprints, yet it has two copies of the 20-bp IR in the *oriV-trfA* and *tra-trb* regions and one in *klcB* and between *traM* and *kfrC* (Fig. 1) (26). The objective of our study was to empirically test the three hypotheses by performing transposition experiments using a Tn21 derivative (designated Tn21/Km) and the cryptic IncP-1 $\beta$  plasmid pBP136 and subsequently analyzing the behavior of plasmid variants with transposon insertions in various sites. Our empirical results and detailed sequence analysis of known IncP-1 plasmids support the third hypothesis, i.e., that region-specific insertion of transposons such as Tn21-like and Tn402/5053-like transposons, in combination with selection for transferable and sta-

ble plasmids, can account for the common architectural features of IncP-1 plasmids.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Escherichia coli* strains used in this study were DH10B [*F<sup>-</sup> mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\phi$ 80d $\Delta$ lacZ $\Delta$ M15  $\Delta$ lacX74 *deoR recA1 endA1 araD139*  $\Delta$ (*ara leu*)7697 *galU galK*  $\lambda^-$  *rpsL nupG*] (TaKaRa BIO), EC100 [*F<sup>-</sup> mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\phi$ 80d $\Delta$ lacZ $\Delta$ M15  $\Delta$ lacX74 *recA1 endA1 araD139*  $\Delta$ (*ara leu*)7697 *galU galK*  $\lambda^-$  *rpsL nupG*] (Epicentre Biotechnologies), S17-1 (*recA1 endA1 thi hsdR*, RP4-2 Tc::Mu::Tn7) (40), a Mu phage lysogen of DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (designated DH1Mu), and a spontaneous rifampin-resistant (Rif<sup>r</sup>) mutant of MG1655 (designated K12R). The strains were cultured at 37°C. Plasmids used in this study are listed in Table 1. Plasmid pBP136 was provided to us in *E. coli* DH10B by K. Kamachi. Plasmid pBP136Km is a pBP136 derivative in which a kanamycin resistance (*Km<sup>r</sup>*) gene derived from the cloning vector pUC4K (47) was inserted into the unique XbaI site of pBP136. Plasmid pMT1247 is a pACYC184 derivative carrying Tn21 (8, 56). This plasmid was digested with BamHI, and the

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pARO190	Ap <sup>r</sup> , pMB1 <sup>ori</sup> , pUC19 derivative with the RP4 <sup>oriT</sup> sequence	34
pBP136	IncP-1 $\beta$ , self-transmissible, no transposons or their footprints	26
pBP136Km	Km <sup>r</sup> , pBP136 derivative with a Km <sup>r</sup> determinant at the unique XbaI site between <i>traM</i> and <i>kfrC</i>	26
pBPTn/A-1	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km in <i>kfrB</i> (position 1209–1210 of pBP136, 26 bp downstream from the 5' end of <i>kfrB</i> )	This study
pBPTn/B-1	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>oriV</i> and <i>trfA</i> (position 9085–9086 of pBP136)	This study
pBPTn/C-2	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>parA</i> and <i>traC</i> (position 26974–26975 of pBP136)	This study
pBPTn/D-5	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km in <i>parA</i> (position 26320–26321 of pBP136, near center of <i>parA</i> )	This study
pBPTn/D-6	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>oriV</i> and <i>trfA</i> (position 9369–9370 of pBP136)	This study
pBPTn/E-2	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>klcA</i> and <i>ORF1</i> (position 7595–7596 of pBP136, 156 bp upstream of the 5' end of <i>klcA</i> )	This study
pBPTn/F-3	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km in <i>traC</i> (position 29473–29474 of pBP136, near center of <i>traC</i> )	This study
pBPTn/G-5	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km in <i>kfrA</i> (position 1410–1411 of pBP136, 7 bp upstream from the 3' end of <i>kfrA</i> )	This study
pBPTn/J-1	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km in <i>ORF1</i> (position 7628–7629 of pBP136, 33 bp downstream from the 5' end of <i>ORF1</i> )	This study
pBPTn/J-2	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>oriV</i> and <i>trfA</i> (position 9524–9525 of pBP136)	This study
pBPTn/J-3	Km <sup>r</sup> , pBP136 derivative carrying Tn21Km between <i>kfrA</i> and <i>korB</i> (position 2367–2368 of pBP136, 95 bp upstream from the 5' end of <i>kfrA</i> )	This study
pMS0220	Ap <sup>r</sup> , pARO190 derivative that carry the 2-kb <i>oriV-trfA</i> fragment of pUO1 (see text and Fig. 2)	This study
pMT1247	Cm <sup>r</sup> , p15A <sup>ori</sup> , pACYC184 derivative carrying Tn21	56
pMT1247Km	Cm <sup>r</sup> , Km <sup>r</sup> , pMT1247 derivative in which a Km <sup>r</sup> determinant is inserted at the BamHI site of Tn21 (designated Tn21Km)	This study
pUC4K	Ap <sup>r</sup> , Km <sup>r</sup> , cloning vector	47
pUO1	IncP-1 $\beta$ , haloacetate degradation, mercury resistance	44

pUC4K-derived Km<sup>r</sup> gene was inserted into the BamHI site, generating pMT1247Km, which carried a Km<sup>r</sup> Tn21 derivative (designated Tn21Km). Two regions of the IncP-1 $\beta$  plasmid pUO1 (positions 1 to 901 and 16,511 to 17,610; accession no. AB063332) (44) were amplified by PCR using appropriate primer sets, and the two PCR products were cloned together between EcoRI and HindIII sites of the cloning vector pARO190 (34). The resulting plasmid, pMS0220, carried the potentially ancestral *oriV-trfA* region of the plasmid, since the Tn21-like transposon TnHad2 between *oriV* and *trfA* (43) was removed. Luria-Bertani broth (LB) and LB agar (LBA) (3) were used throughout this study. The antibiotics added to the media were as follows: Km, 100 mg/liter; nalidixic acid (Nal), 20 mg/liter; Rif, 50 mg/liter; and streptomycin (Sm), 100 mg/liter.

**DNA methodology.** Standard methods were used for extraction of the plasmid DNA, DNA digestion with restriction endonucleases, ligation, gel electrophoresis, and transformation of *E. coli* cells (3). PCR amplification was carried out with *ExTaq* DNA polymerase (TAKARA BIO) or AccuPrime Pfx DNA polymerase (Invitrogen). Purification of the PCR-amplified DNA fragments was done with GFX PCR DNA and the Gel Band purification kit (GE Healthcare) according to the manufacturer's protocol.

**Transposition assays.** Transposition of Tn21Km into pBP136 was detected by the "mating-out" experiments described previously (55). For this purpose, we introduced pMT1247Km into the DH10B derivative harboring pBP136. Ten transformants were selected and cultured to the stationary phase in LB containing Km [LB(Km)] and then mixed with K12R on membrane filters. After 6 hours of incubation at 37°C, the cells were harvested, and appropriate dilutions were plated on LBA(Km, Rif) to select for Km<sup>r</sup> K12R transconjugants. Plasmids from three to six Km<sup>r</sup> transconjugants in each of the 10 mating experiments were analyzed to identify the insertion sites of Tn21Km in pBP136, using the primer Tn21-F19170 (5'-TTTGGATTGGATAGCGTAAAC-3'), which annealed to the *mpA*-distal end of Tn21 (accession no. AF071413) in the outward direction. Transposition of Tn21Km into pMS0220 was also detected by mating-out experiments. Plasmids pMT1247Km and pMS0220 were introduced into *E. coli* S17-1 (40), and the resulting strain was mated with DH1Mu. The steps after the mating were done as described above except for the use of Nal instead of Rif.

**Plasmid stability experiment.** Stability of pBP136 derivatives carrying Tn21Km (pBPTn plasmids) was investigated as follows. The EC100 derivative harboring a pBPTn plasmid was grown to the stationary phase in 5 ml LB(Km) (defined as generation 0 cells). The cells were collected by centrifugation, rinsed in saline, and resuspended in 5 ml of saline; 4.88  $\mu$ l of this cell suspension was transferred into 5 ml LB without Km, the cells were cultured for 16 h, and this procedure was repeated daily (ca. 10 generations per day). At regular time points

the cells were plated on LBA, and colonies thus formed were picked onto LBA(Km) to determine the fraction that showed the Km<sup>r</sup> phenotype.

**Estimates of plasmid maintenance costs.** Pairwise competition experiments were performed between EC100 and EC100(pBPTn). Both strains were pre-cultured for 12 h in LB(Sm) and LB(Km, Sm), respectively. After being rinsed in saline, the cells were inoculated into LB(Sm) at a 1:1 ratio (calculated from optical density at 600 nm) and were grown for 24 h at 30°C and 200 rpm. Appropriate dilutions of the initial and final culture media were plated on LBA(Sm) and LBA(Km, Sm) using Autoplate 4000 (Spiral Biotech), and the number of colonies was counted with QCount (Spiral Biotech). The relative fitness (*W*) was determined as described previously (7, 10) using the following formula:  $W_{ij} = \log_2[N_i(1)/N_i(0)] / \log_2[N_j(1)/N_j(0)]$ , where  $N_i(0)$  and  $N_j(0)$  are the initial densities of EC100(pBPTn) and EC100, respectively, and  $N_i(1)$  and  $N_j(1)$  are their corresponding final densities.

**Statistical analyses.** The standard Student *t* test with Bonferroni correction was used to determine the significance of any differences between means of the plasmid costs. Differences between means of plasmid stability were assessed with a Dunnett multiple-comparison test (15). The  $\chi$ -square test was used for testing differences between observed and expected frequencies of transposon insertions (25). Since the  $\chi$ -square test requires an expected frequency of at least five in each category and there were 48 plasmids with transposon insertions, the 41-kb sequence of pBP136 (accession no. AB237782) was divided into eight regions, representing eight categories (expected frequency = 6 per region). In each analysis, *P* values of <0.05 were considered significant.

**DNA sequence analysis.** The nucleotide sequencing was performed with an ABI PRISM model 3730 sequencer (Applied Biosystems). The computer analysis of the DNA sequences was performed with the software programs GENETYX 13 (SDC Inc.) and BLAST (National Center for Biotechnology Information).

## RESULTS

**Transposition of Tn21Km to pBP136.** To test the null hypothesis that insertion of Tn21 transposons in IncP-1 plasmids is random, we conducted transposition experiments using the transposon-free IncP-1 $\beta$  plasmid pBP136 (26) as the target for Tn21Km, a Km<sup>r</sup> derivative of Tn21 (13). A total of 48 plasmids were analyzed to identify the insertion sites of Tn21Km. Thirty-two of the 48 plasmids (67%) had the Tn21Km insertion near

the *oriV* sequence, while Tn21Km was found in the control and maintenance regions (*kfrA*, *kfrB*, between *kfrA* and *kfrB*, and between *kfrA* and *korB*) of four plasmids, in the transfer genes (*trbD*, *trbE*, *trbG*, *traC*, *oriT*, and *traL*) of nine plasmids, and between the *tra* and *trb* operons (*parA*, between *parA* and *traC*) of three plasmids (Fig. 1). A  $\chi$ -square test (25) confirmed that there were significantly more insertions in the *oriV* region than under the assumption of random insertions and that there were significantly fewer insertions in the two transfer regions and in the control and maintenance regions ( $P < 0.001$ ). Therefore, our results rejected the null hypothesis that transposition was random throughout the plasmid. However, since not all insertions were found around *oriV* or between the *tra* and *trb* operons, the hypothesis that the current IncP-1 plasmid structures can be completely explained by region-specific transposition is also rejected.

At least two hypotheses have previously been formulated to explain the specific architecture of IncP-1 $\beta$  plasmids. First, it has been hypothesized that the 20-bp IRs may attract transposons (24, 52). However, although pBP136 has two 20-bp IRs in both the *oriV-trfA* and the *tra-trb* intergenic regions and one in *kfcB* and between *traM* and *kfrC*, the Tn21Km insertions were clustered only in the *oriV-trfA* region (Fig. 1). It has also been shown that Tn3-like transposons such as Tn3 and Tn21 tend to transpose into AT-rich sequences (38). Therefore, we plotted the G+C content of pBP136 (the average calculation span was 100 bp) and indicated the insertion sites on the plot (Fig. 1). Although the pBP136 sequence has several AT-rich regions (G+C content of  $<50\%$ ), the Tn21Km insertions were not clustered in these regions but in rather high-G+C ( $>65\%$ ) regions. The region with the highest density of insertions (downstream of *oriV*) had  $>70\%$  G+C content (Fig. 1). These results suggest that (i) the 20-bp IRs are not directly involved in acquisition of transposons and (ii) region-specific location of transposons is not correlated with the G+C content of pBP136.

**Experimental bias.** During the transposition and subsequent mating experiments that were performed, only plasmid variants that could still replicate and transfer with or without the help of coresident plasmids would have been retrieved in the transconjugants. Therefore, our method may not have detected every possible plasmid derivative, but there may have been a bias for those plasmids that still efficiently replicated and transferred. Due to the short incubation times (24 h or less) and the presence of antibiotics in the selection media, we assume that there was no bias for plasmid variants with higher stability or lower fitness cost (see below). We tested the transferability of pBP136 derivatives with insertions in *trbD*, *trbE*, *trbG*, *traC*, and *traL* and near *oriT*. The plasmids with an insertion in *traC* or near *oriT* were able to transfer into recipient cells at a frequency similar to that of pBP136Km (ca.  $10^{-2}$  per recipient), but those with an insertion in *trbD*, *trbE*, *trbG*, or *traL* were not transferable ( $<10^{-8}$  per recipient). These results indicate that our experimental system can detect transfer-deficient pBP136 derivatives even if Tn21Km was inserted into the regions encoding plasmid transfer, probably due to mobilization by coresident plasmid variants. Thus, only nonreplicating plasmids and plasmids that are no longer transferable even in the presence of transfer-proficient plasmids would have been lost from our detection system. Therefore, we conclude

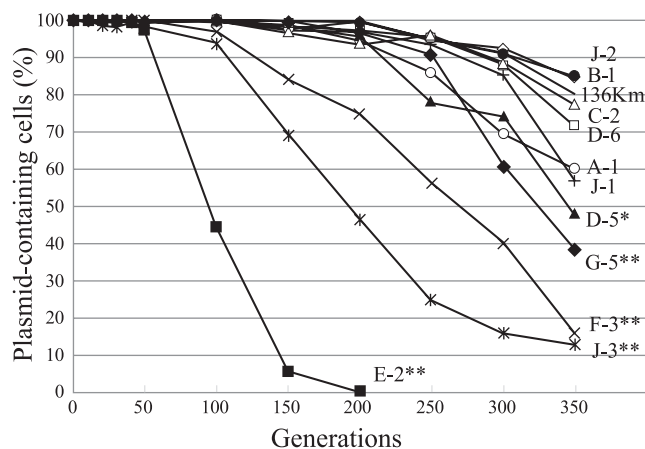


FIG. 2. Stability of pBP136 derivatives with Tn21Km insertions. The detailed insertion sites of Tn21Km in pBP136 are shown in Table 1 and Fig. 1. The percentages of plasmid-containing cells are averages from five independent stability assays. Standard deviations are not shown in this figure for simplicity. Asterisks indicate statistically significant differences ( $P < 0.05$ ) between the means of stabilities as assessed by Dunnett's multiple-comparison test (15): \*, significantly different from the four most stable plasmids, pBP136Km, pBPTn/B-1, pBPTn/C-2, and pBPTn/J-2; \*\*, significantly different from the five most stable plasmids, pBP136Km, pBPTn/B-1, pBPTn/C-2, pBPTn/J-2, and pBPTn/D-6.

that the high concentration of the Tn21Km insertions found in the *oriV* region (67%) cannot be entirely due to experimental bias but must at least in part be due to region specificity of the transposition itself.

**Can selection of specific plasmid variants explain the common IncP-1 plasmid architecture?** There are three major factors that contribute to the long-term persistence of self-transmissible plasmids: (i) a low segregational loss rate, (ii) a small plasmid cost, and (iii) a horizontal plasmid transfer rate that is high enough to compensate for plasmid loss and cost (12, 41). We examined whether impairment of these key characters in some plasmid variants can explain the conserved plasmid architecture. We already showed that most of the pBP136 derivatives with insertions in *tra* and *trb* genes were no longer transferable in subsequent conjugation assays (see "Experimental bias" above). Such transfer-deficient plasmids would thus likely be lost from bacterial communities and not be found in the extant IncP-1 plasmid pool.

To test the effect of the location of transposon insertions on the stability of plasmids, we compared the stability in *E. coli* EC100 of 11 pBPTn plasmids during 350 generations of growth. These plasmids carried the transposon in *kfrA* (possible plasmid maintenance; pBPTn/G-5), *kfrB* (possible plasmid maintenance; pBPTn/A-1), *parA* (plasmid-multimer resolution; pBPTn/D-5), *traC* (DNA primase; pBPTn/F-3), *ORF1* (pBPTn/J-1), between *kfcA* and *ORF1* (pBPTn/E-2), between *kfrA* and *korB* (pBPTn/J-3), between *parA* and *traC* (pBPTn/C-2), and between *oriV* and *trfA* (pBPTn/B-1, pBPTn/D-6, and pBPTn/J-2) (Table 1 and Fig. 1). Of the 11 plasmids tested, those with an insertion between *oriV* and *trfA* (pBPTn/B-1, pBPTn/D-6, and pBPTn/J-2) or between the *tra* and *trb* operons (pBPTn/C-2) were significantly more stable than at least four of the seven plasmids with insertions outside of these regions (Fig. 2). Three of these seven plasmids, pBPTn/E-2,

TABLE 2. Relative fitnesses for pBP136 derivatives carrying Tn2/Km

Plasmid	Insertion site of Tn2/Km or Km <sup>r</sup> determinant	Mean relative fitness of plasmid-carrying to plasmid-free cells (SE, no. of replicates) <sup>a</sup>
pBPTn/A-1	<i>kfrB</i>	0.95235 (0.01365, 10)**
pBPTn/B-1	Between <i>oriV</i> and <i>trfA</i>	0.96315 (0.01316, 10)*
pBPTn/C-2	Between <i>parA</i> and <i>traC</i>	0.99008 (0.01469, 10)
pBPTn/D-5	<i>parA</i>	0.90980 (0.01560, 10)***
pBPTn/D-6	Between <i>oriV</i> and <i>trfA</i>	1.03237 (0.01194, 20)
pBPTn/E-2	Between <i>klaC</i> and <i>ORF1</i>	0.93581 (0.01134, 20)***
pBPTn/F-3	<i>traC</i>	1.00948 (0.01675, 10)
pBPTn/G-5	<i>kfrA</i>	0.95019 (0.01588, 10)***
pBPTn/J-1	<i>ORF1</i>	0.95113 (0.01540, 10)***
pBPTn/J-2	Between <i>oriV</i> and <i>trfA</i>	0.96912 (0.01327, 10)*
pBPTn/J-3	Between <i>kfrA</i> and <i>korB</i>	0.93896 (0.01563, 10)***
pBP136Km	Between <i>traM</i> and <i>kfrC</i>	0.96757 (0.00918, 10)*

<sup>a</sup> The relative fitness was measured by pairwise competition experiments using EC100(pBPTn) and EC100. Asterisks indicate *P* values from Student's *t* test with Bonferroni correction for pairwise comparisons of the relative fitness between pBPTn/D-6 and one of the others: \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001. No asterisk, not significantly different from the value for pBPTn/D-6.

pBPTn/F-3, and pBPTn/J-3, which had an insertion between *klaC* and *ORF1*, in *traC*, and between *kfrA* and *korB*, respectively, were very unstable. These results indicate that pBP136 derivatives with transposons inserted in the two typical regions for IncP-1 plasmids, i.e., *oriV-trfA* and *tra-trb*, are much more likely to persist longer in bacterial communities than plasmid variants with insertions in other regions. This may explain why these other IncP-1 plasmid variants have not been detected so far among natural bacterial populations.

Since the fitness cost that a plasmid confers to its host negatively affects long-term plasmid persistence in the absence of selection for plasmid-encoded traits (6, 7, 10, 29), pBP136 derivatives with the lowest cost would be more likely to persist and thus represent the IncP-1β plasmids we know today. To test whether insertion sites of Tn2/Km in pBP136 affected the plasmid cost, we estimated the relative fitness (*W*) of plasmid-containing versus plasmid-free isogenic *E. coli* EC100 hosts in pairwise competition experiments during 10 generations and attributed the relative decrease in host fitness to the plasmid cost (1 - *W*). Since all pBPTn plasmids used were stably maintained in EC100 within this period (>99.6% on average [Fig. 2]), instability did not confound the plasmid cost data. For 9 of the 12 plasmids tested, the relative fitness of the plasmid-

containing host was 3 to 9% lower than that of plasmid-free cells, indicating a plasmid cost (Table 2). Of the three plasmids with insertions between *oriV* and *trfA*, pBPTn/D-6 was the only one that conferred no cost to its host (it even conferred a potential benefit), whereas the other two variants decreased the host fitness by more than 3%. The cost of plasmid pBPTn/D-6 was also significantly lower than that of plasmids carrying inserts outside the two typical regions (except for the *traC* insertion). Interestingly, one other pBP136 derivative that did not significantly lower the host fitness (*W* = 0.99) carried an insertion in the *tra-trb* region (Table 2). These results indicate that some but not all of the Tn2/Km insertions in the two typical insertion regions of IncP-1 plasmids resulted in a lower plasmid cost than insertions in other regions. Selection for low plasmid cost can thus only partially explain the conserved architecture of currently known IncP-1 plasmids.

**Transposition of Tn2/Km to the *oriV-trfA* region of pUO1.**

To investigate whether the finding that Tn2/Km insertions are concentrated around *oriV* is limited to plasmid pBP136 or is more generally true for IncP-1β plasmids, a similar transposition experiment was performed using pMS0220. This vector is a pUC19-based plasmid that carries the 2-kb potential *oriV-trfA* region from the IncP-1β plasmid pUO1 (44), which shows 92% nucleotide identity to the corresponding sequence of pBP136. *E. coli* S17-1(pMS0220)(pMT1247Km) was mated with DH1Mu, and Km<sup>r</sup> NaI<sup>r</sup> transconjugants were selected. Forty pMS0220::Tn2/Km plasmids from 10 independent experiments were sequenced to identify the insertion sites of the transposon. In 29 plasmids (73%), Tn2/Km was inserted in or near (±100 bp) the 2-kb fragment. Sixteen of these were close to iteron sequences, and 13 were near a truncated *trfA* gene (Fig. 3). As was the case with pBP136, the 20-bp IRs of pUO1 did not seem to be directly involved in acquisition of Tn2/Km (Fig. 3). In the control experiment using the cloning vector pARO190 as the Tn2/Km target, the transposon was inserted randomly into the pUC19-derived portion of the plasmid (Fig. 3). These results together with those for pBP136 suggest that the *oriV-trfA* sequence of IncP-1 plasmids attracts insertions of Tn2/Km.

**DISCUSSION**

One of the most striking features of the more than 18 complete nucleotide sequences of IncP-1 plasmids deposited in

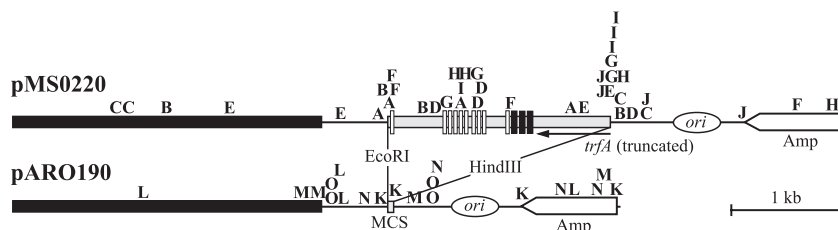


FIG. 3. Transposition of Tn2/Km into plasmids with and without the *oriV-trfA* region of pUO1. The meaning of the letters is the same as described in the legend of Fig. 1. The shaded bar indicates the potential *oriV-trfA* region of pUO1 from which the Tn2-like transposon TnHad2 (43), located in this region, was removed. The horizontal black bar represents the RP4-derived fragment containing its *oriT* sequence in the pUC19-based vector pARO190 (34). The vertical white and black boxes show the iteron sequences and 20-bp IRs, respectively. Plasmid pUO1 has three copies of the 20-bp IRs, whereas pBP136 has two in the corresponding sequence. The replication origin of pARO190 is shown by the oval. Abbreviations: MCS, multicloning site; Amp, gene for resistance to ampicillin.



FIG. 4. Nucleotide sequence between the *parA* and *traC* genes of pBP136. The first nucleotide of the *parA* gene was redefined in this study and thus differs from the previously defined location (26). The numbers shown on the left of the sequence indicate nucleotide positions of pBP136. The insertion sites (inverted triangles) of Tn402/5053-like transposons in various IncP-1 $\beta$  plasmids are shown at homologous locations of the pBP136 sequence. Boxed sequences indicate three resolvase-binding regions (sites I to III) in the putative *res* site of the ParA resolvase of pBP136. Putative  $-10$  and  $-35$  sequences for *parA* are underlined. The sequence in bold and underlined represents the 20-bp IR. Sequence data for pB3, pB4, pB8, pB10, R751, pTP6, and pUO1 are compiled from the DDBJ/EMBL/GenBank databases: pB3, accession no. AJ639924; pB4, AJ431260; pB8; AJ863570; pB10, AJ564903; R751, U67194; pTP6, AM048832; and pUO1, AB063332.

databases today is the location of transposons and/or their remnants in one or both of two specific regions, namely, the regions between *oriV* and *trfA* (*oriV-trfA*) and between the *tra* and *trb* operons (*tra-trb*). Our study provides the first empirical evidence that region-specific insertion of transposons in combination with selection for transferable and stable plasmids explains this conserved architecture. We first showed that after transposition of Tn21Km into plasmids pBP136 and pMS0220, followed by plasmid transfer, a very large proportion of plasmids carried the transposon in the *oriV* region. We then demonstrated that several of the pBP136 plasmid derivatives with Tn21Km insertions outside the two typical regions were either not self-transferable, less stable, or more costly to the host than the plasmids with insertions limited to these two regions. Although our studies employed only IncP-1 $\beta$  plasmids, we believe most of the results can be generalized to all subgroups of IncP-1 plasmids. All IncP-1 plasmids have the same conserved architecture, and they differ only in terms of the accessory genes they carry, sequence divergence and presence or absence of particular backbone genes, and presence of the 20-bp IRs.

In the past, three hypotheses have been formulated that may explain the conserved IncP-1 plasmid architecture. One possible explanation for the region-specific insertions into IncP-1 $\beta$  plasmids is the involvement of the 20-bp IRs present in these two regions in the acquisition of transposons (24, 52). A proposed role of the IRs is to provide cleavage of DNA somewhere within the 20-bp sequence or at their flanking restriction sites, which may facilitate insertion of other DNA fragments (52). At present, we have no idea whether such cleavage took place in pBP136 before transposition. However, our experiments indicated that none of the six 20-bp IRs were directly involved in the acquisition of transposons. This is consistent with the fact that only the  $\beta$  subgroup of IncP-1 plasmids has the 20-bp IRs, while the plasmid architecture is also conserved within the other IncP-1 subgroups. A second hypothesis was the preferential insertion of class II transposons in AT-rich regions. Although class II transposons are known to transpose into AT-rich sequences (38), our data showed that there was no relationship between the insertion sites of Tn21Km and the G+C content of the pBP136 sequence (Fig. 1). Conforma-

tional change of plasmids is a third possible factor that may allow transposons to insert in specific regions. For example, another type of transposon, Tn7, inserted near the *oriT* region of the IncF plasmid pOX-G during conjugation (60). However, conformational change is probably not the case in our study, since Tn21Km transposed near the *oriV* sequence regardless of its activity (Fig. 3). Given that a different factor(s) must attract Tn21-like transposons into this region, we propose that the unique secondary structure of the *oriV-trfA* region, resulting from repeated iteron sequences, may be very important for the target specificity of Tn21-like transposons (Fig. 3). This idea is consistent with previous reports that that DNA secondary structure plays an important role in target site selection for transposons (11, 19, 60).

To allow transposon insertion in a limited number of specific regions might be beneficial for promiscuous and frequently transferable plasmids to persist in bacterial communities, because these plasmids may often be targeted by various transposons. The currently known *oriV-rep* (replication protein gene) regions may thus have evolved as "attractants" for transposons. Although the *oriV-trfA* region is vital for vegetative replication, it is also the most flexible region of the IncP-1 plasmids in that (i) it has a much longer noncoding sequence (ca.1.5 kb) than other open reading frame-intervening sequences and (ii) it is independent from operons encoding plasmid maintenance and transfer and their tight regulatory circuits. This "attractant" hypothesis is supported by the fact that in many promiscuous plasmids of the IncP-1, IncQ, IncW, and IncN groups (but not those of the IncP-7 and IncP-9 groups, which have a less wide host range and lower transfer frequencies), the *oriV* sequence is found downstream of the *rep* gene (17, 35, 39, 45). This enables these plasmids to normally express the Rep proteins even if transposons are inserted near *oriV*. In the case of the IncP-1 group, these ideas are consistent with the complicated structures of accessory fragments in these regions, which show many past DNA rearrangements caused by various transposons and are in stark contrast with the strongly conserved backbones.

Since several Tn21Km insertions in pBP136 were also found outside the two typical regions and since others may have

escaped our detection system, we postulate that the absence of similar plasmid constructs in the currently known IncP-1 plasmid pool is due to impairment of key plasmid characteristics, such as stability, fitness cost, and self-transferability. First, immobilization of a plasmid by insertion of transposons into an essential transfer gene, as shown for several pBP136 variants, gives a direct competitive disadvantage in bacterial communities, since conjugative transfer is thought to contribute to plasmid persistence (41). Second, plasmids that are unstable in the absence of selection would have a disadvantage to persist in bacterial communities. As we have shown, pBP136 derivatives with Tn21/Km insertions in the central control region (pBPTn/E-2 and pBPTn/J-3) were much less stable than those with insertions in other positions (Fig. 2), even though they did not disrupt any genes. The low stability of these two plasmids might be due to the disturbance of the tight regulation of the plasmids (28, 49, 61). In fact, in pBPTn/J-3, Tn21/Km was inserted in one of the binding sites for the regulation protein KorB (insertion site and consensus sequence, TTTA ↓ GCGG CTA AA). This may inhibit transcription of the *kfr* operon downstream, which was recently shown to be involved in plasmid stability (1). This finding is also compatible with our previous observation that IncP-1β plasmids have transposons in regions where they do not disrupt transcription of genes and operons (44). Finally, our data also showed to some extent how minimizing the plasmid cost by acquisition of a transposon at a specific site can be the third mechanism that allows the IncP-1 plasmids with the typical structure to persist longer than other cognate plasmids and thus accumulate in the currently known pool of structurally similar plasmids (Table 2).

Although most IncP-1 plasmids, regardless of their subgroups, have a second "hot spot" for insertion of transposons in the *tra-trb* region (2, 44, 52), we detected only a few Tn21/Km insertions in this region of pBP136 (Fig. 1). We have previously reported that a different type of transposons, the Tn402/5053 group, are predominantly located in this region of IncP-1β plasmids (44). Moreover, the resolution (*res*) sites of resolvases have been shown to be a specific target for these transposons (27, 31), and many IncP-1 plasmids have a resolvase gene, *parA*, between the *tra* and *trb* operons (4, 23, 24, 26, 33, 37, 42, 46, 48). The reports for RP4 ParA and other resolvases (16, 22, 32) helped us to identify the putative *res* site for the ParA protein of pBP136 (Fig. 4). This identification subsequently clarified the insertion sites of Tn402/5053-like transposons in IncP-1β plasmids in or around the putative *res* site (Fig. 4). Such Tn402/5053-like insertions are also found in the *res* site for the ParA resolvase of the IncP-1α plasmid pTB11 (48) and upstream of the resolvase genes of the IncP-1γ plasmid pQKH54 (21) and the recently described IncP-1 plasmid pKJK5 (4). These observations suggest that the specific insertion of the Tn402/5053-like transposons in the *tra-trb* region of IncP-1 plasmids is most likely due to the presence of the *res* site and ParA resolvase. This idea is consistent with the reports that (i) the target specificity of Tn402 (=Tn5090) to the *res* site of the IncP-1α plasmid RP1 (=RP4) depends on the presence of both ParA and the *res* site (27) and (ii) the IncN plasmid R46 carries a Tn402-like insertion just upstream of the *resP* resolvase gene (AY046276). Since several IncP-1 plasmids have transposons that are not related to Tn402/5053-like transposons in the *tra-trb* regions (30, 54), the *res* site for the *parA*

gene might form a unique DNA structure that also attracts other transposons. This hypothesis is compatible with the fact that other *parA* homologue-carrying plasmids, pK245 (accession no. DQ449578) and pSC138 (9), also have transposons just upstream of their *parA* genes (data not shown).

It should be noted that IncP-1β plasmids have one more region where a transposon has been found, i.e., between *traM* and *kfrC*. Plasmid pB4 is at present the only example carrying an insertion, Tn5393c, in this region (44, 46). Although the long-term stability of pB4 has not yet been investigated, our stability experiment using pBP136Km, which has a Km<sup>r</sup> determinant between *traM* and *kfrC*, demonstrated that this plasmid was as stable as the plasmids with transposons in the *oriV-trfA* and the *tra-trb* regions (Fig. 2).

We conclude that the conserved architecture of IncP-1 plasmids can be attributed to region-specific insertion of transposons, especially those of the Tn21 and Tn402/5053 groups, in combination with selection for plasmids that are most stable and transferable and least costly. More comparative genome analyses of IncP-1 plasmids will further improve our insight into the structural similarity and evolutionary history of these plasmids.

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