Mode of Replicon Fusion Mediated by the Duplicated Insertion Sequence IS*21* in *Escherichia coli*

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ABSTRACT

The insertion sequence IS*21* (2.1 kb) originating from the broad-host-range IncP plasmid R68 transposes infrequently; by contrast, the IS*21* tandem repeat found on the derivative R68.45 is highly active in transpositional mobilization of other replicons in a variety of Gram-negative bacteria. The mobilized plasmids are joined to R68.45 by single IS*21* copies in direct orientation.—The formation of IS*21* tandem duplications was observed in cointegrates between R68.45 and pBR325::IS*21* and also in an RP1::IS*21* plasmid derivative in which a segment located between two directly repeated copies of IS*21* was deleted spontaneously. We speculate that IS*21* tandem repeats can arise when the termini of two IS*21* elements are specifically joined in a transposition or deletion event.—A resistance gene flanked by two IS*21* elements in direct orientation did not behave as a transposon. The Ω fragment carrying transcription and translation stop signals was inserted into various sites of the IS*21* tandem repeat; in this way it could be shown that the left IS*21* element (which is next to the kanamycin resistance gene in R68.45) was 100 times more active in cointegrate formation than was the right-hand element.—Cointegrates between the conjugative plasmid R751 and pBR325 derivatives carrying IS*21* and IS*21*::Ω in tandem contained a single IS*21* at one replicon junction and a single IS*21*::Ω at the other. In the IS*21* duplications the inner IS*21* ends were preferentially recognized (presumably by IS*21* transposase), whereas the outer termini were not required for cointegrate formation. Based on these findings a conservative (simple) pathway of transposition is proposed for R68.45 and other plasmids with an IS*21* tandem repeat. In this model R68.45 is pictured as a large transposon whose ends are joined together to form a circular molecule which is capable of autonomous replication.

THE prokaryotic 2.1-kb insertion sequence IS*21* occurs naturally in the broad-host-range IncP plasmid R68. This plasmid was first isolated from *Pseudomonas aeruginosa* and is very closely related to the IncP plasmids RP1, RP4 and RK2 (HOLLOWAY and RICHMOND 1973; BURKARDT, RIESS and PÜHLER 1979). IS*21* has the unusual ability to form tandem repeats. An R68 derivative carrying such an IS*21* duplication, R68.45, forms cointegrates with other replicons at frequencies of 10^{-3} to 10^{-5} in *Escherichia coli* (WILLETTS, CROWTHER and HOLLOWAY 1981; RIESS, MASEPOHL and PÜHLER 1983) and efficiently mobilizes the chromosome of many different Gram-negative bacteria (HAAS and HOLLOWAY 1976; HOLLOWAY 1983). In a cointegrate of R68.45 with another plasmid, the two replicons are joined together by single copies of IS*21* in direct orientation (RIESS, MASEPOHL and PÜHLER 1983).

R68 and RP4 form cointegrates with other replicons via IS*21* transposition at low frequencies; a single IS*21* element transposes rarely (DEPICKER *et al.* 1980; WILLETTS, CROWTHER and HOLLOWAY 1981).[1] While it is clear that the enhanced chromosome mobilizing ability of R68.45 is due to the IS*21* tandem repeat (WILLETTS, CROWTHER and HOLLOWAY 1981; CURRIER and MORGAN 1982; HAAS and RIESS 1983), there is little information on how IS*21* tandem duplications arise and how the duplicated IS*21* element achieves cointegrate formation. The fact that R68.45 readily forms cointegrates does not imply that IS*21* itself preferentially transposes via a replicative pathway, which is often termed "cointegrate" pathway. As we show here by an analysis of IS*21*-promoted replicon fusions, cointegrate formation is explained best by a conservative pathway. According to this model the outer IS*21* termini of the duplicated element should be dispensable for cointegrate formation. Our experiments support this view.

MATERIALS AND METHODS

Media, culture conditions, and techniques for DNA isolation, restriction, ligation and transformation have been described (HAAS and HOLLOWAY 1976; ITOH *et al.* 1984; REIMMANN and HAAS 1986; JEENES *et al.* 1986). The *E. coli* strains used were HB101 (*proA2 leu-6 thi-1 hsdS20 recA13 rpsL20 lacY1 galK2 ara-14 xyl-5 mtl-1 supE44*), ED8767 (*metB hsdS recA56 supE supF* [MURRAY, BRAMMAR and MURRAY 1977]), and S17-1 (*pro thi hsdR recA*, chro-

[1] The insertion sequences IS*8* of RP4 (DEPICKER *et al.* 1980) and IS*21* of R68 are largely homologous and perhaps identical. In this paper we use the designation IS*21* for the homologous insertion sequences of R68, RP1 and RP4.

mosomal insertion of RP4.2 [SIMON, PRIEFER and PÜHLER 1983]). The following plasmids have been described previously: R68 and R68.45 (HAAS and HOLLOWAY 1976; HAAS and RIESS 1983), R751 (HIRSCH and BERINGER 1984), pUB307 (BENNETT, GRINSTED and RICHMOND 1977), pBR325 (PRENTKI *et al.* 1981) and pME14 (REIMMANN and HAAS 1986). The other pME plasmids constructed in this study are shown in Figures 1–3. The pBR325::IS*21* derivatives pME16 and pME23 resulted from the resolution of cointegrates between pBR325 and a temperature-sensitive derivative of R68.45 (RELLA 1984) in a *recA*$^+$ *E. coli* background. The temperature-sensitive plasmid was eliminated by growth at 43°.

Assay of cointegrate formation: *E. coli* ED8767(R751) was transformed with the pBR325 derivatives containing one or two IS*21* elements (shown in Figures 1–3). Transformants were purified once on selective medium (nutrient agar + 50 μg trimethoprim/ml + 50 μg chloramphenicol/ml + 100 μg ampicillin/ml), grown to exponential phase from single colonies, diluted appropriately and mated with strain HB101. Selection was then made for transfer of R751 alone (on nutrient agar + 200 μg streptomycin/ml + 100 μg trimethoprim/ml) and for transfer of cointegrates between R751 and the pBR325 derivative (on nutrient agar + 200 μg streptomycin/ml + 50 μg chloramphenicol/ml + 100 μg ampicillin/ml). The frequency of cointegrate formation was calculated as the ratio of cointegrate transfer per R751 transfer; the data of three cultures were averaged. Cointegrate plasmids were isolated by the method of HOLMES and QUIGLEY (1981) followed by phenol-chloroform extraction and isopropanol precipitation. Cointegrate structures were analyzed by appropriate restriction digests, *e.g.*, with *Sma*I or *Hpa*I, and *Hin*dIII; published restriction maps of R751 (54 kb; HIRSCH and BERINGER 1984), pBR325 (6.0 kb; PRENTKI *et al.* 1981) and IS*21* (2.1 kb; WILLETTS, CROWTHER and HOLLOWAY 1981) were used. IS*21* tandem repeats were recognized by typical 2.1-kb bands after digestion with *Sma*I, *Hpa*I, or *Pvu*II (these enzymes each cut IS*21* once). Within the precision of restriction mapping, the DNA between the duplicated IS*21* elements in R68.45, all derivatives of R68.45, and pME29 was <50 bp.

RESULTS AND DISCUSSION

Structures and frequencies of IS*21*-fused plasmids: Transposition of IS*21* was assayed in recombination-deficient (*recA*) *E. coli* strains by measuring the frequency of cointegrates formed between the conjugative IncP plasmid R751 and pBR325 derivatives carrying one or two IS*21* copies (Figure 1). Selection was made for the transfer of the pBR325 markers (resistances to ampicillin and chloramphenicol). The vector pBR325 alone was not mobilizable at detectable frequencies (*i.e.*, $\leq 10^{-7}$) by R751. When IS*21* was present on pBR325, mobilization occurred and was entirely due to IS*21*-promoted cointegrate formation. By a series of restriction digests, we first verified the cointegrate structures of R751 with pME325, a pBR325 derivative which contained the 6.6-kb *Hin*dIII/*Sal*I fragment of R68.45 carrying the IS*21* tandem duplication (*cf.* Figure 2). As predicted by the previous work of RIESS, MASEPOHL AND PÜHLER (1983), the two plasmids were linked together by single IS*21* copies in direct orientation (Figure 1). Cointegrate formation was frequent (*ca.* 1×10^{-3}) and pME325 was found inserted into many different sites of R751 (data not shown). Sometimes two pME325 molecules were integrated into R751.

Are both IS*21* elements required for and equally active in transposition? To test this, the Ω fragment, an artificial element consisting of transcription and translation stop signals and a selectable streptomycin/spectinomycin resistance marker (PRENTKI and KRISCH 1984), was inserted into the duplicated IS*21* element at four different positions giving plasmids pME41-pME44 (Figure 1). Insertion of Ω into the right-hand IS*21* element reduced the frequency of cointegrate formation less than tenfold, whereas Ω insertion into the left IS*21* copy had a much more severe effect (more than 100-fold reduction; Figure 1). It thus appears that the transposition functions of both IS*21* elements contribute to cointegrate formation but the left IS*21* element is much more active and sufficient for high frequency cointegration. SCHURTER and HOLLOWAY (1986) have shown that a promoter located at the left end of IS*21* reads outward. These authors have argued that in $(IS21)_2$ the promoter of the right-hand element may activate the transpositional activity of the left element. Our data are in agreement with this hypothesis but do not prove it.

In the cointegrates of R751 with pME41-pME44 the two replicons were always fused by a single IS*21* copy at one junction and a single IS*21*::Ω element at the other junction (Figure 1). Three independent cointegrates of each plasmid pair were analyzed. The cointegrate structures are consistent with a conservative (simple) pathway of IS*21*-mediated insertion. When strains carrying R751 and one of the Ω containing plasmids (pME41-pME44) were used as donors and the conjugative transfer of the Ω element was selected (on media with spectinomycin at 50 μg/ml), 100% of the transconjugants were also resistant to ampicillin and chloramphenicol. (The following numbers of transconjugants were tested: 4 with pME41, 100 with pME42, 20 with pME43, 100 with pME44.) We conclude that simple transposition of IS*21*::Ω into R751 did not occur and that plasmid cointegrates were always formed.

A single IS*21* element (*e.g.*, on pME36) also led to cointegrate formation albeit at a very low frequency (Figure 1). Like other IS elements (GRINDLEY and REED 1985), IS*21* might occasionally use a replicative pathway of insertion. Alternatively, the single IS*21* copy of pME36 might form a tandem repeat spontaneously and the duplicated IS*21* might be responsible for cointegrate formation. The question of how IS*21* tandem repeats may arise is discussed below.

Do resistance markers flanked by single IS*21* copies in direct orientation behave as transposons? In

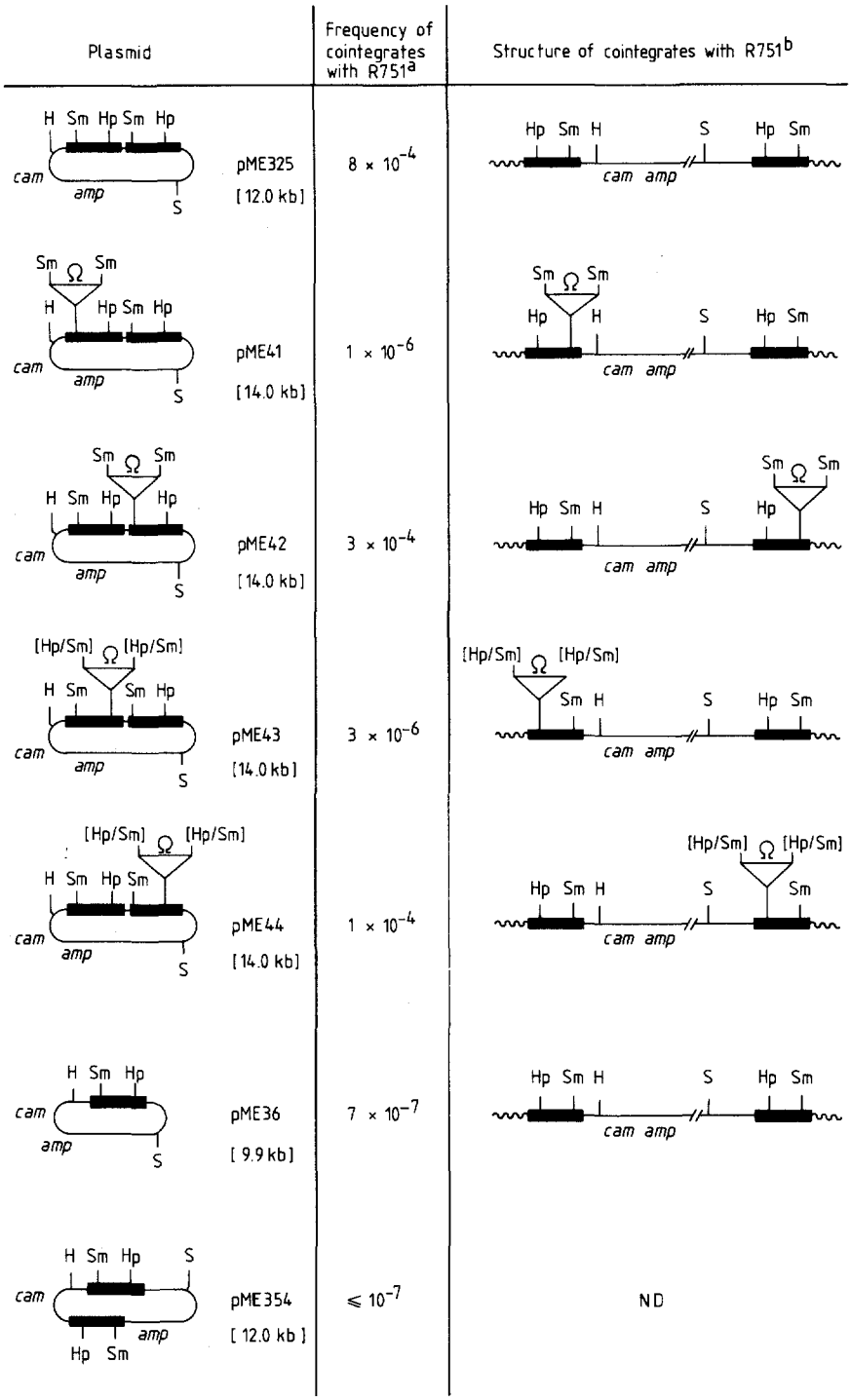

FIGURE 1. Structures and frequencies of cointegrates between R751 and pBR325 derivatives carrying IS*21*. IS*21* elements are depicted by ■ and R751 sequences by ∿. The 6.6-kb *Hin*dIII/*Sal*I fragment of R68.45 (Figure 2A) was cloned into pBR325 giving pME325. Partial digestion of pME325 with *Sma*I or *Hpa*I followed by ligation in the presence of *Sma*I-cut Ω fragment produced pME41-pME44. The 4.5-kb *Hin*dIII/*Sal*I fragment of R68 was inserted into pBR325 and into pME23 (= pBR325::IS*21* at 4.3 kb); the resulting recombinants were pME36 and pME354, respectively. Abbreviations for restriction sites are: H, *Hin*dIII; Sm, *Sma*I; Hp, *Hpa*I; S, *Sal*I; for resistance markers: *cam*, chloramphenicol; *amp*, ampicillin. The 2.0-kb Ω element (PRENTKI and KRISCH 1984) carries a streptomycin/spectinomycin determinant and a *Hin*dIII site at each terminus (not shown). (ND = not determined.) [a] The frequency of cointegrates is calculated as the frequency of *amp* and *cam* transfer per frequency of R751 (trimethoprim resistance) transfer. R751 transfer was 1×10^{-3} per donor. [b] At least three cointegrates from each cross were examined by diagnostic cuts with *Sma*I, *Hin*dIII, and, in some cases, *Hpa*I.

pME354 (Figure 1) the chloramphenicol and ampicillin resistance genes situated between IS*21* elements transposed at frequencies of $\leq 10^{-7}$. Thus, the juxtaposition of two IS*21* elements seems to be important for high transposition frequency.

Formation of IS*21* tandem repeats: The mechanism by which IS*21* has duplicated in R68 to give R68.45 is not known. However, we observed a striking tendency of IS*21* elements to form tandem repeats under different experimental conditions. When R68.45 mobilized pME16 (= pBR325::IS*21*) in *recA* *E. coli* strains, the two plasmids were found to be joined via IS*21* ends resulting in two types of cointegrates (Figure 2A). The frequency of mobilization was 10^{-4}. Of ten independent cointegrates analyzed, four had configuration *a* and six had configuration *b*. All were stable in strain HB101 and all carried the three IS*21* elements in the same orientation. In a simplistic view cointegrates *a* or *b* could arise from specific double strand cuts at site © on R68.45 and at sites ⓐ or ⓑ on pME16, respectively (Figure 2A). We assume that the IS*21*-encoded transposase is responsible for

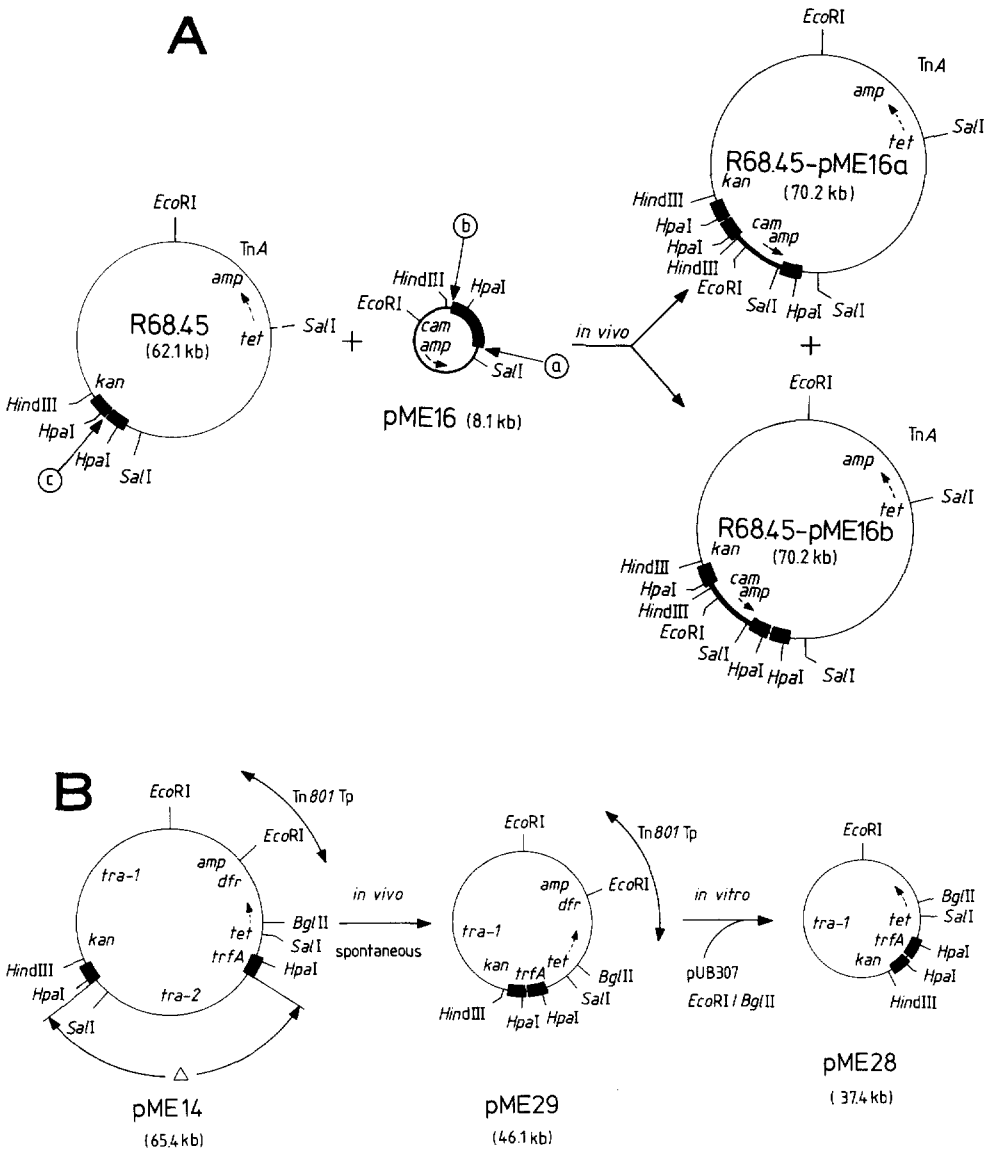

FIGURE 2. Formation of IS*21* tandem repeats. *A*, Mobilization of pME16 (= pBR325::IS*21* at 0.2 kb) by R68.45 gave cointegrate types R68.45-pME16a and R68.45-pME16b. The arrows ⓐ, ⓑ, and ⓒ indicate the sites where IS*21*-transposase is thought to introduce double strand breaks (according to the cut-and-paste model; see text). *B*, The spontaneous deletion (Δ) in pME14 was formed *in vivo*. The 16.4-kb *Eco*RI/*Bgl*II fragment carrying Tn*801*Tp was replaced *in vitro* by the homologous 7.7-kb *Eco*RI/*Bgl*II fragment from pUB307 without Tn*801*. Two *Hin*dIII and two *Sal*I sites in Tn*801*Tp have been omitted. Abbreviations are the same as in Figure 1, with the addition of: *tet*, tetracycline resistance; *kan*, kanamycin resistance; *dfr*, trimethoprim resistance; *trfA*, *trans*-acting replication control; *tra-1*, *tra-2*, transfer gene regions. The direction of plasmid replication is indicated by – – →. The distance between the tandemly repeated IS*21* elements in the trans-position products was <50 bp.

these cuts and the subsequent "paste" process but this hypothesis was not tested experimentally; no functional analysis of IS*21*-encoded proteins is currently available.

An alternative hypothesis can be put forward. The R68.45-pME16 cointegrates might arise by a recombinational exchange between one IS*21* element on R68.45 and the IS*21* element on pME16, even in a Rec$^-$ background. It has been shown that intermolecular recombination between two IS*5* elements occurs in *recA* strains at considerable frequencies (*ca.* 10^{-5}; TIMMONS, LIEB and DEONIER 1986). If transposition were more important than recombination in the generation of R68.45-pME16 cointegrates, then one might expect that R68 should mobilize pME16 at a lower frequency than did R68.45. A control experiment showed, however, that pME16 was mobilized by R68 at high frequency (*ca.* 10^{-4}) and, therefore, there is no *prima facie* evidence against recombinational exchanges being involved in the formation of R68.45-pME16 cointegrates (Figure 2A). A more detailed interpretation was complicated by the fact that mobilization of pME16 by R68 did not produce the expected simple replicon fusions with one IS*21* element at each junction but instead gave a high percentage (50%) of plasmids with IS*21* tandem repeats and these cannot be explained by recombinational exchanges.

Further mobilization experiments were done with R68.45 and three other pBR325::IS*21* derivatives that carried IS*21* insertions at various sites and in either orientation. In all cases cointegrates were formed which contained an IS*21* tandem repeat at one junction and a single IS*21* element at the other junction. The cointegrates between R68.45 and one pBR325::IS*21* derivative, pME36 (Figure 1), were structurally unstable, for unknown reasons. Some of these cointegrates were seen to decay into a large plasmid (≈R68) and a small derivative of pME36

carrying a tandem repeat of IS*21* (data not shown). Similar observations were recently made by DANILEVICH and KOSTYUCHENKO (1985) who studied the interaction of pRP19.6 (an RP1 derivative carrying an IS*21* duplication) with pBR325::IS*21*. These authors also observed the preferential formation of pBR325 derivatives with tandem repeats of IS*21*.

Another instance of spontaneous formation of an IS*21* tandem repeat was found in the RP1 derivative pME14 (Figure 2B). This plasmid is replication-deficient because of an IS*21* insertion in the RP1 replication control gene *trfA* but capable of autonomous replication in *E. coli* strain S17-1 which carries a *trfA*$^+$ gene in the chromosome (REIMMANN and HAAS 1986). The IS*21* elements on pME14 are present in the same orientation. In one of 50 cultures of strain S17-1-(pME14) a spontaneous deletion between the two IS*21* copies of pME14 eliminated the tra-2 region and gave pME29, a plasmid with a tandem repeat of IS*21* (Figure 2B). A derivative of pME29 without transposon Tn*801*Tp (REIMMANN and HAAS 1986) was then constructed. The resulting plasmid pME28 (Figure 2B) was transfer-deficient (Δtra-2), replication-deficient (but complemented by *trfA*$^+$ *in trans*) and yielded IS*21*-dependent cointegrates analogous to those formed with R68.45. Transposition of pME28 into target replicons could proceed in the absence of vegetative pME28 replication, *i.e.*, without complementation by *trfA*$^+$ (data not shown).

Taken together, these results suggest that plasmids with $(IS21)_2$ have a tendency to transpose into sites at or very near (<50 bp) the termini of IS*21* on another replicon and in this respect formally resemble Tn*802*. This transposon, which is probably identical to Tn*1* and Tn*801*, preferentially inserts near the termini of Tn*1*-like elements (GRINSTED *et al.* 1978). We also speculate that IS*21* transposase is involved in the generation of IS*21* tandem repeats (*e.g.*, those shown in Figure 2) but this point requires further experiments.

IS*21* termini required for transposition of $(IS21)_2$: The IS*21* nucleotide sequence (2132 bp) shows terminal 11 bp inverted repeats with one mismatch (N. WILLETTS, personal communication). If the model of conservative $(IS21)_2$ transposition is correct, the IS*21* transposase can be expected to recognize preferentially the IS*21* termini at the junction of the duplicated element (site ⓒ in Figure 2A) and the outer IS*21* termini should be dispensable for cointegrate formation. This hypothesis was tested with the IS*21* duplication originating from pME28 (Figure 3). Most of the right-hand IS*21* of pME35 (= pBR325::$(IS21)_2$) could be deleted without effect on cointegrate formation; the 0.38-kb fragment containing the left end of the right-hand IS*21* in pME45 was sufficient for transposition (Figure 3). When 55 ± 10 bp of the left terminus of the left IS*21* were also deleted as in pME49, cointegrate formation was still efficient (Figure 3). Larger deletions in the left IS*21* abolished transposition (data not shown). These results confirm that only the inner IS*21* termini are needed for cointegrate formation.

CONCLUSIONS

There is growing evidence that some prokaryotic insertion sequences such as IS*10* and IS*50* can transpose by a nonreplicative "cut-and-paste" mechanism (BERG 1977; GRINDLEY and REED 1985; BENDER and KLECKNER 1986). Although the interaction of R68.45 and related plasmids with other replicons produces cointegrates, plasmids with a duplicated IS*21* element appear to transpose via a conservative pathway. The main evidence comes from the structures of the cointegrates between R751 and the pME325::Ω plasmids (Figure 1). A replicative mode of $(IS21)_2$ transposition would probably result in cointegrates having two IS*21* copies at one or both replicon junctions. It is possible that such structures were produced transiently but lost subsequently because of instability. We do not think that this event is likely since in different experiments—with pBR325::IS*21* and R68.45 as interacting plasmids—we were able to recover cointegrates carrying two IS*21* copies at one plasmid fusion site and one IS*21* element at the other site (Figure 2A).

If we adopt the cut-and-paste model, which are the preferred cleavage sites of IS*21* transposase? In the donor replicon the outer IS*21* termini are not required for cointegrate formation and the recognition preferentially takes place at the junction of the duplicated IS*21* element (Figure 3). The apparent cut ⓒ at the IS*21* junction (Figure 2A) may in fact consist of two double strand cuts within <50 bp. Nucleotide sequencing data should clarify this point. Since the junction in $(IS21)_2$ seems to be a highly favored site for IS*21* transposase, this may explain why R68.45 is such a good transposon in many Gram-negative bacteria. In a target replicon not carrying IS*21*, the IS*21* transposase seems to act on (almost) random sites, as shown by the analysis of R68.45-pACYC184 cointegrates (RIESS, MASEPOHL and PÜHLER 1983). When the target replicon contains an IS*21* element the cut seems to be made preferentially at either end of IS*21* (cuts ⓐ and ⓑ in Figure 2A) and the IS*21* elements are joined in head-to-tail configuration. We would like to point out that plasmids with $(IS21)_2$ could in some cases also insert into target replicons at sites far from the IS*21* termini (our unpublished data). Simple IS*21* insertions may also occur. An example is pME14, an RP1 derivative which carries two IS*21* copies in the same orientation but not adjacent to each other. Plasmid pME14 probably arose by insertion of a single IS*21* copy into the plasmid's *trfA* locus while the

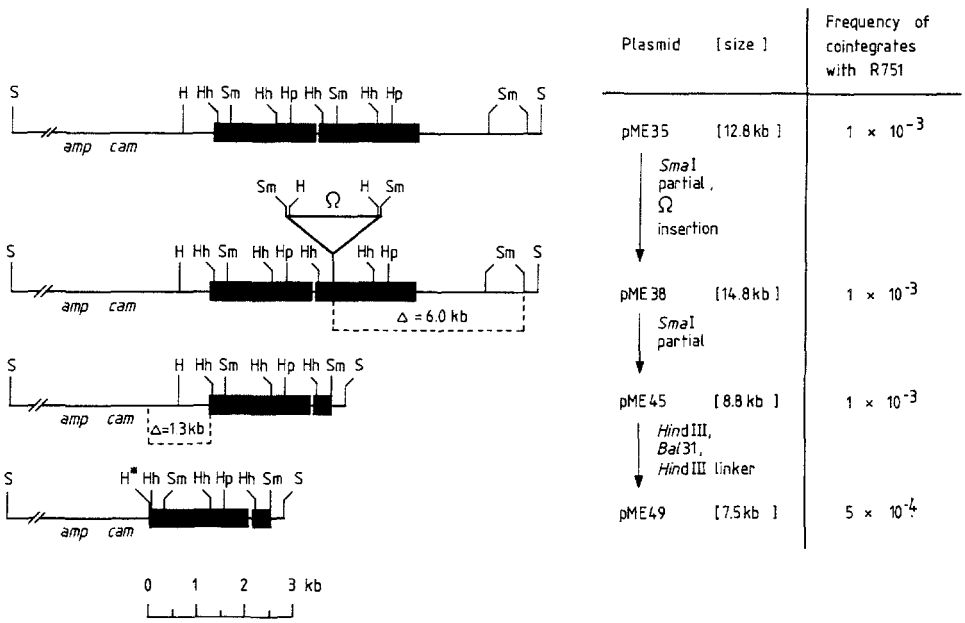

FIGURE 3.—Effects of deletions of the outer termini in (IS*21*)$_2$. The (IS*21*)$_2$ of pME28 was cloned on a 7.4-kb *Hin*dIII/*Sal*I fragment into pBR325 resulting in pME35. For the construction of pME49, pME45 was cleaved with *Hin*dIII, digested progressively with nuclease Bal31 under the conditions specified by the supplier (Boehringer), extracted with phenol-chloroform (1:1), dephosphorylated with alkaline phosphatase (Boehringer), extracted again with phenol-chloroform, and ligated in the presence of a fivefold excess of phosphorylated *Hin*dIII linker (Pharmacia P-L Biochemicals) in ligation buffer containing 15% (w/v) polyethylene-glycol (PHEIFFER and ZIMMERMAN 1983). Linearized plasmid maps are given with IS*21* sequences as ■. Abbreviations are the same as in Figure 1, with the following additions: H*, *Hin*dIII linker, Hh, *Hph*I. Only the *Hph*I sites in IS*21* are shown, those in the vector are omitted.

plasmid was integrated in the *P. aeruginosa* chromosome (REIMMANN and HAAS 1986).

R68.45 may be regarded as a large transposon whose ends are joined together to form a circular molecule. It is interesting to note that circular intermediates in transposition have been postulated by several authors. IYOBE, KATO and MITSUHASHI (1982) have found that the Pseudomonas transposon Tn*2001* forms minicircular DNA which cannot replicate but readily transposes into the bacterial chromosome. MORISATO and KLECKNER (1984) have demonstrated the presence of circularized Tn*10* molecules in a transposase-overproducing strain. ISBERG and SYVANEN (1985) have proposed a pathway of Tn*5* transposition which involves excision and circularization of the transposon before transposition. Seen in this context, R68.45 would not need to form a circular transposition intermediate—after the initial transposase cut(s) R68.45 itself is the intermediate.

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