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Transposition of Tn*904* Encoding Streptomycin Resistance into the Octopine Ti Plasmid of *Agrobacterium tumefaciens*

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A transfer-deficient derivative of plasmid RP1-pMG1 was isolated after insertion of Mu *cts*62. The Tra⁻ R plasmid was used to donate Tn*904*, encoding streptomycin resistance, to Ti plasmid pAL102 harbored by *Agrobacterium tumefaciens* Ach5. Under conditions promoting high Ti transfer frequencies, 155 strains were isolated in which the streptomycin marker coupled with the Ti plasmid in further transfer experiments. These isolates represent stable insertions of Tn*904* into the Ti plasmid. In addition, 19 strains were isolated in which the insertion of Tn*904* was apparently unstable. The frequency of stable Tn*904* transpositions was estimated to be 3×10^{-4} per transferred Ti plasmid. Evidence was obtained that Tn*904* readily may transpose from the Ti plasmid into the bacterial chromosome. The strains carrying Ti plasmids with stable insertions were characterized with respect to virulence, octopine degradation, octopine synthesis in induced tumors, and Ti plasmid transfer. Thirteen of the strains were found to be affected in tumor-inducing ability.

Plant tumors induced by *Agrobacterium tumefaciens* are a disease that interests molecular biologists, as it provides an opportunity to study regulatory processes in the plant cell. It has recently been shown that the development of plant tumors is accompanied by the transfer of *A. tumefaciens* DNA to the plant cell, where it is stably inherited and transcribed (5, 8; A. M. Ledeboer, Ph.D. thesis, University of Leiden, Leiden, The Netherlands, 1978). The transferred DNA consists of a particular piece of the Ti plasmid. Ti plasmids are carried by all virulent *A. tumefaciens* strains (38). It has already been established that the octopine-type Ti plasmids confer upon their bacterial hosts the ability to degrade octopine (4, 28), to induce tumors (4, 34, 37), to induce a tumor-specific octopine-synthesizing enzyme (4, 28), and to exclude bacteriophage AP1 (32). When octopine is added to the medium, Ti plasmids behave as self-transmissible plasmids (9, 13, 16). It has been demonstrated that octopine degradation and Ti transfer are negatively controlled by a common repressor gene (19, 20, 31). These functions have been positioned on a gross map of the octopine Ti plasmid (25). The identification and localization of more markers is desirable. Transposons can be employed for this purpose (24).

(i) They may induce new mutations on the Ti plasmid affecting functions that are not manipulatable with standard microbiological techniques. This is, e.g., important for the isolation of avirulent mutants and of mutants that induce altered tumors.

(ii) Transposons can be localized by using restriction endonuclease fragmentation of plasmid DNA and subsequent agarose gel electrophoresis.

(iii) Once inserted, transposons may induce deletions in adjacent DNA. This property facilitates plasmid mapping.

(iv) Insertions in the piece of Ti DNA that is transferred into the plant cell provide the opportunity to study the expression of the inserted procaryotic DNA in a eucaryotic cell.

Hernalsteens and co-workers (12) have studied the insertion of Tn*7* in RP4::Ti cointegrate plasmids and have isolated several mutants. In the present study, we employed a transfer-deficient plasmid mutant as the donor for the streptomycin transposon Tn*904* that is present in the R plasmid RP1-pMG1 (R. H. Olsen, personal communication). As the Ti plasmid to be studied, we chose the derepressed plasmid mutant pAL102 derived from the octopine Ti plasmid harbored by wild-type *A. tumefaciens* strain Ach5. This plasmid renders its host constitutive for octopine degradation and Ti transfer (19). It was also selected for study because bacteria harboring it have been found to be more efficient in the recently developed in vitro transformation of cell wall-regenerating *Nicotiana tabacum* protoplasts (27).

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As a result of our study several mutant Ti plasmids were isolated that exhibited a decrease or loss of virulence. The corresponding mutations were probably caused by the insertion of transposon Tn*904*.

MATERIALS AND METHODS

Bacterial strains are listed in Tables 1 and 2. All incubations with *A. tumefaciens* strains were at 29°C. Incubations with *Escherichia coli* were as indicated.

Media. SM medium has been described previously (17). LC medium (17) was employed as a rich medium. For *E. coli* strains, the medium of Vogel and Bonner (36) was used.

Isolation of Mu *cts*62 insertions in RP1-pMG1. A phage stock of Mu *cts*62 was prepared after thermal induction at 43°C of *E. coli* strain KMBL1614. Strain AB1932-1 (carrying RP1-pMG1) growing logarithmically at 32°C in LC enriched with thymine (20 mg per liter), 2.5 mM $MgSO_4$, and 2.5 mM $CaCl_2$ was infected with Mu *cts*62 at a multiplicity of infection of four phages per bacterium. After lysis, the culture was incubated overnight at the same temperature to select for stable lysogens. Sixty percent of the surviving population was lysogenic for Mu *cts*62. These colonies were killed at 43°C. The lysogenized culture was mated on a bacterium filter with KMBL1666 at 32°C for 5 h. Thereafter the mixture was resuspended and plated at 37°C on the medium of Vogel and Bonner (36) supplemented with the amino acid requirements of KMBL1666 and with tetracycline to select for RP1-pMG1-carrying transconjugants. Two thousand of the appearing colonies were streaked on plates with the same medium and, after overnight incubation, were replica-plated onto LC plates with a soft-agar overlay containing KMBL1368. In this way, 11 colonies were shown to produce Mu phage. They were purified and rechecked for phage production. The auxotrophic markers were that of KMBL1666. When these strains were used as donors with nonlysogenic recipients, transfer frequency was low (about 10^{-7}) and most Tc^r recipients were thermosensitive.

TABLE 1. *E. coli strains*[a]

Strain	Genotype	Source
KMBL1368	*thyA301 bio-87 serA101 recA36*	P. van de Putte
KMBL1614	*thi-8 trp-704*::(Mu *cts*62) *galK1381 pon*	P. van de Putte
KMBL1666	*thi-22 thy-22 pyrF101* Δ*tonB trp-701*::Mu1 Δ*S lac-22*	P. van de Putte
AB1932-1[b]	*arg met lac nal* (Cb Km Tc Sm)	G. A. Jacoby
KA923[c]	*thi-22 thy-22 pyrF101* Δ*tonB trp-701*::Mu1 Δ*S lac-22* (Cb Km Tc Sm Mu *cts*62)	This study

[a] For resistance markers, see Materials and Methods.
[b] AB1932-1 carries the IncP1 type R plasmid RP1-pMG1 (15), which confers Cb^r Km^r Tc^r Sm^r. The Cb^r is coded for by TnA or Tn*801* (11), whereas the Sm^r determinant forms part of Tn*904* (R. H. Olsen, personal communication).
[c] KA923 carries the RP1-pMG1-derived plasmid pRL150 with Mu *cts*62 inserted near the Km^r locus.

Selection of octopine-utilizing bacteria. This was performed as described previously (19). Colonies were tested for octopine utilization by streaking them on octopine-bromothymol blue medium (13).

Mating conditions were as described previously (19). Because LBA4015 contains the transfer-derepressed Ti plasmid pAL102, conjugation was carried out in the absence of octopine. With LBA89, conjugation was on rich medium. In all experiments, mating on filters was used.

Virulence tests were performed on *Kalanchoë daigremontiana* stems as described (4) and on the hypocotyls of 2-weeks-old *Lycopersicon esculentum* (tomato, var. Rutgers) seedlings.

Plasmid DNA isolation and restriction fragmentation was performed according to Koekman et al. (25).

Lysopine dehydrogenase activity was determined by following the protocol of Otten and Schilperoort (30). Tumor tissue pieces were crushed in extraction buffer with a glass rod and, after centrifugation in an Eppendorf table centrifuge for 2 min, the supernatant fluid was incubated with a test mixture containing

TABLE 2. *A. tumefaciens strains*

Strain	Phenotypic properties[a]	Plasmid(s)	Plasmid properties[a]	Source
LBA89[b]	Met^- Ile^- Rif^r	pAL112	Uad^+ Cb^r Km^r Tc^r	This laboratory
LBA4011	Rif^r			This laboratory
LBA4013[c]		pAL102	Uad^c Tra^c	This laboratory
LBA4015[d]		pAL102; pRL151	Uad^c Tra^c; Cb^r Tc^r Sm^r	This study
LBA4020	Ery^r Spc^r			This laboratory

[a] Met^- and Ile^-, Requirement for methionine and isoleucine, respectively. Uad^+, Degradation of octopine (octopinic acid and lysopine; 18); Uad^c, constitutivity for this trait. Tra^c, Constitutive Ti transfer (19). For resistance markers, see Materials and Methods.
[b] pAL112 is a cointegrate plasmid consisting of RP4 and Ti-B6S3 (21). Ti-Ach5 and Ti-B6S3 are identical with respect to DNA homology and restriction endonuclease fingerprints (33).
[c] LBA4013 carries Ti plasmid pAL102 derived from Ti-Ach5. The strain is constitutive for transfer of Ti (Tra^c) as well as for octopine degradation (Uad^c) as a result of a repressor mutation (20).
[d] pRL151 is a transfer-deficient Km^s derivative of pRL150. The isolation of this plasmid is described in this study.

NADH, pyruvate, and arginine. Samples were subjected to paper electrophoresis. Staining was with phenanthrenequinone reagent. Tests were scored as positive when a clear yellow-green fluorescent spot was present at the same position as reference octopine.

Selection for antibiotic resistance. Selection was on the following concentrations (values in milligrams per liter). For *A. tumefaciens*: 1,500 streptomycin (Sm^r), 1 tetracycline (Tc^r), 10 carbenicillin (Cb^r), 100 kanamycin (Km^r), 500 spectinomycin (Spc^r), 20 rifampin (Rif^r), and 20 erythromycin (Ery^r). The last three of these markers are chromosomally located. For *E. coli* the following amounts were used (values in milligrams per liter): 25 Sm^r, 10 Tc^r, 500 Cb^r, and 50 Km^r.

Chemicals. D-(+)-Octopine was purchased from Sigma Chemical Co., streptomycin came from Brocacef, and carbenicillin (Pyopen) was purchased from Beecham. Kanamycin, rifampin, and spectinomycin were generous gifts from Kanematsu Gosho, Ciba Geigy, and The Upjohn Co., respectively.

RESULTS

In *A. tumefaciens*, transfer of R plasmids usually proceeds at a higher frequency than transfer of Ti plasmids. Therefore, to be able to select in transfer experiments for Ti plasmids carrying insertions of Tn*904*, we had to isolate a transfer-deficient derivative of R plasmid RP1-pMG1 on which Tn*904* is located. It is known that genomes with bacteriophage Mu insertions are prone to the formation of deletions (14). This is especially the case when such plasmids are transferred from *E. coli* into *Rhizobium meliloti* or *A. tumefaciens* (7, 35).

We have isolated 11 *E. coli* strains carrying insertions of Mu *cts*62 in RP1-pMG1 (see above). On the genetic map of RP4, which is closely related to RP1, Km^r marker has been localized between the two clusters of *tra* genes (3). Therefore, strain KA923 (Table 1) was selected for further use, because upon conjugation with *E. coli* acceptors, this strain gave rise to 5% Km^s transconjugants and thus probably had an insertion of Mu *cts*62 near the Km^r and *tra* genes. KA923 (pRL150) was used in crosses with *A. tumefaciens* LBA4013 at 32°C. The transfer frequency of pRL150 was very low (about 10^{-7} per acceptor bacterium) and when transconjugants were selected on Tc^r, 60% of the colonies were Km sensitive. About one in every two Km^s Tc^r clones was deficient for transfer of Tc^r. It could, therefore, be concluded that Mu *cts*62 was inserted in a site near Km^r and between the *tra* genes of RP1-pMG1. One Km^s Tc^r Tra^- clone derived from a KA923 × LBA4013 cross was selected for further use. This strain was called LBA4015 and the Tra^- R plasmid was catalogued as pRL151. No transfer of the Tc^r marker was ever observed under conditions that are optimal for RP1-pMG1 transfer and inhibitory for Ti plasmid transfer (namely mating on rich medium).

Procedure for the selection of insertions of Tn*904* in the Ti plasmid. When LBA4015 was used as a donor in mating experiments on SM medium with LBA4011 as the acceptor, the transfer frequency of pAL102 was usually about 10^{-1} per acceptor cell. If the Sm^r marker was transferred, two possibilities existed: (i) transposition of Tn*904* to the Ti plasmid or (ii) mobilization of the Tra^- R plasmid by the transfer-proficient Ti plasmid. These alternatives were distinguished by the remaining pRL151 markers Tc^r and Cb^r. The results of a typical mating experiment are given in Table 3. The colonies arising after plating on streptomycin selection medium were tested for Tc^r and Cb^r. About one in three Sm^r colonies was Tc^s and Cb^s. The other colonies were always both Tc^r and Cb^r. The latter bacteria were of two types; one type harbored both pAL102 and pRL151, and the other type harbored pRL151 only. In 10 strains of the first type neither Tc^r, nor Cb^r, nor Sm^r was linked with the Uad^+ marker in transfer experiments with LBA4020 as the acceptor. Thus, in these cases cointegrate plasmids had not been formed. In the second case the plasmid pRL151 was still transfer deficient. As expected, no Tra^+ revertants had been selected. This possibility was already unlikely because pRL151 was not only transfer deficient, but had also lost the kanamycin resistance and, thus, most probably had a deletion.

The colonies arising on streptomycin medium after the cross of LBA4015 with LBA4011 were roughly of two size classes present in equal proportions. The majority of the Sm^r Cb^s Tc^s colonies were, for unknown reasons, smaller. They were purified as being candidates for having Ti plasmids with Tn*904* insertions. A total number of 194 of such clones, recovered from 39 independent experiments, were further analyzed. Twenty of the strains were Uad^- as well as avirulent. Since it is known that octopine deg-

TABLE 3. *Mating of LBA4015 × LBA4011*[a]

Donor-acceptor ratio	Number of resistant colonies on SM Rif Sm medium (per ml)	Frequency
Donor only	0	$<10^{-8}$
Acceptor only	210	2×10^{-7}
Donor + acceptor (1:1) before mating	100	2×10^{-7}
Donor + acceptor (1:1) after mating	48×10^5	9×10^{-4}

[a] The transfer frequency of pAL102 was 2×10^{-1} per acceptor cell in this experiment.

radation and virulence are separate plasmid functions (18, 19, 28), it was concluded that the latter strains had a large Ti plasmid deletion or had a chromosomal mutation for streptomycin resistance. These strains were not further analyzed.

Characterization of putative insertion mutants. The cotransfer of Sm^r and Uad$^+$ was used for testing whether the remaining 174 Uad$^+$ Sm^r isolates had Ti plasmids with Tn*904* insertions. These strains were used as donors in crosses with LBA4020 as acceptor. After selection on octopine-spectinomycin medium, at least 20 Uad$^+$ transconjugant colonies were tested for streptomycin resistance. In 155 of the strains, all Uad$^+$ colonies were Sm^r. For 14 of these strains, we tested 100 transconjugant colonies per experiment, and all 1,400 colonies examined were Sm^r (Table 4). The remaining 19 Sm^r Uad$^+$ donor strains, however, gave one or more Sm^s Uad$^+$ transconjugant colonies. These isolates may, therefore, carry unstable Tn*904* insertions.

Alterations in plasmid-controlled phenotypic properties of the bacteria were taken as a second indication for the insertion of Tn*904* into pAL102. As described below, 13 of the isolates showed an impairment of tumor-inducing ability. The structure of plasmid DNA was used as a third criterion. By using several restriction endonucleases and agarose gel electrophoresis, it was possible to demonstrate and to localize the insertion of a piece of DNA in the Ti plasmids of 20 strains studied (for an example see Fig. 1; G. Ooms et al., manuscript in preparation). For a total number of 155 strains it was thus safe to assume a translocation of Tn*904* towards the Ti plasmid.

TABLE 4. *Cotransfer of Uad$^+$ and Sm^r in matings employing LBA4020 as the acceptor strain*[a]

Donor strain	Uad$^+$ transconjugants that were also Sm^r (%)	Remark
LBA4202	100	Classified as stable insertions of Tn*904* into pAL102
LBA4219	100	
LBA4224	100	
LBA4227	100	
LBA4222	0	Classified as unstable insertions (19 out of 174 isolates)
LBA4230	25	
LBA4235	72	
LBA4236	6	
LBA4237	23	
LBA4237	12	
LBA4239	13	

[a] More than 100 colonies were tested in every experiment.

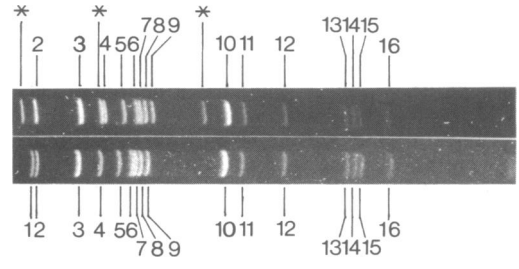

FIG. 1. *Agarose gel electrophoresis of plasmid pAL237 DNA from LBA4219 treated with restriction endonuclease SmaI from S. marcescens. Upper lane: Ti plasmid DNA from LBA4219 carrying an insertion of Tn904 in SmaI restriction fragment 1. Bottom lane: wild-type Ti plasmid DNA from Ach5 strain treated in a similar way. Newly appearing bands are indicated by asterisks. See Chilton et al. (6) for data concerning physical map.*

Mating with LBA89. To confirm that the Sm^r marker was Ti plasmid located in these isolates, the cointegrate plasmid pAL112 was introduced into them by mating with LBA89 and selecting for Tc^r. It is known that pAL112 is incompatible with Ti plasmids. Therefore the resident plasmid with the Sm^r insertion should disappear as a consequence of the entrance of pAL112 unless the Sm^r marker was rescued by recombination with pAL112 or with the chromosome. Previous experiments (21) predicted that the rescue frequency would be 5 to 10% in a Rec$^+$ background. In several strains the Sm^r character was maintained for about 15% (Table 5). In the remaining cases, however, all Tc^r transconjugants were Sm^r. This outcome suggested that the cells were diploid for Sm^r, presumably with a second insertion of Tn*904* in the chromosome. One of these strains, LBA4202, was therefore studied in more detail. When the Ti plasmid was transferred from LBA4202 to LBA4020, 100% linkage of Uad$^+$ and Sm^r was observed (Table 4). A purified transconjugant clone (LBA4233) was again mated with LBA89. In consecutive experiments the proportion of Sm^r Tc^r colonies was 34 and 46%. It appeared that the percentage was increasing. Ten single colonies were tested thereafter in analogous experiments. In one case the fraction of Sm^r Tc^r was 17%; in the other nine cases it was 100%. Finally the plasmid was transferred from LBA4233 into LBA4301, which is Rec$^-$ (21), and the mating with LBA89 was repeated. In this experiment the observed proportion of Sm^r Tc^r transconjugants was again 100%. Apparently a process of diploidization for the Sm^r marker was occurring, which proceeded independently of the bacterial *rec* functions.

Character of strains showing less than

100% linkage of Sm^r and Uad$^+$. Nineteen Sm^r Uad$^+$ strains derived from LBA4015 × LBA4011 crosses showed the appearance of Sm^s transconjugant colonies when mated with LBA4020. The ratio of Sm^r to Sm^s colonies varied with the different strains (Table 3). One strain readily gave rise to Sm^s bacteria without losing Uad$^+$ when cultured under nonselective conditions. In the other 18 strains the Sm^r marker was stable when cultured this way. It was assumed that in the first case (one strain) Tn*904* was unstably inserted. In the latter cases (18 strains) the Sm^r was a stable character of the cells. Therefore, the fact that there was a high but not absolute linkage of Sm^r with the Ti plasmid had to be explained.

Matings were performed with LBA89 similar to those described in the preceding paragraph. Introduction of pAL112 did not remove the Sm^r in all strains tested (Table 5).

When plasmid DNA from 2 of the 18 strains was analyzed with respect to restriction patterns, no inserted DNA was found. The DNA of one such strain was extracted according to the method of Casse et al. (4a) and subjected to electrophoresis on agarose gels; only plasmid DNA with the molecular weight of the Ti plasmid was detected. Thus, no evidence was obtained for insertion of the Sm^r marker in another replicon other than the Ti plasmid or the chromosome. We conclude that in the isolates in question, Tn*904* was initially inserted in the Ti plasmid, but that it was gradually lost as a result of instability. At the same time Tn*904* gave rise to new, stable insertions into the bacterial chromosome, which could not be removed by introduction of pAL112. For plasmid DNA isolation large numbers of cells have to be extracted. It is, therefore, likely that in unstable strains the proportion of plasmid DNA with insertions will decrease, after the many generations required to produce these quantities, to a point at which the DNA inserted will no longer be detectable.

TABLE 5. *Disappearance of Sm^r after the introduction of pAL112 by mating with LBA89 and selection for Tc^r colonies on SM medium*[a]

Acceptor strain	Tc^r transconjugant acceptor colonies that remain Sm^r (%)	Remark
LBA4210	100	Strains classified as stable insertions of Tn*904* into pAL102 (Table 3)
LBA4212	15	
LBA4217	100	
LBA4219	14	
LBA4220	18	
LBA4221	23	
LBA4226	100	
LBA4227	100	
LBA4228	16	
LBA4231	100	
LBA4232	62	
LBA4202	100	
LBA4233	100 (9 times)	Uad$^+$ Sm^r transconjugants from LBA4202×LBA4020.
LBA4233	17 (1 time)	
LBA4309	100	Plasmid of LBA4202 in Rec$^-$
LBA4015	13	Parental strain with Sm^r in pRL151
LBA4222	100	Strains classified as unstable insertions of Tn*904* into pAL102
LBA4224	100	
LBA4230	100	
LBA4235	100	
LBA4236	100	
LBA4237	100	
LBA4239	100	

[a] In all cases at least 200 colonies were tested.

Transposition frequency. When it is assumed that the 155 strains described, which showed 100% cotransfer of Sm^r and Uad$^+$ (and of which 13 had detectable mutations), indeed represent transpositions of Tn*904* from pRL151 into the Ti plasmid pAL102, the estimate for the transposition frequency amounts to 3×10^{-4} per transferred Ti plasmid.

Transposition of Tn*801*. RP1-pMG1 carries, besides Tn*904*, Tn*801* conferring carbenicillin resistance (11). When we looked for Cb^r Sm^s Tc^s colonies after mating of LBA4015 with LBA4011, it was found that none of 500 Cb^r transconjugants had those features. Compared to Tn*904*, transposition of Tn*801* into the Ti plasmid seems to occur at a much lower frequency ($< 3 \times 10^{-6}$).

Properties of insertion mutants. Although a few strains gave variable results, none of the 155 stable strains was unable to transfer the Ti plasmid. In all strains plasmid transfer was independent of the presence of octopine. All were sensitive to homooctopine, a property only exhibited by bacteria harboring Uad^c plasmids like pAL102 (19, 31).

Virulence was tested on *K. diagremontiana* and *L. esculentum*. Thirteen strains showed alterations of tumor-inducing ability (Table 6). Retransfer experiments demonstrated that the mutations in all these cases were located on the Ti plasmid. Strain LBA4210 induced tumors on *Kalanchoë* stems with more roots than found when the parental strain Ach5 was used, whereas tumor formation was weaker. Tomato tumors of all strains were tested for the presence of lysopine dehydrogenase activity (30). All tumors were positive, although weak reactions

TABLE 6. *Tumor-inducing properties of insertion strains*[a]

Strain	Virulence on *L. esculentum* (tomato)	Virulence on *K. daigremontiana*	Remark
LBA4202	±	±	
LBA4210	±	±	Increase of root formation on *Kalanchoë*
LBA4212	±	+	
LBA4217	±	±	Very weak on both plant species
LBA4219	−	−	
LBA4220	−	−	
LBA4221	±	+	
LBA4226	−	+	
LBA4227	−	+	
LBA4228	±	±	
LBA4231	−	±	Very weak on *Kalanchoë*
LBA4232	−	−	
LBA4234	±	+	

[a] +, As wild type and LBA4013; ±, intermediate but significantly lower than LBA4013; −, no tumor formation.

were scored for some of the mutants. It was, however, difficult to quantitate these differences.

DISCUSSION

The results reported make clear that the streptomycin resistance determinant present on RP1-pMG1 is able to transpose in *A. tumefaciens* into the octopine Ti plasmid. RP1-pMG1 originated as a recombinant plasmid between RP1 and the IncP2 type R plasmid pMG1 harbored by a *Pseudomonas aeruginosa* strain (15). By transfer to *E. coli,* an RP1 derivative was selected that had acquired the genetic determinant for streptomycin resistance. Compared to RP1 its mass was increased by 6 megadaltons (15). R. H. Olsen has obtained evidence that the Sm^r gene forms part of a transposon, which may jump to different replicons in *E. coli* (personal communication). Our results confirm this for *A. tumefaciens.* The observed transposition frequency was rather high (3×10^{-4}), whereas Tn*801* transposition was not detected. It is, however, known that transposition of Tn*1* (most probably identical to Tn*801*) into the Ti plasmid is possible (13).

The results described illustrate that, by the use of Tra^- R plasmids as transposon donors, good results can be obtained, provided that the frequency of translocation is high enough compared to the level of mobilization of the R plasmid. The use of Mu *cts*62 insertions in the R plasmid to generate deletions was an application of findings reported by Dénarié et al. (7) and Van Vliet et al. (35). This approach may also be of use to obtain Tra^- R plasmids in other gram-negative bacteria and would be especially important for those bacterial species into which transduction or transformation of Tra^- plasmids has failed.

It is interesting that the Ti plasmid mobilized the Tra^- R plasmid efficiently. This may perhaps mean that Ti plasmids also promote the transfer of chromosomal DNA. The mobilization was not accompanied by transposition of Tn*801* or Tn*904* into the Ti plasmid. Cointegrate formation was not found either. In both these respects there is a difference with a comparable study in *P. aeruginosa* by Olsen (29).

Thirteen insertion mutants were recovered with an impaired ability to induce tumors on one or both of the test plant species. In addition, one strain induced significantly more root formation at the tumor than the wild-type parental strain. It may be that in the latter mutant Tn*904* has hit certain Ti plasmid genes that control the morphology of the plant tumor. These virulence mutants have been studied with respect to insertion site of the transposon (G. Ooms et al., manuscript in preparation).

No mutants other than the virulence type were isolated (e.g. Uad^- mutants). Given the size of the Ti plasmid this may partially be explainable by the restricted number of insertions studied. It is likely that the genes involved in octopine metabolism are low in number; therefore, there may be also a low probability of hitting them. On the other hand there is also the phenomenon of site preference observed for several transposons (10, 22, 26). It is thus important to study the Ti plasmid by using a range of different transposons.

We have found indications that Tn*904* translocated to secondary sites, presumably on the bacterial chromosome, as has been observed with other transposons (2, 11, 23). The resulting homology between the Ti plasmid and the chromosome may prove to favor chromosome mobilization during Ti-controlled conjugation. The phenomenon of translocation to secondary chromosomal sites will perhaps facilitate the isolation and localization of auxotrophic mutations (23).

In one case the Sm^r coded by Tn*904* was lost under nonselective conditions. In the other

cases, the bacteria remained Sm^r. There were 18 strains that showed a high, but not absolute, linkage of Sm^r and Uad^+ in transfer experiments, whereas pAL112 did not remove the Sm^r marker. It is likely that these 18 represent a class of insertion mutants with unstable Ti-carried Tn*904* insertions, which had already established a second insertion in the bacterial chromosome by the time they were analyzed. The instability of some Tn*904* insertions has also been observed in *E. coli* by R. H. Olsen (personal communication). This is an interesting aspect of Tn*904* transposition. To study this phenomenon, it is preferable to use *E. coli* with its well-developed genetical technology as a host system.

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LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
2. **Barth, P. T., N. Datta, R. W. Hedges, and N. J. Grinter.** 1976. Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistances from R483 to other replicons. J. Bacteriol. **125**:800–810.
3. **Barth, P. T., and N. J. Grinter.** 1977. Map of plasmid RP4 derived by insertion of transposon C. J. Mol. Biol. **113**:455–474.
4. **Bomhoff, G. H., P. M. Klapwijk, H. C. M. Kester, R. A. Schilperoort, J. P. Hernalsteens, and J. Schell.** 1976. Octopine and nopaline: synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. **145**:177–181.

4a. **Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Dénarié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**:229–242.

5. **Chilton, M.-D., M. H. Drummond, D. J. Merlo, D. Sciaky, A. L. Montoya, M. P. Gordon, and E. W. Nester.** 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell **11**:263–271.
6. **Chilton, M.-D., A. L. Montoya, D. J. Merlo, M. H. Drummond, R. Nutter, M. P. Gordon, and E. W. Nester.** 1978. Restriction endonuclease mapping of a plasmid that confers oncogenicity upon *Agrobacterium tumefaciens* strain B6-806. Plasmid **1**:254–269.
7. **Dénarié, J., C. Rosenberg, B. Bergeron, C. Boucher, M. Michel, and M. Barate de Bertalmio.** 1977. Potential of RP4::Mu plasmids for in vivo genetic engineering of gram-negative bacteria, p. 507–520. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. **Drummond, M. H., M. P. Gordon, E. W. Nester, and M.-D. Chilton.** 1977. Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumours. Nature (London) **269**:535–536.
9. **Genetello, C., N. van Larebeke, M. Holsters, A. de Picker, M. van Montagu, and J. Schell.** 1977. Ti plasmids of Agrobacterium as conjugative plasmids. Nature (London) **265**:561–563.
10. **Grinsted, J., P. M. Bennet, S. Higginson, and M. H. Richmond.** 1978. Regional preference of insertion of Tn*501* and Tn*802* into RP1 and its derivatives. Mol. Gen. Genet. **166**:313–320.
11. **Hedges, R. W., and A. Jacob.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132**:31–40.
12. **Hernalsteens, J. P., M. De Greve, M. van Montagu, and J. Schell.** 1978. Mutagenesis by insertion of the drug resistance transposon Tn7 applied to the Ti plasmid of *Agrobacterium tumefaciens*. Plasmid **1**:218–225.
13. **Hooykaas, P. J. J., C. Roobol, and R. A. Schilperoort.** 1979. Regulation of the transfer of TI plasmids of *Agrobacterium tumefaciens*. J. Gen. Microbiol. **110**:99–109.
14. **Howe, M., and E. Bade.** 1975. The genetics and molecular biology of bacteriophage Mu-1. Science **190**:624–632.
15. **Jacoby, G. A., A. E. Jacob, and R. W. Hedges.** 1976. Recombination between plasmids of incompatibility groups P-1 and P-2. J. Bacteriol. **127**:1278–1285.
16. **Kerr, A., P. Manigault, and J. Tempé.** 1977. Transfer of virulence *in vivo* and *in vitro* in Agrobacterium. Nature (London) **265**:560–561.
17. **Klapwijk, P. M., A. J. R. de Jonge, R. A. Schilperoort, and A. Rörsch.** 1975. An enrichment technique for auxotrophs of *Agrobacterium tumefaciens* using a combination of carbenicillin and lysozyme. J. Gen. Microbiol. **91**:177–182.
18. **Klapwijk, P. M., P. J. J. Hooykaas, H. C. M. Kester, R. A. Schilperoort, and A. Rörsch.** 1976. Isolation and characterization of *Agrobacterium tumefaciens* mutants affected in the utilization of octopine, octopinic acid and lysopine. J. Gen. Microbiol. **96**:155–163.
19. **Klapwijk, P. M., T. Scheulderman, and R. A. Schilperoort.** 1978. Coordinated regulation of octopine degradation and conjugative transfer of Ti plasmids in *Agrobacterium tumefaciens:* evidence for a common regulatory gene and separate operons. J. Bacteriol. **136**: 775–785.
20. **Klapwijk, P. M., and R. A. Schilperoort.** 1979. Negative control of octopine degradation and transfer genes of octopine Ti plasmids in *Agrobacterium tumefaciens*. J. Bacteriol. **139**:424–431.
21. **Klapwijk, P. M., P. van Beelen, and R. A. Schilperoort.** 1979. Isolation of a recombination deficient *Agrobacterium tumefaciens* mutant. Mol. Gen. Genet. **173**:171–175.
22. **Kleckner, N.** 1977. Translocatable elements in procaryotes. Cell **11**:11–23.
23. **Kleckner, N., R. K. Chan, B. K. Tye, and D. Botstein.** 1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. J. Mol. Biol. **97**:561–575.
24. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics. J. Mol. Biol. **116**:125–159.
25. **Koekman, B., G. W. Ooms, P. M. Klapwijk, and R. A. Schilperoort.** 1979. Genetic map of an octopine Ti plasmid. Plasmid **2**:347–357.
26. **Kopecko, D. J., J. Brevet, and S. N. Cohen.** 1976. Involvement of multiple translocating DNA segments and recombinational hot-spots in the structural evolution of bacterial plasmids. J. Mol. Biol. **108**:333–360.
27. **Márton, L., G. J. Wullems, L. Molendijk, and R. A.**

Schilperoort. 1979. *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens.* Nature (London) **277**:129–131.

28. **Montoya, A. L., M.-D. Chilton, M. P. Gordon, D. Sciaky, and E. W. Nester.** 1977. Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumor cells: role of plasmid genes. J. Bacteriol. **129**:101–107.
29. **Olsen, R. H.** 1978. Evolution of *Pseudomonas* R-plasmids: consequences of Tn*1* insertion and resultant partial diploidy to chromosome and Tra⁻R-plasmid mobilization. J. Bacteriol. **133**:210–216.
30. **Otten, L. A. B. M., and R. A. Schilperoort.** 1978. A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim. Biophys. Acta **527**:497–500.
31. **Petit, A., and J. Tempé.** 1978. Isolation of Agrobacterium Ti-plasmid regulatory mutants. Mol. Gen. Genet. **167**:147–156.
32. **Schell, J.** 1975. The role of plasmids in crown-gall formation by *Agrobacterium tumefaciens,* p. 163–181. *In* L. Ledoux (ed.), Genetic manipulations with plant material. Plenum Publishing Corp., New York.
33. **Sciaky, D., A. L. Montoya, and M.-D. Chilton.** 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid **1**: 238–253.
34. **Van Larebeke, N., Ch. Genetello, J. Schell, R. A. Schilperoort, A. K. Hermans, J. P. Hernalsteens, and M. van Montagu.** 1975. Acquisition of tumor inducing ability by non-oncogenic *Agrobacterium* as a result of plasmid transfer. Nature (London) **255**:742–743.
35. **Van Vliet, F., B. Silva, M. van Montagu, and J. Schell.** 1978. Transfer of RP4::Mu plasmids to *Agrobacterium tumefaciens.* Plasmid **1**:446–455.
36. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. **218**:97–106.
37. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens.* J. Bacteriol. **123**:255–264.
38. **Zaenen, I., N. van Larebeke, H. Teuchy, M. van Montagu, and J. Schell.** 1974. Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. J. Mol. Biol. **86**:109–127.