Characterization of the Replication and Stability Regions of *Agrobacterium tumefaciens* Plasmid pTAR

DANIEL R. GALLIE, DAVID ZAITLIN, KEITH L. PERRY,[†] AND C. I. KADO*

Davis Crown Gall Group, Department of Plant Pathology, University of California, Davis, California 95616

Received 29 August 1983/Accepted 1 December 1983

A 5.4-kilobase region containing the origin of replication and stability maintenance of the 44-kilobase *Agrobacterium tumefaciens* plasmid pTAR has been mapped and characterized. Within this region is a 1.3-kilobase segment that is capable of directing autonomous replication. The remaining segment contains the stability locus for maintenance of pTAR during nonselective growth. Approximately 35% of pTAR shares sequence homology with pAg119, a 44-kilobase cryptic plasmid in grapevine strain 1D1119. However, no homology was detected between pTAR DNA and several Ti plasmids or several other small cryptic plasmids in many *A. tumefaciens* strains. A recombinant plasmid containing the origin of replication and stability maintenance region of pTAR was compatible with pTiC58, pTi15955, and pTi119 and incompatible with pAg119. A new compatibility group, Inc Ag-1, is discussed.

Virulent *Agrobacterium tumefaciens* strains harbor large Ti plasmids, which are responsible for crown gall disease (4, 26). Besides Ti plasmids, there are numerous cryptic plasmids ranging from 1 to over 250 megadaltons (8, 17, 27, 32). Most of these plasmids have no known functions, but their presence in *A. tumefaciens* may be of ecological significance for the host bacterium. Many of these plasmids coexist with a Ti plasmid in the same bacterial cell, suggesting that the cryptic plasmid and the pTi are compatible. For example, pAgK84, a plasmid responsible for agrocin 84 biosynthesis (7), harbored by *Agrobacterium radiobacter* K84, can coexist with a Ti plasmid, and shares no sequence homology to pTiC58 or pTi15955 (28). The grapevine strain 1D1422, an unusual biotype 1 Hungarian isolate (31), is able to utilize L-(+)- and DL-tartaric acid (D. Zaitlin, unpublished results). This strain is nononcogenic and harbors the 44-kilobase (kb) plasmid (pTAR) that confers on its host the ability to catabolize tartrate and is compatible with the Ti plasmids pTiC58 and pTiAch5 (J. Kao, personal communication), suggesting that it is functionally different from the Ti plasmids. Preliminary evidence indicates that pTAR may be one representative of a distinct class of *A. tumefaciens* plasmids.

Although the origin of replication of certain Ti plasmids have been mapped in relation to other known functional elements (14), no extensive studies of other *A. tumefaciens* plasmids have been made. In this communication we report on the replication and stability maintenance region of a new *A. tumefaciens* plasmid, pTAR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used are described in Table 1. Phage P1::Tn5 was kindly provided by A. D. Kaiser. Cloning vectors pSa151 and pSa734 were described elsewhere (33). Medium 523 (11) and L-broth (18) were used for liquid cultures and with 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) for solid medium. The plasmid hybrids were maintained in *Escherichia coli* HB101 *recA leu proB1 lacY* (6) received from F. Bolivar. *A. tumefaciens* strains in transformation experiments were grown at 30°C in YEB medium (13).

* Corresponding author.

[†] Present address: Biotechnology Group, Chevron Chemical Co., Richmond, CA 94804.

Ampicillin, carbenicillin, chloramphenicol, kanamycin, neomycin, rifampin, and spectinomycin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of plasmid DNA. *A. tumefaciens* cells were grown to the mid-log phase, at which time carbenicillin was added to a final concentration of 100 µg/ml. The culture was incubated for 60 min at 30°C with shaking, and then plasmid DNA was isolated and purified by the method of Kao et al. (13). Hybrid plasmids of pTAR and pCK1Z were amplified in *E. coli* HB101 with 200 µg of spectinomycin per ml. These and pTAR-pSa151 hybrid plasmids were purified as described by Bolivar (5). The rapid plasmid purification procedures described previously (12, 35) were also used for small quantity isolation of plasmid DNA, which was stored in TE buffer (10 mM Tris, pH 7.4, 1 mM disodium EDTA) at 4°C.

Enzymes. Restriction endonucleases, T4 ligase, and DNA polymerase I were purchased from New England Biolabs (Beverly, Mass.) and Bethesda Research Laboratories (Gaithersburg, Md.). The reaction conditions were as recommended by the supplier. *EcoRI* was purified by S.-T. Liu.

Agarose and polyacrylamide gel electrophoresis. Horizontal slab gels of 0.8 to 1.3% agarose (Sea-Kem) in E buffer (0.08 M Tris-hydrochloride-0.04 M sodium acetate-4 mM disodium EDTA adjusted to pH 7.9 with glacial acetic acid) were used to electrophoretically separate restricted DNA fragments. Plasmid DNA fragments were purified by electroelution from preparative gels (37) and used in further restriction studies. *HindIII* and *HpaI* restriction digests of λ DNA were used as standards in determining the molecular weights of the pTAR restriction fragments.

Vertical slab gels were prepared as 5 to 10% gradient or uniform 5% polyacrylamide (30:0.8 [wt/wt] acrylamide-bisacrylamide) in E buffer. Electrophoresis was carried out at 4 V/cm for 3 h. The standards for molecular weight determination were as described above. DNA fragments under 1 megadalton could be sized with this method.

DNA labeling and molecular hybridization. Plasmid DNA, purified from *A. tumefaciens* strain 1D1119 by CsCl-ethidium bromide buoyant density gradient centrifugation was radiolabeled by nick translation as described by Rigby et al. (24). Samples of 12.5 µCi each of [α -³²P]dCTP and [α -³²P]dATP (specific activity, 1,500 Ci/mmol each) were used in the reaction, which proceeded at 17°C for 30 min. Approximately 60% of the radionucleotides were incorporat-

TABLE 1. *A. tumefaciens* strains used in this study

Strain	Relevant characteristics	Size of plasmids (megadaltons)	Source
12D12	Biotype 1 (var. <i>radiobacter</i>)		This paper via M. Schroth
LBA4301	Rec ⁻ Rif ^r UV ^s	>200 ^a	P. Hookaas and R. A. Schilperoort
CS11	Biotype 1, pTiC58-cured C58	>200 ^a	C. L. Schardl
1D1422 ^b	Biotype 1 (var. <i>radiobacter</i>)	29 ^a	S. Sule
1D1119 ^b	Biotype 1	29 ^a , >120	This paper
15955 ^b	Biotype 1	118	J. E. DeVay
NT1 ^b	Biotype 1	>200 ^a	M.-D. Chilton
29-1 ^b	Biotype 1	27 ^a , 120	This paper
Lodi 1 ^b	Biotype 1	21 ^a , 23 ^a , 120, >200 ^a	This paper
Lodi 3 ^b	Biotype 1	21 ^a , 23 ^a , 120, >200 ^a	This paper
Sinton 4 ^b	Biotype 1	29 ^a , 120, >200 ^a	This paper
C58 ^b	Biotype 1	120, >200 ^a	R. Dickey
27 ^b	Biotype 1	127 ^a , 153	E. W. Nester
9-3 ^b	Biotype 3	124, >200 ^a	This paper
14-1 ^b	Biotype 3	119, >200 ^a	This paper
23-4 ^b	Biotype 3	137, >200 ^a	This paper
24-2 ^b	Biotype 3	136, >200 ^a	This paper
37G4 ^b	Biotype 3	136, >200 ^a	This paper
52BA1 ^b	Biotype 3	120, >200 ^a	This paper
56A2 ^b	Biotype 3	136, >200 ^a	This paper
58-1 ^b	Biotype 3	65 ^a , 135, >200 ^a	This paper
70D4 ^b	Biotype 3	102, 127 ^a , >200 ^a	This paper
73B4 ^b	Biotype 3	142, >200 ^a	This paper
77B2 ^b	Biotype 3	131, >200 ^a	This paper

^a Cryptic plasmid.^b Strains used in plasmid DNA sequence homology study with pTAR.

ed into acid-precipitable material, and the specific activity of the DNA was calculated to be 3×10^7 dpm/ μ g. Highly purified pTAR plasmid DNA was prepared for use as a hybridization probe by end labeling of fragments using [γ -³²P]ATP (specific activity, 1,800 Ci/mmol) and T4 polynucleotide kinase as described by Berkner and Folk (2). A 3- μ g sample of plasmid DNA was hydrolyzed with *Eco*RI and *Hind*III, phenol extracted, and precipitated with 2 volumes of ice-cold ethanol. Labeling was performed under 5'-phosphate exchange conditions in the presence of an excess of ADP. The final specific activity of the DNA was 1.2×10^6 dpm/ μ g.

In situ denaturation and elutive transfer of the electrophoretically separated DNA fragments to nitrocellulose paper were performed by the method of Southern (29). Hybridization conditions were as described by Maniatis et al. (15). Labeled 1D1119 plasmid DNA (5×10^5 dpm per filter) and pTAR DNA (8×10^5 dpm per filter) were used as probes. Filters were incubated for 12 to 15 h at 42°C and were extensively washed in 2 $\times$ SSC (0.3 M NaCl, 0.03 M sodium acetate)–0.5% sodium dodecyl sulfate, followed by 0.1 $\times$ SSC–0.5% sodium dodecyl sulfate at room temperature. The final washes were in the latter solution at 67 to 70°C with three changes over 4 h with gentle agitation. X-ray film (Kodak X-Omat AR) was exposed to the filters at –70°C for 1 to 4 days with a single Dupont Cronex intensifying screen.

Construction of the cloning vehicle pCK1Z. Phage P1 DNA carrying the transposon Tn5 was digested with *Hind*III and *Xho*I. pBR325 plasmid DNA was digested with *Hind*III and *Sal*I. After complete digestion, both DNA samples were purified by phenol extraction and isopropanol precipitation and ligated under conditions outlined by New England Biolabs. Transformation into *E. coli* HB101 was as described by Morrison (19), and the transformed cells were selected on 50 μ g of kanamycin per ml. Restriction digests of purified

plasmids showed that the pBR325 plasmid had acquired the appropriate fragment of the P1::Tn5 DNA that confers high-level kanamycin and neomycin resistance.

Transformation of *A. tumefaciens*. Preparation and transformation of *A. tumefaciens* strains were as described by Holsters et al. (9), but with modifications. Cells were grown at 30°C in 5 ml of YEB until the mid-log phase (5×10^8 cells per ml). After pelleting, the cells were frozen in dry ice-ethanol for 5 min and thawed at 37°C in water for 3 min. Plasmid DNA (4 μ g in no more than 100 μ l of TE buffer) was added to the cells, which were subjected to a second round of freeze and thaw steps of 5 and 25 min, respectively. After the addition of 1 ml of YEB, the cells were incubated at 30°C for 1 h, pelleted, suspended in water, and plated on medium 523 supplemented with the appropriate antibiotics.

RESULTS

Mapping pTAR. To locate the origin of replication on pTAR, an extensive restriction map was constructed (Fig. 1). The unique *Hpa*I site is designated as the 0/44-kb position. *Sma*I did not cut pTAR DNA. Restriction fragment size totals gave a molecular size estimate of 44 kb for pTAR, in close agreement with previous physical contour length measurements by electron microscopy (data not shown). In the methods employed, all fragments greater than 100 base pairs in size have been observed and mapped.

The restriction sites of each enzyme were mapped primarily by way of single, double, and triple enzyme digests and confirmed by way of double digestion with each of the other 13 enzymes. In the case of *Bst*EII, *Pvu*II, *Hind*III, and *Eco*RI, it was necessary to clone pTAR DNA fragments to map the sites of these enzymes, which cut frequently enough to avoid elucidation by double digests alone. As necessary, pTAR fragments were cloned into pSa151, pSa734, and

FIG. 1. Circular restriction map of pTAR. The unique *Hpa*I site has been assigned as 0 kb, and the locations of the other cleavage sites are positioned relative to it. The scale in kb is indicated at the outer perimeter of the map. The letters correspond to the restriction enzymes listed beside the map. The numbers contained within the map designate the pTAR fragments according to size after digestion with an endonuclease. The total size of pTAR is 44 kb.

pCK1Z (see above). *Eco*RI fragments 18, 14a, and 17 were localized to their present position, but these were not ordered in relation to each other as were *Eco*RI fragments 16 and 11.

Within pTAR *Sal*I fragment 2 there appears to be a partially modified *Sal*I recognition site. *Sal*I digests of pTAR DNA regardless of the plasmid preparation or the source of *Sal*I enzyme, resulted in a five-fragment pattern and a size of 55 kb for pTAR. Purified pTAR DNA from 1D1422, when transformed into 15955 or 12D12 and subsequently purified from these strains, yielded identical results. *Sal*I fragment 2 could be cloned as a single piece into *E. coli* vectors pSa734 or pBR325. Digestions with *Sal*I always yielded, in addition to the vector, two fragments identical in size to the two smallest *Sal*I fragments. This suggests a modifying function which acts partially at this particular *Sal*I site within *Sal*I fragment 2 and exists in *A. tumefaciens* 1D1422, 15955, and 12D12, but not *E. coli* HB101. This site is indicated as a dashed line on the map in Fig. 1.

Replication origin region of pTAR. Because the efficiency of transformation of *A. tumefaciens* strains ranges from 10^3 - to 10^4 -fold lower than that of *E. coli* K-12, it was desirable to manipulate pTAR within an *E. coli* host. We were unable to successfully introduce and maintain pTAR or pTAR derivatives in *E. coli* strains. In addition, no intact *A. tumefaciens* Ti or cryptic plasmids have been successfully transferred to *E. coli*, further suggesting that *A. tumefaciens* plasmid

origins do not function in *E. coli*. Conversely, repeated attempts to introduce pBR325 into several *A. tumefaciens* strains also failed, indicating that the pMB1 origin (3) does not function in *A. tumefaciens*. Therefore, an *E. coli* plasmid vector might be used in cloning the pTAR origin, assuming a nonoperative pMB1 origin in *A. tumefaciens*, so that only the clone containing the intact pTAR origin would be able to function. pCK1Z was chosen because of the presence of the neomycin phosphotransferase II gene of Tn5. Many *A. tumefaciens* strains are quite sensitive to neomycin, providing easy selection of transformants. A *Sal*I-*Xho*I double digest of purified pTAR DNA yielding roughly equal-sized fragments was cloned into the *Sal*I site of pCK1Z, only one of which, once transformed into plasmidless strain 12D12 (C. I. Kado, unpublished results), gave rise to many neomycin-resistant colonies. This fragment maps at approximately 24 to 31.5 kb on the pTAR map in Fig. 1. The new plasmid, pUCD400, with both the pTAR origin and pMB1 origin, seems to contain all the plasmid-encoded functions necessary for autonomous replication (Fig. 2).

To eliminate the possibility that this pTAR fragment was enabling the pMB1 origin to function in *A. tumefaciens*, a partial *Hind*III digest of purified pUCD400 DNA was ligated and transformed directly as open circular DNA into 12D12. The resulting plasmid, pUCD450, retains the kanamycin and neomycin resistance genes and the pTAR DNA or pUCD400 and replicates in *A. tumefaciens*, but no longer contains any

FIG. 2. Linear restriction map of pUCD400 and its derivatives showing the location of the pTAR replication region and the stability locus. One of the four *Hind*III sites has been arbitrarily designated as the 0-kb coordinate. The bar above and the legend to the right of the restriction map indicate the source of the different regions of pUCD400. The pUCD400 derivatives are indicated below the map, where a line represents pUCD400 DNA maintained in the derivative. An arrow with pMB1ori represents the insertion of a pMB1 replication origin; an arrow with Nm^r represents the insertion of the Tn5 kanamycin and neomycin resistance gene; pUCD400 derivatives 461, 462, 463 are pUCD460 with the Tn5 kanamycin and neomycin resistance gene inserted at the appropriate arrow. The designations given to each derivative is listed to the right of each derivative as part of a table showing size, drug resistance markers, and relative stability of each derivative in *A. tumefaciens*. The solid black bar above the restriction map indicates the replication origin region, and the line to the right of the black bar represents the area involved in conferring stability to the plasmid in *A. tumefaciens*. a, *Sau*3A partially digested pBR325 DNA was ligated to *Bam*HI digested pUCD450 and transformed in *E. coli* strain HB101, resulting in a 1.8-kb pMB1 origin insert in the *Bam*HI site. b, *A. tumefaciens* var. *radiobacter* strain 12D12 containing a pTAR derivative plasmid was grown for up to 50 generations in 523 broth without selective pressure, at which point the culture was diluted and plated on medium 523. Colonies which appeared were picked onto appropriate drug plates, from which the relative stability of the plasmid was observed. +, Plasmid retained; -, plasmid lost after 50 generations.

pBR325 DNA. It is represented as a bar below the linear restriction map in Fig. 2.

Since pUCD450 represents about 7.5 kb of pTAR DNA, further reductions in size were desired. pUCD460 is a *Hind*III fragment deletion derivative of pUCD400 that no longer contains the neomycin resistance gene and replicates in *A. tumefaciens*. Into separate and partial digests of pUCD460 with *Hind*III and *Eco*RI were ligated *Hind*III and *Eco*RI fragments containing the kanamycin and neomycin resistance gene, respectively. These fragments containing the Tn5 antibiotic resistance gene bordered by either *Hind*III or *Eco*RI sites are approximately 1.4 kb (T. Close and D. Zaitlin, unpublished data). The one *Hind*III site and the two *Eco*RI sites of pUCD460 into which this gene was inserted are shown as arrows along the pUCD460 plasmid in Fig. 2, resulting in the derivatives pUCD461, pUCD462, and pUCD463. In no case did the introduction of the antibiotic gene interfere with pUCD460 replication. Complete digestion of pUCD461 with *Eco*RV, *Eco*RI, *Hind*III, and *Ava*II followed by ligation and transformation into the Rec⁻ strain LBA4301 resulted in plasmids pUCD470, pUCD471, pUCD472, and pUCD473, respectively. Complete digestion

of pUCD462 with *Eco*RI and *Hind*III followed by ligation and transformation into LBA4301 also resulted in plasmids pUCD471 and pUCD472, respectively. In addition to pUCD470, the *Eco*RV derivative of pUCD460, pUCD480, containing two contiguous *Eco*RV pTAR DNA fragments, was also obtained in the transformation into LBA4301.

Because LBA4301 harbors a large cryptic plasmid that might aid pUCD470 in replication by supplying a *trans*-acting function, pUCD470 DNA was isolated from its host, LBA4301, and transformed into strain 12D12. In this host, pUCD470 replicated autonomously as well. The same results were found for pUCD473.

The limits of the origin region deduced from the functional pUCD400 derivatives above are shown by the solid bar above the map in Fig. 2 and in Fig. 4. This region represents 1.3 kb of pTAR DNA. The fact that the complete *Hind*III digestions of pUCD461 and pUCD462 did not yield a plasmid composed of a single *Hind*III fragment suggests that the *Hind*III site exists within or separates integral component(s) required in the replication process.

Characteristics of the pTAR replication region. pUCD400 was readily transformed and maintained in all *A. tumefa*-

ciens strains tested including 12D12, LBA4301, 15955, and CS11. When pUCD400 was transformed into 12D12(pTAR) or 15955(pTAR) and the host was grown under selective pressure for pUCD400, the pTAR plasmid band was no longer observed in agarose gels. The virulent Californian strain 1D1119, a tartrate-utilizing biotype 1 grapevine gall isolate, possesses a 44-kb cryptic plasmid, pAg119, and an octopine type Ti plasmid, pTi119. When 1D1119 was selected for neomycin resistance after transformation with pUCD400, it no longer harbored its native pAg119. This suggests that incompatibility exists between pUCD400 and pTAR and between pUCD400 and pAg119 and that the incompatibility function has been maintained in pUCD400.

Although it has been reported for a number of plasmids (10, 16, 21, 23), the process of stable plasmid inheritance is not well understood. Hosts containing pTAR can be routinely cultured without selective pressure for pTAR with no apparent loss of the plasmid. To test whether pUCD400 or its derivatives (Fig. 2) exhibit the same level of stability, 12D12 containing a pTAR derivative was grown in medium 523 without selective pressure for at least 50 generations. After dilution and plating on medium 523, colonies that appeared were picked onto plates containing the appropriate antibiotic. pUCD400 exhibited complete stability (Fig. 2). Colonies that grew on the antibiotic medium were randomly chosen for quick plasmid isolation to confirm the phenotypic screen. Several pUCD400 derivatives were stably inherited, including pUCD461, pUCD462, and pUCD463. Consistent with the observation that the pMB1 origin is inactive in *A. tumefaciens*, the presence or absence of the pMB1 origin in the pTAR derivatives appears to have little effect on the plasmid.

pUCD410 and pUCD420 were completely lost by the 50th generation, suggesting high instability as was also noted for pUCD470, pUCD471, and pUCD473, plasmids which do not contain the pMB1 origin. This suggests that a region not far from the pTAR origin aids the plasmid in stable inheritance. This region is designated by a line above the restriction map in Fig. 2.

Homology between pTAR and other *A. tumefaciens* plasmids. To determine whether pTAR shared any sequence homology with cryptic *A. tumefaciens* plasmids, a survey revealed several grapevine strains harboring plasmids (Table 1). From these strains, plasmid DNA was isolated, blotted onto nitrocellulose, and probed with nick-translated pTAR DNA. Hybridization was detected weakly with total NT1 DNA and strongly with pAg119 (data not shown).

Purified plasmids isolated from 1D1119 were digested with *Eco*RI, *Hind*III, *Kpn*I, *Sal*I, and *Bgl*II. Whole pTAR DNA was end labeled and used as the hybridization probe. Significant portions of pAg119 DNA have sequence homology with pTAR (Fig. 3). In Fig. 3, lane 5, pAg119 remains uncut by *Bgl*II, and pTi119 is digested into many fragments. The agarose gel containing the digests of total 1D1119 plasmid DNA was not acid treated before blotting onto nitrocellulose to maintain the integrity of DNA fragments of less than 2 kb; therefore the small fragments transferred well, but the whole uncut pAg119 plasmid band in Fig. 3, lane 5, was not transferred efficiently. Even so, the accompanying hybridization lane reveals that pTAR DNA hybridization is limited to pAg119. Thus, pTAR shares no detectable homology with pTi119, and the bands showing homology in lanes 1 through 4 are likely due to sequences contained entirely on pAg119.

The reciprocal hybridization was then performed, and the regions of sequence homology were mapped. pTAR DNA was digested with *Xba*I, *Eco*RI, *Hind*III, and *Pvu*II. Purified

FIG. 3. Analysis of homologous regions between pTAR and pAg119. (A) Ethidium bromide-stained 1% agarose gels and autoradiograms of Southern blots containing 1D1119 plasmid DNA digested with *Eco*RI (lanes 1), *Hind*III (lanes 2), *Kpn*I (lanes 3), *Sal*I (lanes 4), and *Bgl*II (lanes 5). The bright bands correspond to fragments from pAg119 in the gel photograph. The Southern blot was hybridized with γ -³²P-end-labeled pTAR DNA fragments. *Bgl*II cleaves only pTi119, leaving pAg119 as a supercoiled band at the top of lane 5; (B) Purified pTAR DNA digested with *Xba*I (lanes 6), *Eco*RI (lanes 7), *Hind*III (lanes 8), and *Pvu*II (lanes 9). The Southern blot was hybridized with α -³²P-labeled, nick-translated 1D1119 plasmid DNA.

1D1119 plasmid DNA was nick translated and used to probe the pTAR DNA fragments. Figure 3 shows that all four pTAR *Xba*I fragments, *Eco*RI fragments 1, 2, 3, and 6, *Hind*III fragments 3, 4, 5, 6, and 10, and *Pvu*II fragments 1, 2, and 3 share homology with pAg119. *Hind*III fragments 7 and 8b do not hybridize, *Eco*RI fragment 3 hybridizes moderately, and *Hind*III fragment 10 hybridizes weakly, suggesting that these are the limits to the shared homology. The consensus, therefore, indicates a contiguous region of shared homology representing approximately 35% of pTAR, shown as the shaded area in the simplified linear restriction map of pTAR in Fig. 4. The remaining 65% region of pTAR exhibits no detectable homology with 1D1119 plasmid DNA.

DISCUSSION

In the study of initiation and control of *A. tumefaciens* plasmid replication, we chose plasmid pTAR for its compatibility with Ti plasmids and its interesting ability to confer L(+) and DL-tartrate utilization on *A. tumefaciens*. The molecular size is 44 kb and an extensive restriction map of pTAR was determined that revealed a partially modified *Sal*I site and the regions containing the origin of replication and stability maintenance. These regions were isolated by cloning pTAR fragments in *E. coli* with pCK1Z. This recombinant plasmid and a series of deletion and insertion derivatives of this plasmid were subsequently introduced into *A. tumefaciens*, enabling us to separate the pTAR origin of replication region and determine its interaction with the pMB1 origin of pCK1Z. Several small plasmids containing only pTAR DNA and the kanamycin and neomycin resistance gene of Tn5 were then constructed that replicated autonomously in *A. tumefaciens*. These pTAR derivatives define a maximum region necessary for autonomous replication of 1.3 kb. Preliminary experiments with a cell-free coupled transcription-translation system of *A. tumefaciens* (M. Hagiya, T. J. Close, R. C. Tait, and C. I. Kado, submit-

FIG. 4. Simplified linear restriction map of pTAR for five enzymes showing the common DNA region from the hybridization of pAg119 to pTAR. The large shaded region is the consensus of the hybridization data indicating a high degree of sequence homology between the two plasmids. The dotted lines on either side of the shaded region define the limits of sequence homology. The small black bar above the restriction map indicates the replication origin region for pTAR.

ted for publication) indicated that no proteins were synthesized within this region. In comparison, 580 base pairs is required for autonomous replication for pMB1 (1), 2.3 kb is required for the F plasmid (36), 2.5 kb is required for R100 (25), and 1.9 kb is required for pSa (34). Generally, low-copy-number plasmids require 2- to 2.5-kb of DNA for autonomous replication, whereas high-copy-number plasmids require about 1 kb (20). Many low-copy-number plasmids require a coding region for a positive replication function (*rep*), e.g., R6K, *pir* (30); pSa, *repA* (34); pT181, *repC* (22), resulting in a 1- to 1.5-kb increase in size for the minimal amount of DNA required for autonomous replication. Often *rep* proteins exist at low levels, making detection difficult. In addition, the copy number of pTAR remains unaffected by chloramphenicol treatment. Thus, the existence of a pTAR *rep* protein remains possible.

We also found a region near, but not contained within, the region of the origin of replication involved in conferring stability to the plasmid. When present, it enables the plasmid to be stably inherited in the absence of any outside selective pressure for the plasmid. When deletions were made within this locus, noticeable reductions in plasmid maintenance were observed. In like manner, unstable derivatives of pSC101 (16), F (23), R1 (21), or RMS201 (10) are thought to be defective in a partition function (*par*), the mechanism ensuring stable plasmid inheritance to the daughter cells.

Although the stability locus is involved in the stable inheritance of pTAR, it is not possible at this point to describe the region as involved in plasmid partitioning. It is equally plausible that this region specifies an accessory *rep* function, although not essential, allowing for efficient plasmid replication. Deletions affecting this accessory *rep* function would then result in inefficient plasmid replication and the eventual loss of the plasmid from the bacterial population.

Detectable hybridization was found between pTAR and an *SalI* fragment total NT1 DNA and with pAg119, but not between pTAR and the pTi or other cryptic plasmids in *A. tumefaciens*. Strain NT1 harbors the large cryptic plasmid of C58, which upon *SalI* digestion might have yielded this *SalI* fragment. If so, and although the hybridization with pTAR DNA is weak, it is possible that pTAR may have originated as a much larger plasmid.

The strong hybridization of pTAR DNA to pAg119 suggests that pTAR is not unique among *A. tumefaciens* plas-

mids. The hybridization of the two plasmids occurs in a continuous region that comprises 35% of pTAR, with the remaining 65% exhibiting no detectable homology. The region of DNA homology contains the entire replication region for pTAR. Also, pUCD400 was found to be incompatible with pAg119 in 1D1119. Therefore we suggest Inc Ag-1 as a designation for an *A. tumefaciens* incompatibility group, the first two members of which are pTAR and pAg119.

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