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Analysis of *Agrobacterium tumefaciens* Plasmid pTiC58 Replication Region with a Novel High-Copy-Number Derivative

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The origin of replication, *ori*, of the nopaline tumor-inducing plasmid, pTiC58, mapped in a region that shares sequence homology with octopine plasmids pTiAch5 and pTiB6. Within this region, the minimum amount of DNA necessary for maintaining autonomous replication was a 2.6-kilobase region, which also comprised the incompatibility function *inc*. pTiC58 derivatives containing *inc* were incompatible with *Agrobacterium tumefaciens* plasmids pTiC58, pTiD1439, pTiAch5, pTi15955, and pTiA5 and were compatible with *A. rhizogenes* plasmid pRi12. Situated adjacent to the origin region was a 1.5-kilobase *par* segment involved in stable inheritance of pTiC58 under nonselective growth conditions. When *par* was present, plasmid maintenance approached that of the wild-type pTiC58. Rapid loss from the cell population was observed for plasmids not containing this locus. Another 1.5-kilobase region, *cop*, positively regulated pTiC58 copy number, enabling certain pTiC58 derivatives to exist at a copy number up to 80 times higher than that of wild-type pTiC58. Deletions within the *cop* locus resulted in reduced copy number. The *ori/inc* regions were flanked on either side by the *par* and *cop* loci.

The tumor-inducing (Ti) plasmid is now known for conferring on *Agrobacterium tumefaciens* the ability to incite crown gall tumors in plants, for opine production and catabolism, and for variations in tumor morphology and plant host range (1, 27). Yet little is understood about the replication process of the Ti or any other class of *A. tumefaciens* plasmids (9, 17). The location of the origin of replication for one octopine-type plasmid (pTiB6) and loci involved in incompatibility, stable plasmid inheritance, and the exclusion of certain incoming Ti plasmids have been reported (12, 17). Nopaline and octopine Ti plasmids share serveral regions of sequence homology (8, 11). Although a 16-kilobase (kb) region of the nopaline-type plasmid pTiC58 shares homology with the region containing the origin of replication of the octopine-type pTiB6, this region has not been shown definitively to actually contain the origin of replication of pTiC58 or any associated functions.

We report here the isolation of the replication region of pTiC58. Within this region, we mapped the regions necessary for autonomous replication, plasmid stability, incompatibility, and copy number control. Useful in the determination of the last function was a novel pTiC58-origin derivative that was maintained at high copy levels in *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains used are listed in Table 1. pSa and pSa734 (3, 32) and pBR325 (2) are described elsewhere. pKNR-H, constructed by T. Close and D. Zaitlin (unpublished data), contains the neomycin/kanamycin resistance gene (NPT II gene) from Tn*5*, which was isolated and fitted with various linkers. Medium 523 (14) and L broth (20) were used for liquid cultures and were used with 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) for solid medium. For transformation of *A. tumefaciens*, strains were grown at 30°C in YEB medium (16). Kanamycin, neomycin, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo.

Plasmid DNA purification. Preparative isolation of plasmid DNA from *A. tumefaciens* has been described elsewhere (16). Rapid screening of *A. tumefaciens* for plasmid DNA and the isolation of small quantities of plasmid DNA from *Escherichia coli* has been described elsewhere (15, 32).

Transformation. Transformation procedures for *A. tumefaciens* (9) and *E. coli* (21) have been described previously.

Enzymes. T4 DNA ligase and the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I were prepared by B. Froman and R. C. Tait. Other restriction enzymes were purchased from New England Biolabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Gaithersburg, Md. The reaction conditions used were as recommended by the suppliers.

Plasmid segregation. A fresh stationary culture (10 μl) of *A. tumefaciens* grown in 5 ml of medium 523 supplemented with 75 μg of neomycin per ml for 24 h at 30°C was then transferred every 24 h thereafter for the length of the study to 5 ml of fresh medium 523 containing no antibiotics. Portions (10 μl) of stationary-phase cultures were serially diluted with sterile distilled water, plated on medium 523, and incubated at 30°C until colonies appeared. One hundred colonies were transferred to medium 523 containing 75 μg of neomycin per ml. The retention of a plasmid in a given cell population was determined as the percentage of colonies that grew on the antibiotic medium. The number of generations to reach stationary phase was determined by viable cell count immediately after the inoculation of fresh medium and after 24 h of growth.

Virulence assay. Using the young leaves of eight-week-old *Kalanchoe daigremontiana*, we tested virulence by wounding one half of a leaf with the wild-type strain and the other half with the same strain, now harboring pUCD500. After 2 months, the final data were collected. Strain 1D1439 was tested on the stems of young tomato plants (*Lycopersicum esculentum* cv. Bonny Best).

Copy number estimation. A 5-ml sample of medium 523 supplemented with 75 μg of neomycin per ml was inoculated

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TABLE 1. Bacterial strains used

Strain		Characteristics	Source
E. coli	HB101	*recA leu pro B1 lacY*	F. Bolivar
A. tumefaciens	LBA4301	Rec$^-$ Rif^r UV^s *vir*$^-$	R. A. Schilperoort
A. tumefaciens	15955	*oct*^{c} *vir*$^+$	J. E. DeVay
A. tumefaciens	C58	*nop*^{c} *vir*$^+$	R. Dickey
A. tumefaciens	Ach5	*oct*^{c} *vir*$^+$	R. Dickey
A. tumefaciens	A6	*oct*^{c} *vir*$^+$	R. Manasse
A. tumefaciens	1D1439	*nop*^{c} *oct*^{c} *vir*$^+$	K. L. Perry
A. rhizogenes	3D12	*manopine*^{c} *vir*$^+$	This laboratory

with 10 μl of freshly grown LBA4301 cells harboring the plasmid to be tested. The cells were grown at 30°C to a cell density of 1×10^9 cells per ml, and 0.35-ml portions were then taken for plasmid isolation (15). Samples (70 μl) of the purified plasmid preparations with 0.001, 0.005, 0.01, 0.05, and 0.1 μg of pUCD500 DNA were resolved in separate lanes by electrophoresis in a 0.7% agarose gel of 10 V/cm for 3 h. The DNA was stained with 1 μg of ethidium bromide per ml for 1 h, rinsed 30 min with distilled water, and photographed with Type 55 Polaroid Land Film. The negative was traced densitometrically at A_{550} with a Beckman model 3220 recording spectrophotometer equipped with a film scanner. The quantity of plasmid DNA and plasmid copy number were estimated with the pUCD500 DNA standards and the cell density of the culture.

RESULTS

A pTiC58 region homologous to the origin region of pTiAch5 has been suggested to contain the pTiC58 origin of replication (*ori*) (37). Fine restriction mapping of this region precisely positioned restriction sites in pTiC58 relative to each other (Fig. 1). For example, the *Kpn*I site mapped by Depicker et al. (5) to be in *Hin*dIII fragment 13 actually was contained in *Hin*dIII fragment 32. The 23-kb *Kpn*I fragment 2, containing the homology region, was cloned into the unique selectable *Kpn*I site of pUCD500P, a cloning vehicle derived from pSa734 (Fig. 2). The resulting hybrid molecule, pUCD500P-2, was digested completely with *Hin*dIII, and the fragments were ligated with a fragment containing the NPT II gene (see above). Strain LBA4301 was transformed with the ligated DNA and selected on medium 523 containing 75 μg of neomycin per ml. Transformants were screened for plasmids, the smallest of which was 6.0 kb and designated pUCD510. Restriction analysis of pUCD510 indicated that it contained, in addition to the NPT II gene fragment, a single fragment corresponding to pTiC58 *Hin*dIII fragment 13 (data not shown).

The restriction map of pTiC58 by Depicker et al. (5) indicated that 4.5-kb *Hin*dIII fragment 13 contains an *Eco*RI site (Fig. 1). The procedure used in the construction of pUCD510 was repeated with pUCD500P-2 DNA digested with *Eco*RI followed by ligation to the NPT II gene fragment fitted with *Eco*RI linkers. After transformation, one neomycin-resistant LBA4301 transformant contained a 6.6-kb plasmid designated pUCD500. Restriction analysis of pUCD500 revealed that it included the NPT II gene fragment, *Eco*RI fragment 27, and a third 2.9-kb fragment. Hybridization of α-^{32}P-labeled pUCD500 DNA to *Eco*RI-digested pTiC58 DNA that had been blotted onto nitrocellulose by the Southern procedure (29) confirmed that *Eco*RI fragment 27 was present in pUCD500 and that the third fragment hybridized to *Eco*RI fragment 25 (data not shown). *Eco*RI fragments 27 and 25 are adjacent to each other (Fig. 1). The *Kpn*I site within *Eco*RI fragment 25 is the terminus of pTiC58 *Kpn*I fragment 2, the cloned fragment of pUCD500P-2 used

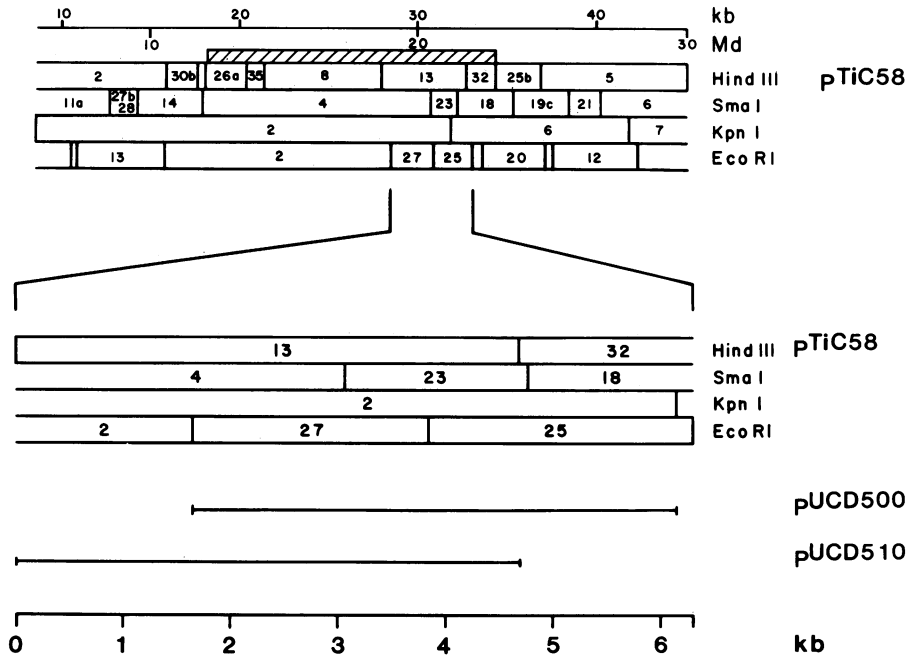

FIG. 1. Fine mapping of the pTiC58 region containing the replication region. Above is a partial pTiC58 restriction map by Depicker et al. (5). The hatched area represents the pTiC58 region which shares sequence homology to pTiAch5. The expanded map below is a corrected version of the above map for the region indicated. Below the expanded map are the regions of pTiC58 DNA contained in pUCD500 and pUCD510.

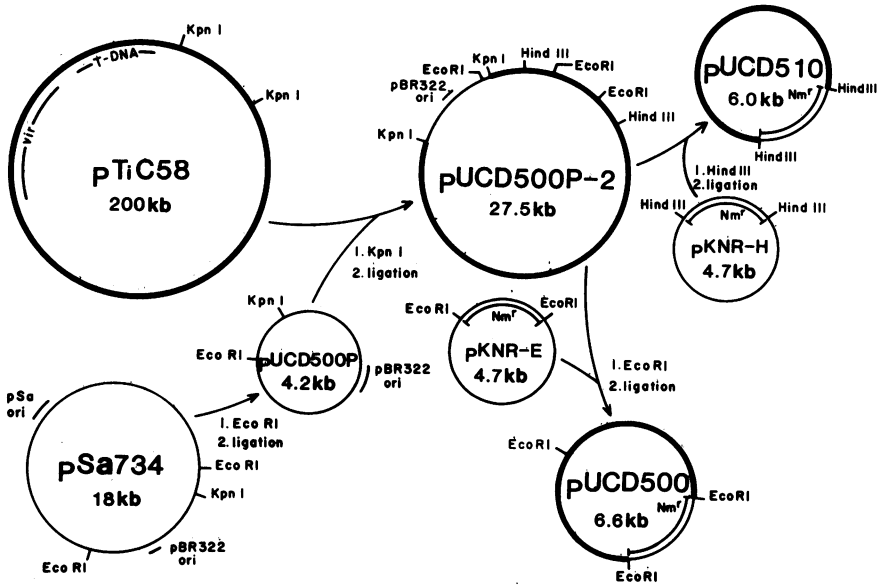

FIG. 2. Construction of pUCD510. pSa734 DNA cleaved with *Eco*RI was cloned in a 4.2-kb pSa734 derivative, pUCD500P, which contained the pBR322 origin of replication and an unique *Kpn*I site. pUCD500P was used for the cloning of pTiC58 *Kpn*I fragment 2 from *Kpn*I-digested pTiC58 DNA. The resulting clone, pUCD500P-2, digested completely with *Hin*dIII and ligated to a 1.4-kb *Hin*dIII-ended fragment coding for neomycin resistance gave rise to pUCD510. pUCD500 was made by the ligation of *Eco*RI-digested pUCD500P-2 DNA with a 1.4-kb *Eco*RI-ended fragment coding for neomycin resistance.

to make pUCD500. An *Eco*RI site is located approximately 0.6 kb from this *Kpn*I site in the vector DNA (30). Therefore, the 2.9-kb *Eco*RI fragment of pUCD500, which hybridizes to pTiC58 fragment 25, is composed of 2.3 kb of *Eco*RI fragment 25 and 0.6 kb of vector DNA.

pUCD500 was found to be a high-copy-number mutant. To determine whether the junction between *Eco*RI fragments 27 and 25 had been maintained in the proper orientation, restriction sites for several endonucleases were mapped on pUCD500 and pUCD510 (Fig. 3). A comparison of the restriction maps shows that the two pTiC58 DNA-containing *Eco*RI fragments of pUCD500 are in the same orientation with respect to one another as they are in pTiC58.

To further delineate the limits of the pTiC58 origin, several deletion derivatives of pUCD500 were made (Fig. 3). pUCD501, a *Hin*dIII-deletion derivative, functions as an autonomous replicating unit as do pUCD502 and pUCD503, which are *Sac*II-deletion derivatives. When LBA4301 cells were transformed with pUCD504, which contains only *Eco*RI fragment 27 of pTiC58 cloned into pBR325, no transformants

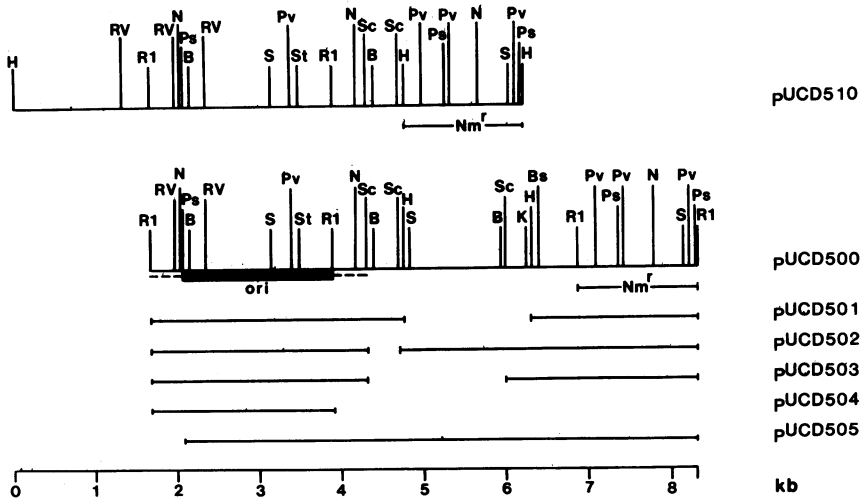

FIG. 3. Restriction map of pUCD510 and pUCD500 and its derivatives. pUCD510 and pUCD500 are positioned to overlap regions of common DNA. Below pUCD500 are the deletion derivatives of pUCD500. Deleted regions are represented by the absence of a line. DNA from pSa maps from coordinate 6.2 to 6.8. ori, Origin; Nm^r, neomycin resistance-encoding region. H, *Hin*dIII, RI, *Eco*RI; RV, *Eco*RV; N, *Nco*I; Ps, *Pst*I; B, *Bam*HI; S, *Sma*I; Pv, *Pvu*II; St, *Stu*I; Sc, *Sac*II; K, *Kpn*I; Bs, *Bst*EII. pUCD504 and pUCD505 are regions cloned in pBR325.

TABLE 2. Relative plasmid copy number and segregation

Plasmid	Relative copy number[a]	Plasmid segregation[b]
pTiC58	1[c]	0
pUCD510	1	0.26
pUCD500	80	0, 3.9[d]
pUCD501	6	3.8
pUCD502	70	0, 3.5[d]
pUCD503	10	3.6

[a] In strain LBA4301.
[b] Measured as percentage of plasmid loss per generation.
[c] Relative copy number of pTiC58 is designated as a value of 1 (28).
[d] The two values reflect the biphasic nature of segregation for this plasmid.

were obtained. These data suggest that the 0.4-kb region between the *Eco*RI site (map coordinate 3.9) and the first *Sac*II site (map coordinate 4.3) contains information necessary for autonomous replication. pUCD505, a *Pst*I fragment of pUCD500 cloned into the *Pst*I site of pBR325, does not replicate in LBA4301 cells, suggesting that the region from the *Pst*I site (map coordinate 1.7) to the neomycin-resistance gene (0.4 kb distant) contains necessary information for replication. Therefore, the consensus of the pUCD500 derivatives suggests that a region of 2.6 kb contains the information required for autonomous plasmid replication (Fig. 3).

Copy number. pUCD500 is present at high copy levels in *A. tumefaciens*. Plasmid isolation of pUCD500 from 1 liter of LBA4301 culture yielded 70 times more plasmid DNA than did pUCD510 from the same amount of cells. Amplifiable plasmids such as ColE1 (6) can exist at an elevated copy number as a result of protein synthesis inhibition. Because ColE1 does not require continued protein synthesis to replicate (6), when prevented from further growth its host cell will accumulate a large ColE1-plasmid population. To determine whether the high copy number of pUCD500 was a result of amplification, a culture of LBA4301 cells containing the plasmid was tested as described previously (31). LBA4301 cells containing pUCD500 were grown to mid-log phase in medium 523 at 30°C, at which point chloramphenicol was added to a concentration of 200 μg/ml. Samples were removed throughout a 24-h period, and the plasmid was isolated and analyzed by agarose gel electrophoresis. No increase in plasmid DNA was detected (data not shown), suggesting that the high copy nature of pUCD500 was not due to amplification and that pUCD500 DNA initiation or replication or both requires the continued synthesis of protein.

Koekman et al. (17) isolated a pTiAch5 origin-containing plasmid which could exist at increasingly higher copy numbers, up to 20 copies per cell, as greater selection pressure was applied for the presence of the plasmid in its host. To determine whether the copy number of pUCD500 had any relation to the amount of antibiotic pressure applied for its presence, LBA4301 cells containing pUCD500 were grown in medium 523 with 10 μg of neomycin per ml (twice the base level of neomycin resistance for LBA4301) overnight at 30°C. Portions (10 μg) of this culture were then transferred to fresh medium 523 containing 10, 20, 40, 80, 250, and 1,000 μg of neomycin per ml and were allowed to grow overnight at 30°C. Portions containing the same cell mass were taken for plasmid isolation and agarose gel electrophoresis analysis. No difference in copy number was detected.

Widely used techniques in the determination of plasmid copy number (25, 36) rely on whole DNA isolation followed by the separation of supercoiled DNA from linear DNA. Even after a shearing step, whole DNA preparations of *A. tumefaciens* yielded a viscous product that inhibited the isolation of all the chromosomal DNA from the plasmid DNA. As a measure of copy number, resistance to neomycin was unreliable for *cop* mutants that had a copy number increased more than a few fold over the wild type. Therefore, an approximation of the relative copy number (relative to a pTiC58 copy number of 1 [28]) of pUCD510 and pUCD500 and its derivatives was determined by the copy number technique described above. The relative copy number of pUCD500 in strain LBA4301 was estimated to be 80 times that of pTiC58 (see Table 2 and Fig. 4). In comparison, pUCD510 was estimated to be present at one copy per cell, much like the copy number of the parent plasmid pTiC58. The relative copy number for the pUCD500 deletion derivative pUCD502 was estimated to be 70, similar to that of pUCD500. pUCD501 and pUCD503 exhibited intermediate relative copy numbers of 6 and 10 per cell, respectively, in LBA4301 cells. These deletion derivatives define a region in pUCD500 that is involved in the regulation of copy number. This 1.5-kb region, which is not included in

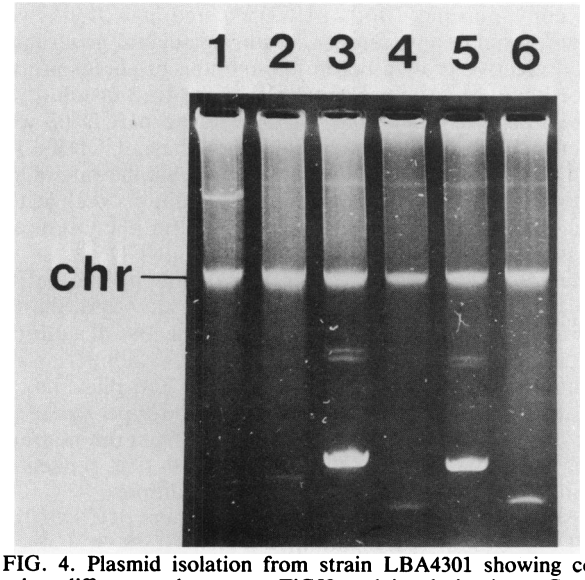

FIG. 4. Plasmid isolation from strain LBA4301 showing copy number differences between pTiC58 and its derivatives. Growth conditions and plasmid isolations were identical for each strain (see text). Lanes: 1, pTiC58; 2, pUCD510; 3, pUCD500; 4, pUCD501; 5, pUCD502; 6, pUCD503. chr, chromosomal DNA. Open circle and linear forms of plasmid DNA are also present in lanes 3 and 5.

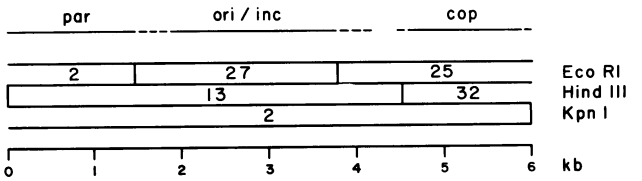

FIG. 5. Linear restriction map of a portion of pTiC58 containing the region of replication and maintenance functions. Shown above the map are the regions involved in: *par*, stable plasmid inheritance; *ori*, origin of replication; *inc*, incompatibility with other *inc*Rh-1 members; and *cop*, copy number control.

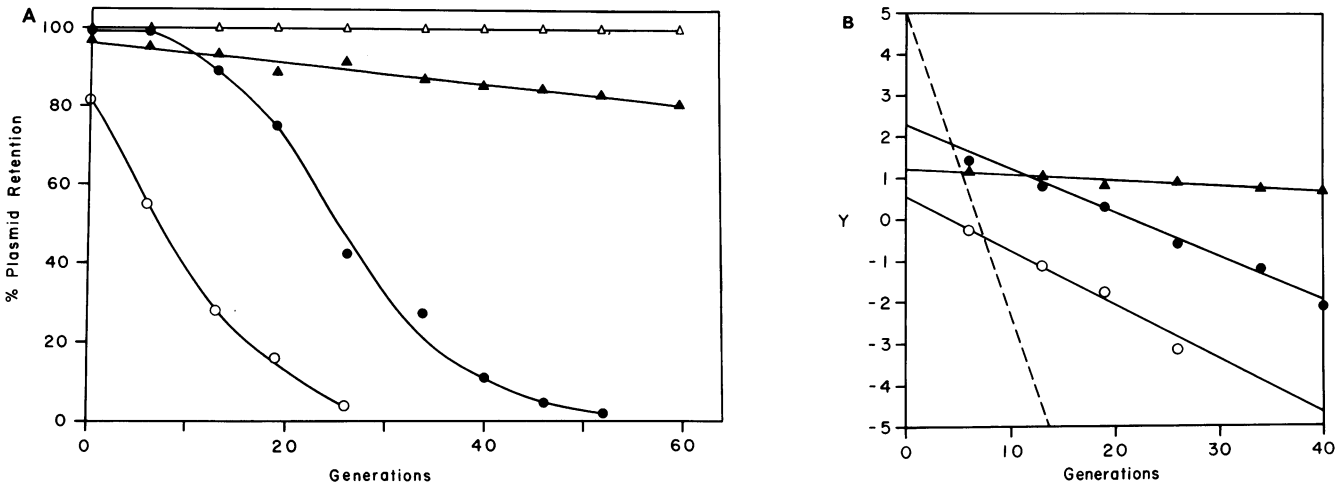

FIG. 6. Segregation of pTiC58 and its derivatives. A. Percentage of LBA4301 cells retaining plasmid plotted as a function of the number of generations of growth under nonselective conditions. The method for determining frequencies is described in the text. B. Replotting of the same data where $Y = \log_e\log_e (1/(1-F_m))$ and F_m is the fraction of the cell population containing plasmid (7). For A. and B.: △, pTiC58 and pTiC58::Tn*5*; ▲, pUCD510; ○, pUCD501 and pUCD503; ●, pUCD500 and pUCD502.

the region containing the minimal information for autonomous replication, is designed *cop* (Fig. 5).

Regions containing *cop* isolated from pUCD500 as an *Sma*I or an *Hin*dIII fragment were cloned into the pSa-derived cloning vector pUCD2 (3). These plasmid constructs showed no change in copy number from that of pUCD2. Complementation of pUCD510 or pTiC58 in trans with the *cop* containing plasmids resulted in no increase of copy number for either pTiC58 or pUCD510, suggesting that *cop* functions solely in a cis relationship.

The copy number of pUCD500 differed from strain to strain. In strain C58, the copy number of pUCD500 was one-fourth the copy number found for pUCD500 in strain LBA4301. In strains Ach5, 15955, A6, 3D12, and 1D1439, the pUCD500 copy number was 1.1, 1.6, 2.0, 1.2, and 2.5 times greater, respectively, than that in strain C58. These differences were not observed with pUCD510 or pTiC58 in these strains, although a twofold increase in copy number would be difficult to detect for a plasmid which exists at one copy per cell. Therefore, it is not known whether these apparent differences were due to actual interaction with the different hosts or were a result of strain-dependent efficiency of plasmid isolation.

Stability. Cultures of *A. tumefaciens* can be routinely grown at 30°C without any observed loss of the Ti plasmid. With growth on nopaline (the sole carbon and nitrogen source), as a phenotypic marker for the presence of pTiC58, no loss of pTiC58 was detected from an LBA4301 culture (0% per generation) (see Fig. 6 and Table 2). The same results were obtained with LBA4301 cells containing pTiC58::Tn*5*, with neomycin resistance as the phenotypic marker. A slow segregation rate of 0.26% per generation was observed for an LBA4301 culture containing pUCD510 which contained only *Hin*dIII fragment 13 of pTiC58, suggesting that some information necessary for complete stable inheritance was lost in the construction of pUCD510. In contrast, pUCD501 was completely lost from LBA4301 cells after 30 generations (3.8% per generation). pUCD510 contained an additional 1.5-kb region of pTiC58 that was not present in pUCD501. This region involved in conferring stability to the pTiC58 origin was designated as *par* (Fig. 5). The *par* region, when present in a pTAR-derived *par*$^-$-cloning vector, was found to stabilize the vector, which possesses an unrelated *ori* (D. Gallie, S. Novak, and C. I. Kado, manuscript in preparation).

The rate of loss per generation of pUCD503 was similar to that of pUCD501 (Table 2). The loss of pUCD500 from LBA4301 cells was phenotypically biphasic, i.e., for the first seven generations of growth under nonselective conditions, the culture exhibited no phenotypic loss of the plasmid (0% per generation), identical to that of the intact parent pTiC58, but by the 13th generation, the rate of loss began to be measurable and soon approached a loss per generation (3.9%) similar to that of pUCD501 (3.8%). After 52 generations of nonselective growth, pUCD500 was completely lost from the cell population. pUCD502 exhibited a rate of loss similar to that of pUCD500. This phenotypically biphasic pattern of plasmid segregation was not surprising for a *par*$^-$ high copy plasmid. Both pUCD500 and pUCD502 were present in high copy number, requiring several generations of nonselective growth before the number of copies present per cell could be diluted enough that the true instability of the plasmid was unmasked. pUCD501 and pUCD503 were present at a much lower copy number than pUCD500 and pUCD502, and therefore, they exhibited a similar rate of loss per generation earlier in nonselective conditions such as that which was seen in the second phase of the phenotypically biphasic pattern noted for pUCD500 and pUCD502.

The rate loss for pUCD500 (*par*$^-$) and its derivatives (*par*$^-$) was replotted as described by Durkacz and Sherratt (7) (Fig. 6B). The broken line represents the loss of a plasmid from a bacterial population after shifting to nonpermissive conditions for plasmid replication. Since the plasmids are *par*$^-$ (pUCD510 coming close to the wild-type *par*$^+$) and their rate loss curves depart significantly from the nonreplicating theoretical curve, the data suggest that replication continues under nonselective growth conditions.

Incompatibility. To determine whether pUCD510 or pUCD500 exhibit incompatibility with pTiC58, *A. tumefaciens* C58 harboring pTiC58 was transformed with pUCD510 or pUCD500 followed by selection for neomycin resistance. *A. tumefaciens* Ach5, 15955, A6, and 1D1439 (a strain capable of catabolizing both octopine and nopaline [24]) and *A. rhizogenes* 3D12 were tested. Figure 7 shows the fate of

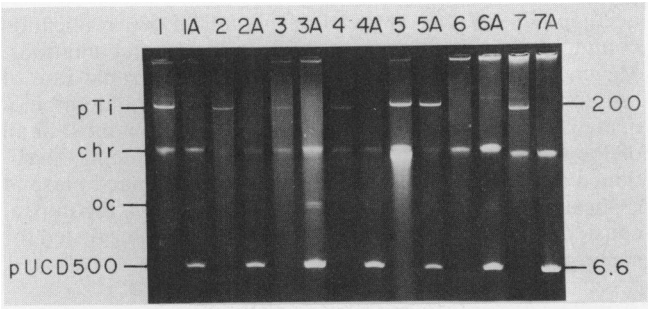

FIG. 7. Incompatibility of pUCD500 with Ti and root-inducing plasmids. Plasmid isolation was carried out by the method of Kado and Liu (15) and separated with agarose gel electrophoresis. Lanes: 1 and 1A, C58 and C58(pUCD500), respectively; 2 and 2A, A6 and A6(pUCD500), respectively; 3 and 3A, Ach5 and Ach5(pUCD500), respectively; 4 and 4A, 15955 and 15955(pUCD500), respectively; 5 and 5A, 3D12 and 3D12 (pUCD500), respectively; 6 and 6A, LBA4301 and LBA4301(pUCD500), respectively; 7 and 7A, 1D1439 and 1D1439(pUCD500), respectively. chr, chromosomal DNA; oc, open circle form of pUCD500. pUCD510 exhibited incompatibility in an identical manner. On the right is a kb scale.

the native plasmids present before and after the introduction of pUCD500. In lanes 1 and 1A, the introduction of pUCD500 into strain C58 resulted in the segregation and loss of pTiC58. Octopine Ti plasmids harbored by strains Ach5, 15955, and A6 in lanes 2, 3, and 4, respectively, were incompatible with pUCD500 as seen in lanes 2A, 3A, and 4A, respectively. On the other hand, pRi12, seen in lanes 5 and 5A, was compatible with pUCD500. This compatibility is consistent with earlier reports (4, 35). Lanes 6 and 6A represent strain LBA4301 before and after the introduction of pUCD500, respectively. As seen in lanes 7 and 7A, pTi1439 was incompatible with pUCD500. In all cases, identical results were obtained when pUCD510 was used in place of pUCD500 (data not shown). Strains C58, A6, Ach5, LBA4301, and 1D1439 contained a cryptic plasmid, which was compatible with pUCD500 or pUCD510.

To test whether the Ti plasmids had completely segregated from their host strains after the introduction and selection for pUCD500, the above strains were assayed for virulence on *K. daigremontiana* or tomato plants. Strains C58, 1D1439, 15955, Ach5, and A6 produced tumors after inoculation, yet after the introduction of pUCD500 no tumors were noted (Table 3). As expected, avirulent strain LBA4301 remained avirulent when harboring pUCD500. Inoculation with strain 3D12 containing pUCD500 continued to produce the hairy root gall symptomatic of *A. rhizogenes* infection. Identical assays with strains harboring pUCD510 in place of pUCD500 gave identical results, suggesting that the curing of Ti plasmids is complete when incompatibility exists.

As pUCD501, pUCD502, and pUCD503 (which contains only the *ori* region) also exhibit incompatibility with pTiC58, the region containing *ori* also contains *inc* (Fig. 5).

DISCUSSION

We mapped the *ori* of pTiC58 and three plasmid maintenance functions: (i) copy number control, (ii) partitioning, and (iii) incompatibility. These functions are clustered, and they flank the 2.6-kb *ori* region of pTiC58 (Fig. 5). In addition to *ori*, this region might encode a specific replication protein, as seen for plasmids such as R1 (18), pSC101 (34), and R6K (10).

Incompatibility. The 2.6-kb region of pTiC58 which contains *ori* also contains the incompatibility function(s), as seen between pTiC58-origin derivatives and various nopaline and octopine Ti plasmids. The hybridization homology of pTiC58 and pTiAch5 is weaker between the origin regions than between the common DNA contained in the T-DNA region of the two plasmids (8). A comparison of the restriction sites between the *ori/inc* region of pTiB6 and pTiAch5 with that of pTiC58 reveals few common sites, also suggesting that the homology within this region is not complete.

Root-inducing and Ti plasmids are compatible in the same host (26, 35), suggesting that they differ extensively in their origin regions. An 8-kb homologous region between pRi2659 and pTiC58 ends just before pTiC58 *Eco*RI fragment 27 (26), a fragment which we found to contain nearly the entire pTiC58 origin region. Therefore, the location of pTiC58 *ori*, situated just beyond a region of homology with a root-inducing plasmid, supports the compatibility data and the idea that the origins of the two plasmids differ extensively.

Copy number. The *cop* mutant, pUCD500, exists at a high level. When the *cop* locus was not present, as in pUCD510 and pUCD501, lower copy levels were observed. The *cop* locus may code for a function involved in the positive regulation of pTiC58 replication. pUCD500 may exist at a high copy level as a result of the deletion during its construction of a function which modulates the expression of the *cop* locus. This modulator would map on pTiC58 outside the region described by pUCD500. Interaction of this putative modulator and the *cop* locus would act to maintain the low copy number of pTiC58. It is not likely that the deletion of the *cop* region, as in pUCD501, impairs a replication function, thus causing greater plasmid instability and simulating lower copy number than that of pUCD500. pUCD510 does not contain the *cop* locus and exhibits low copy number, yet it is quite stable. Deletions made close to *ori* of plasmid pTAR (9) have also resulted in high-copy-number mutant plasmids (D. Gallie, unpublished data), suggesting that similarities in copy number regulation may exist between different *Agrobacterium* plasmids. Although there may be an interaction at some level between *cop* and *par*, they map as separate loci. Also separate are *cop* and *inc*, since the *inc* function is still operational in the absence of the *cop* locus.

The presence of *cop* in a pSa-derived plasmid does not alter the copy number of the plasmid, suggesting that *cop* may be specific as to the type of plasmid it will control. Also, *cop* cannot act in trans to increase the copy number of pUCD510 or pTiC58, indicating that it must be in a cis relationship to function.

The variation of pUCD500 copy number from host to host may indicate an influence of the chromosome or a cryptic

TABLE 3. Curing of Ti and Ri plasmids and virulence of *A. tumefaciens* and *A. rhizogenes* containing pUCD500

Strain	Before introduction of pUCD500:		After introduction of pUCD500:	
	pTi or pRi present	Virulence[a]	Loss of pTi or pRi	Virulence[a]
LBA4301	None	−		−
C58	pTiC58	+	Yes	−
Ach5	pTiAch5	+	Yes	−
A6	pTiA6	+	Yes	−
15955	pTi15955	+	Yes	−
1D1439	pTi1439	+	Yes	−
3D12	pRi12	+	No	+

[a] All strains were tested on *K. daigremontiana*, except for strain 1D1439, which was tested on *L. esculentum*. −, Avirulent; +, virulent.

plasmid, if present, on pUCD500 copy number regulation. Chromosomal mutations within the *Staphylococcus aureus* 1350 genome have led to an increased copy number of pT181, suggesting that chromosomal DNA can play a role in copy number regulation (13). It may also be an indication of the relative efficiency of plasmid isolation from various hosts.

The high copy number of pUCD500 seems to be of a different nature than that of pAL2832, a pTiB6 octopine-plasmid derivative reported to exist at high copy levels (17). The copy number of pAL2832 in strain LBA2832 increased only as the antibiotic concentration, to which it encoded resistance, was increased. Yet at the upper level of antibiotic concentration tested, cell growth was greatly retarded, and cell viability was reduced at least 10-fold (17). These same antibiotic levels did not result in retarded cell growth or loss of cell viability for strains harboring pTiB6-origin derivatives that exhibited with type (i.e., stringent) copy number. In contrast, the high copy number of pUCD500 remained constant regardless of the antibiotic concentration, cell growth of strains containing pUCD500 was not greatly retarded, and cell viability remained unchanged. It may be that pAL2843 is a mutant in a replication or partition function or both. Such a plasmid would be greatly affected in its ability to be stably inherited. The high antibiotic concentration would select for those cells that obtained a greater share of the cell plasmid population as a result of unequal plasmid partitioning. The statistically low chance of this occurrence would account for the low viable count and reduced growth rate of strain LBA4301 containing pAL2832.

Stability. pTiC58 is stably inherited under nonselective conditions, whereas pUCD510 is lost at a rate of 0.26% per generation. That pUCD510 contains a region important in plasmid maintenance is shown by a comparison of its rate of loss to that of pUCD501 (3.8% per generation), a plasmid that is similar to pUCD510 except for the absence of 1.5-kb pTiC58 DNA, the *par* locus, and the fact that this locus can stabilize in cis an unrelated plasmid. Neither pUCD500 nor its derivatives contain the *par* locus of pUCD510. From relative plasmid copy numbers, pUCD500 and its derivatives seem equally unstable. High-copy plasmids that are *par*$^-$ can mimic greater stability or a more efficient partition process simply because random distribution of their numbers can ensure stable inheritance (22). pUCD500, being *par*$^-$, possibly has no mechanism for its active segregation to the daughter cells and, therefore, is distributed randomly to the daughter cell upon host cell divisions. The probability (P_o) of a daughter cell not receiving even a single copy of pUCD500 by the binomial distribution $P_o = 2(1/2)n$ (23) where the copy number $N = 80$ is 1 in 10^{22}, assuming that when a daughter cell receives one or more copies of pUCD500, the copy number is brought up through plasmid replication to 80 copies before the next round of cell division. If this were the case, no loss of pUCD500 from strain LBA4301 should have been detected. Yet after 52 generations under nonselective growth conditions, pUCD500 was completely lost, suggesting either that there are subpopulations of strain LBA4301 which contain lower copy levels of pUCD500 or that pUCD500 replication stops at the onset of nonselective growth conditions. If the latter conclusion was correct, it would take approximately seven generations for the copy number to reach one per cell, after which rapid loss of the plasmid from the cell population would be detected. The loss of pUCD500 initially follows this model since no loss is detected before the seventh generation, but then it diverges significantly from this model once the loss of pUCD500 becomes detectable, suggesting that pUCD500 replication continues after the onset of nonselective growth conditions. Meacock and Cohen (19) have noted a more rapid rate of loss for pSC101 high-copy *par*$^-$ mutants than random binomial distribution would predict. It is possible that most or all of the 80 copies per cell of pUCD500 are randomly partitioned as a unit, and this combined with continued plasmid replication gives a rate loss that differs from theoretical considerations. Such a model has already been suggested for super-*par*$^-$ pSC101 mutants (33).

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