JOURNAL OF BACTERIOLOGY, July 1988, p. 3170–3176
0021-9193/88/073170-07$02.00/0
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Vol. 170, No. 7

Minimal Region Necessary for Autonomous Replication of pTAR

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Received 4 January 1988/Accepted 19 April 1988

The native 44-kilobase-pair plasmid pTAR, discovered in a grapevine strain of *Agrobacterium tumefaciens*, contains a single origin of DNA replication confined to a 1.0-kilobase-pair region of the macromolecule. This region (*ori*) confers functions sufficient for replication in *Agrobacterium* and *Rhizobium* species but not in *Pseudomonas solanacearum*, *Pseudomonas glumae*, *Pseudomonas syringae* pv. *savastanoi*, *Xanthomonas campestris* pv. *campestris*, and *Escherichia coli*. *ori* contains a *repA* gene that encodes a 28,000-dalton protein required for replication. Nucleotide sequencing of *repA* and its promoter region revealed four 8-base-pair palindromic repeats upstream of the *repA* coding region. Deletion of these repeats alters *repA* expression and plasmid copy number. Downstream of *repA* are three additional repeats in a region essential for replication. A locus responsible for plasmid partitioning (*parA*) and a putative second locus regulating plasmid copy number are part of the origin region and are required for stable plasmid maintenance.

The replication of many plasmids depends on host functions and on one or more plasmid-encoded proteins (1, 2, 4, 6, 19, 21, 25, 26, 28, 29, 34–36, 38, 40). Other plasmids, such as ColE1 and CloDF13, replicate in the absence of protein synthesis (9, 43). Still others, such as RSF1010 (29, 31) and RK2 (30), encode replication proteins which are required in some hosts but not in others. Interestingly, as an apparent result of plasmids using the replicative machinery of the host, the amount of plasmid-borne information required is kept to a minimum (<3 kilobase pairs [kb]). Production of replication (Rep) proteins is often regulated to maintain the copy number of the plasmid in the cell population without causing a detrimental metabolic effect on the host. Regulation of the *rep* gene is mediated either by the level of Rep protein interacting with upstream sequences, often in the form of direct repeats (1, 2, 19, 25, 26, 34–36), by the action of an RNA (21, 22, 40, 41), or by the interaction of other proteins (42).

The locus responsible for replication can determine the host range of the plasmid. Plasmids of the incompatibility groups P (7), Q (3), and W (37) display broad-host-range features. These plasmids contain specialized origins of DNA replication that apparently play a significant role in extending their host ranges (29, 30).

In our continued interest in plasmid host range specificity, we have focused on the 44-kb narrow-host-range plasmid pTAR, which is naturally harbored in grapevine strains of *Agrobacterium tumefaciens* (14), and have undertaken detailed studies of the origin of the DNA replicative and partitioning features of this plasmid (12, 14). Plasmid pTAR shares no sequence homology with Ti and Ri plasmids and is completely compatible with these tumor- and root-inducing plasmids in *A. tumefaciens* and *Agrobacterium rhizogenes*. The compatibility feature of pTAR made it suitable for the construction of useful stable cloning vectors for *Rhizobium* and *Agrobacterium* spp. (13). Biologically, the plasmid may be responsible for conferring ecological specificity on *A. tumefaciens* strains that infect grapevines since pTAR confers on these strains the ability to utilize tartrate, a substrate abundantly produced by grapevines (44).

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To develop an understanding of the nature of broad- and narrow-host-range plasmids, we have initiated the studies described in this paper to define the minimal region that is required for the autonomous replication of pTAR.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and media. The descriptions and sources of *A. tumefaciens* LBA4301 Rec$^-$ Rm^r and *Escherichia coli* HB101 were given previously (37). Other bacterial strains were from our laboratory collection. The pTAR derivative, pUCD400, was described elsewhere (14). The IncW plasmid pSa and the IncP plasmid RK2 were from laboratory stocks. T4 DNA ligase was prepared by R. C. Tait. *Bal*31 exonuclease and restriction endonucleases were from New England BioLabs, Inc., Beverly, Mass. DNA polymerase I (Klenow fragment) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. *A. tumefaciens* was grown in medium 523 (16), and *E. coli* was cultured in L broth (23), both at 30°C. YEB medium (20) was used for preparing competent *A. tumefaciens* cells. For plating assays, all media were solidified with 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.).

Plasmid purification and transformation. Preparative isolation of plasmid DNA from *A. tumefaciens* was described elsewhere (20). Plasmid DNA was isolated by rapid miniscreening procedures (17, 39). Methods for transformation of *A. tumefaciens* (14) and *E. coli* (24) were previously published.

Plasmid segregation. *A. tumefaciens* cells were grown in 5 ml of medium 523 containing 25 μg of neomycin per ml at 30°C, and 10 μl of culture was transferred successively every 24 h into fresh medium in the absence of neomycin selection. The number of generations was determined by viable cell counts by taking 10-μl samples from each culture, serially diluting them with sterile distilled water, and plating 100 μl of each diluted cell suspension on medium 523, which then was incubated at 30°C until colonies appeared. Plasmid retention was determined as the percentage of colonies that grew on solid media containing neomycin.

Copy number estimation. Copy number determinations were performed as described previously (11). Cells were grown to log phase at 30°C in medium 523 containing 75 μg of neomycin per ml to a density of 10^9 cells per ml, and 0.35-ml portions were analyzed against known concentra-

tions of pUCD500 DNA by electrophoresis in a 0.7% agarose gel at 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 the plasmid copy number were estimated by using the pUCD500 DNA standards and the cell density of the culture.

DNA sequencing. DNA sequence analyses were according to Sanger et al. (27) by using pUC19 as modified by Chen and Seeburg (5).

Electron microscopy. Plasmid DNA was prepared for transmission electron microscopy as described by Sheikholeslam et al. (32) except that uranium was substituted by platinum-paladium (80:20) for rotary shadow casting of the DNA. Analysis of the plasmid replication loops was done by the method of Tait et al. (38). Transmission electron microscope model AEI was used.

Protein analyses by in vitro coupled transcription-translation. *E. coli* S-30 fraction was obtained from Amersham Corporation, Arlington Heights, Ill., and used by the recommendations of the supplier.

RESULTS

Host range. The pTAR derivative, pUCD400, the construction of which is described by Gallie et al. (14), contains the *ori* and *par* loci of pTAR, an aminoglycoside phosphotransferase II (NPTII) gene of Tn*5*, and the pMB1 *ori* and *bom* sites of pBR325. This plasmid lacks transfer functions but can be mobilized with helper plasmids such as pRK2013 (8). Attempts were made to introduce pUCD400 into *Pseudomonas solanacearum* 10D45R, *Pseudomonas glumae* 33D4, *Pseudomonas syringae* pv. *savastanoi* 2015-32R, and *Xanthomonas campestris* pv. *campestris* 84-81R by triparental matings between *E. coli* HB101(pUCD400), HB 101(pRK2013), and the recipient strain. Transfer and maintenance of pUCD400 was successful with members of the family *Rhizobiaceae* but failed with bacterial species outside this family. These experiments suggest that the host range of pTAR is confined primarily to members of the *Rhizobiaceae*, supporting earlier studies on vectors containing *ori* and *par* of pTAR (13).

Defining the pTAR *ori*. Previous studies identified a 1.3-kb (*Eco*RV-*Ava*II) restriction fragment, which contains the entire origin (Fig. 1) (14). Electron microscopic analyses of replicating pUCD400 molecules purified from *A. tumefaciens* LBA4301 showed several replicating intermediates whose characteristic replicating loop structures mapped within or close to the region previously defined as the *ori* locus (Fig. 2). The average position of the replication loops suggested that the right end of the *ori* might be the initiation site (*oriV*).

Deletion derivatives of pUCD481 (Fig. 1) were constructed by *Bal*31 digestions of the left end of the *ori* region, which were followed by blunt ending with T4 DNA polymerase and adding *Eco*RI linkers. These derivatives were transformed into *E. coli* HB101 after ligation. Derivatives pUCD482 through pUCD486 were capable of replicating in *A. tumefaciens* LBA4301, whereas pUCD487 and pUCD488 were unable to do so.

To determine the functional limit of the right end of the *ori* region, the *Ava*II-*Eco*RI fragment of about 894 base pairs (bp) was removed, resulting in pUCD489 (Fig. 1). This deletion derivative was incapable of supporting plasmid

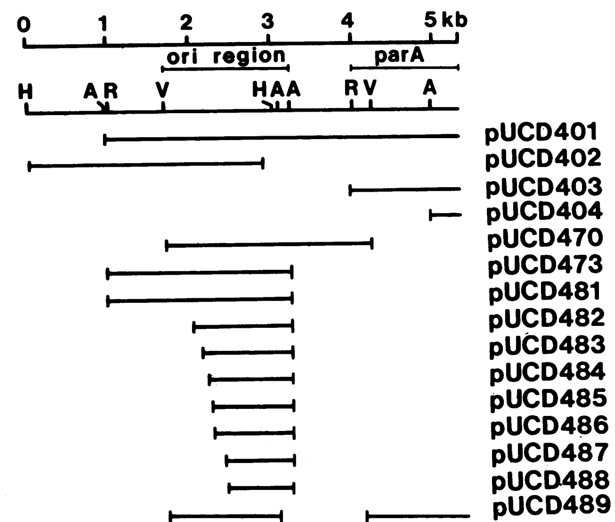


FIG. 1. Plasmid constructs used in pTAR *ori* analysis. Replication region (ori region) and partition locus (parA) as previously delineated (12, 14) are shown above the restriction map of the region. H, *Hin*dIII; A, *Ava*II; R, *Eco*RI; V, *Eco*RV. pTAR DNA present in a construct is represented by a black line. Nondelimited lines extending beyond the 5-kb scale had no origin or partitioning activities. pUCD470, pUCD481, and pUCD489 do not include a pMB1 *ori*, while all other constructs do. pUCD401, pUCD403, and pUCD404 extend approximately 2 kb to the right of *parA*.

replication in LBA4301, while those containing the 125-bp *Ava*II fragment supported replication, suggesting that the *ori* locus extended into this *Ava*II fragment. Restriction analysis of the deletion derivatives described above defined a region of 1.0 kb, represented by pUCD486, that contained all the information necessary for autonomous replication (Fig. 1).

Protein analysis. We used an in vitro *E. coli* coupled transcription-translation system to identify polypeptides encoded by the region containing the origin of DNA replication and other replication-associated functions of pTAR. The plasmid pUCD401, which contains *oriV*, *repA*, and *parA* (12), encodes at least two gene products: *repA* and *parA*. When pUCD401 was used as the template, four proteins of approximately 20,000, 24,500, 25,000, and 34,000 daltons (Da) were synthesized (Fig. 3, lane 1). The protein of 25,000 Da was identified previously as ParA (12), and for reasons given below, the 34,000-Da protein, which is observed above the NPTII gene product in Fig. 3, was designated as the putative RepA protein. A protein of 20,000 Da was found to be encoded by another locus situated to the right of the known *parA* locus and extends beyond the region designated as *ori* in Fig. 1 (data not shown). The function of this protein, which has not been identified, is not required for replication or partitioning. The 24,500-Da protein (as discussed below) was also found not to be required for replication. When the deletion derivative plasmid pUCD403 lacking *ori* (Fig. 1) was used as template, the synthesis of the 34,000-Da product was not detected (Fig. 3, lane 2). When the deletion was extended into the *parA* locus (pUCD404, Fig. 1), the 25,000- and 34,000-Da proteins were not detected (Fig. 3, lane 3).

To confirm that the 34,000-Da protein is indeed encoded by *repA*, plasmid pUCD402 containing a truncated *repA* and no *parA* locus was used as a template. The 34,000-Da protein and the 25,000-Da ParA protein were not synthesized (Fig. 3, lane 4). However, the 24,500-Da protein, previously seen with pUCD401, was detected. For further confirmation,

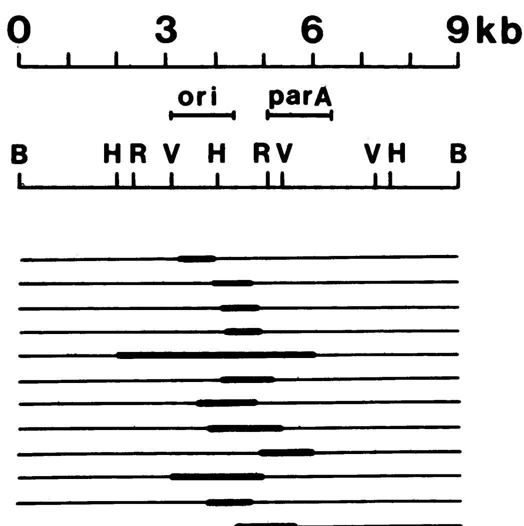


FIG. 2. Analysis of replicative intermediates of pUCD400 derivatives purified from *A. tumefaciens* LBA4301. Replication intermediates of pUCD400 were prepared and digested with *Sal*I (site not shown; cf. reference 12) to generate linear molecules, which were spread in a hyperphase solution containing 50 μl of formamide, 20 μl of 1 M Tris hydrochloride (pH 8.0), 0.1 M EDTA, 40 μl of 1-μg/ml DNA, and 3 μl of 1% cytochrome *c*. Water was used as a hydrophase to maximize linear expansion. The hyperphase was retrieved on a film of 3% Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) on 300-mesh copper electron microscope grids. The grids were immersed in 5 mM uranyl acetate (in 0.05 M HCl in 90% ethanol) for 30 s and then in 95% ethanol for 5 s, air dried, rotary shadowed with platinum-paladium, and examined by electron microscopy. The replication bubbles are represented by a heavy line. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; V, *Eco*V.

pUCD481 and pUCD473 containing only *repA* (Fig. 1) were found to direct the synthesis of the 34,000- and 24,500-Da proteins (Fig. 3, lanes 6 and 9). The deletion derivative pUCD486, containing the right-hand portion of *repA* in the *ori* region (Fig. 1), directed the synthesis of the 34,000-Da

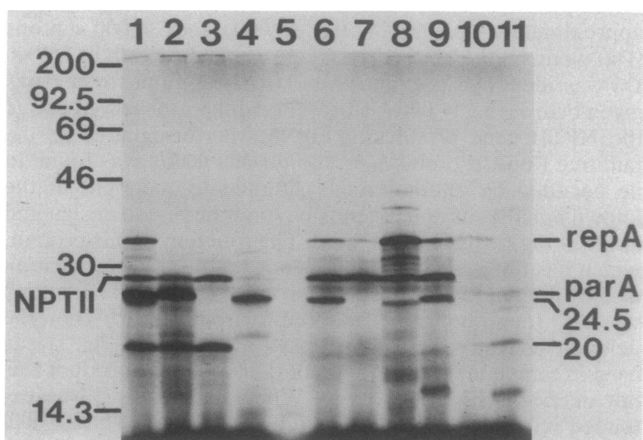


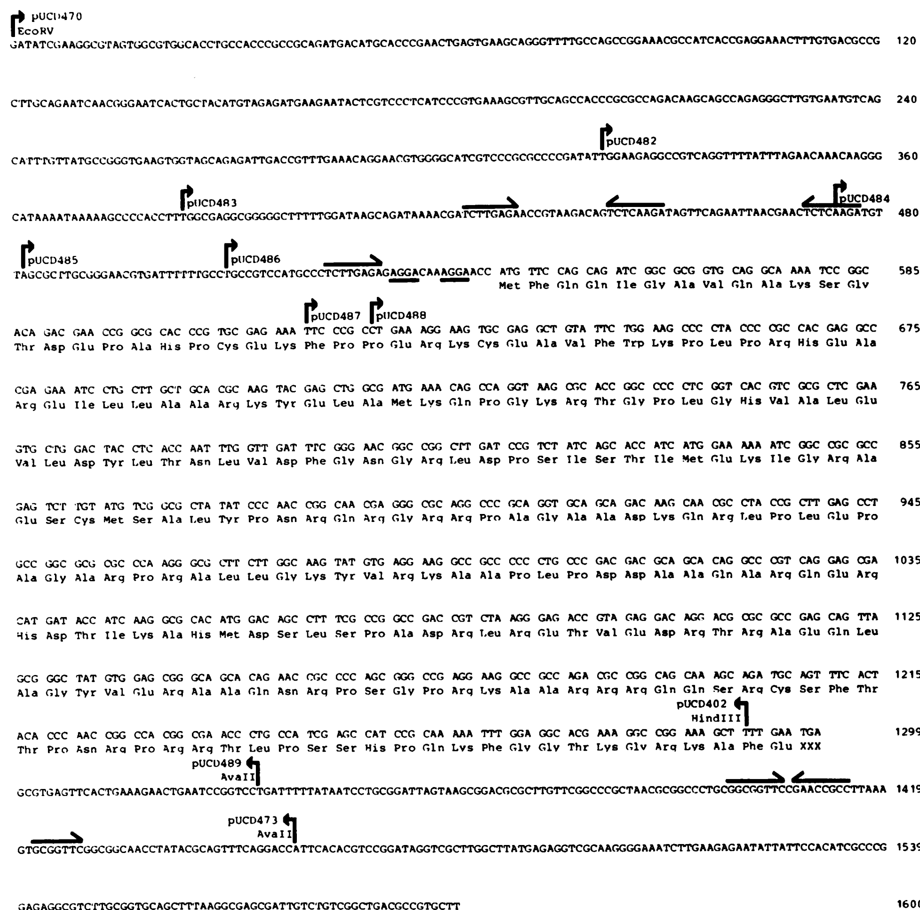
FIG. 3. *E. coli* S-30 in vitro protein analysis of the pTAR *ori* region. Lane 1, pUCD401; lane 2, pUCD403; lane 3, pUCD404; lane 4, pUCD402; lane 5, molecular weight standards with respective weights (in thousands) indicated on the left; lane 6, pUCD481; lane 7, pUCD486; lane 8, pUCD470; lane 9, pUCD473; lane 10, pUCD489; lane 11, pTAR. NPTII is indicated on the left. Proteins encoded by the origin region of pTAR are indicated on the right.

but not the 24,500-Da protein (Fig. 3, lane 7), indicating that this portion encodes the 34,000-Da product. To further define the locus encoding the 34,000-Da RepA protein, pUCD489, containing a 125-bp deletion of the right side of the *ori* region, was used as a template. As observed in lane 10 of Fig. 3, the 34,000-Da protein was synthesized, indicating that RepA is encoded by the *repA* locus in a region of less than 1 kb. The data also indicate that the locus encoding the 24,500-Da protein is to the left of the *repA* locus. For the deletion derivatives of pUCD481 that were unable to support replication in *A. tumefaciens*, RepA was not produced. Constructs which produced RepA but not the 24,500-Da protein remained replication proficient (data not shown). The 24,500-Da protein, therefore, appears to be not required for replication, but its role as an ancillary regulatory element has not been ruled out in these studies.

DNA sequence. The nucleotide sequence of each derivative of pUCD481 was determined to precisely locate the extent of each deletion. To facilitate sequencing, an *Eco*RI site was added to the *Bal*31-deleted end of each derivative, allowing an *Eco*RI-*Hin*dIII fragment from each derivative to be introduced into the *Eco*RI-*Hin*dIII sites of pUC19, in which the fragments were sequenced. The position of each deletion derivative is indicated in Fig. 4. The *Bal*31-created terminus in pUCD486, which contains the largest deletion and yet retains replicative function, begins 509 bp to the right of the *Eco*RV site, whereas the nonreplicative derivative, pUCD487, has an additional 106 bp removed. The replicative-proficient pUCD486 contains 37 bp of pTAR DNA upstream to the start codon of an open reading frame (ORF). The replicative-deficient pUCD487 contains a 23-codon deletion from this start site into the coding region.

The ORF in pUCD486 has a coding capacity for a 28,000-Da protein; this ORF begins at base 547 and finishes just beyond the *Hin*dIII site at base 1299 (Fig. 4). The ORF is in the same position as the locus which encodes the 34,000-Da RepA protein. The obvious discrepancy in the size estimate of this protein (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis) may be explained by the composition of the protein as deduced from the nucleotide sequence. The protein has 20 more basic amino acid residues than acidic ones and would therefore contain a substantial net positive charge, which can cause anomalous migration during electrophoresis.

There are two purine-rich regions (5′-AGGA-3′), 3 and 10 bp upstream of the putative RepA start codon. These regions are homologous in sequence and position to the ribosome-binding site preceding translated reading frames of enteric bacteria (33). Contained within the 125 bp located immediately upstream of the ORF are four 8-bp repeats (designated by half arrows over the sequence in Fig. 4), ordered as two sets of inverted repeats. These repeats are also imperfect palindromes; the three bases on either end of the sequence 5′-TCTTGAGA-3′ of these repeats are complementary. Within this same region, there exist several candidates for the −35 and −10 components of a promoter. The existence of the four repeats in an area which probably also serves as the *repA* promoter suggests their involvement in regulation of the gene. At 120 bp downstream from the stop codon of *repA* are two inverted 8-bp repeats (5′-GGCGGTTC-3′), separated by a single base pair. A truncated third repeat lies 7 bp downstream from the first two. This family of repeats is unrelated in sequence to the four repeats found upstream of *repA* and lacks palindromic potential within the repeat itself. However, this family of repeats is located within the 125-bp *Ava*II fragment known to be essential for replication.



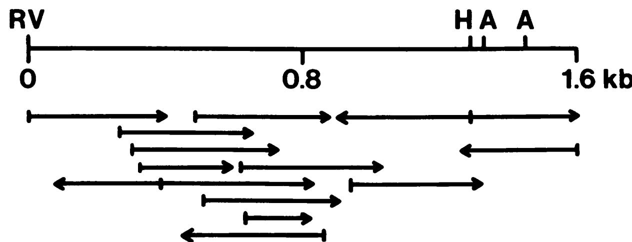


FIG. 4. pTAR *ori* DNA sequence and amino acid sequence of the open reading frame corresponding to *repA*. The sequencing strategy is shown below. The site at which each deletion derivative maps is indicated above the sequence with a bent arrow. Repeats are indicated by straight half arrows. The possible Shine-Dalgarno region is underlined. H, *Hin*dIII; A, *Ava*II; RV, *Eco*RV.

Within the pTAR origin region, there exists no sequence analogous to the primosome assembly site of F, φX174, and ColE1 (5'-GTGAGCG-3') (15). Nor is there any sequence or set of repeats analogous to the family of repeats 5'-(G/T)GAG(A/G)G-3' found in the origin region sequences of bacteriophage lambda and plasmids R6K, mini-F, RK2, and P1 (10). However, the sequence from base 401 to 409 on the opposite strand to that shown in Fig. 4 is similar to the *dnaA*-binding-site sequence 5'-TTAT(A/C)CA(A/C)A-3', which is found in the origin region of pBR322, ColE1, pSC101, R100, R1, CloDF13, P1, F, and *oriC* of the *E. coli* chromosome (18).

Copy number. pTAR exists in *A. tumefaciens* strains under stringent copy number control (14). To determine whether the region upstream of the pTAR *repA* gene might be involved in copy number control, pUCD481 and its derivatives were analyzed for alterations in copy number (Table 1). pTAR is under stringent copy number control in *A. tumefaciens* and is present at 1 to 2 copies per cell. The absence of the pTAR *par* gene leads to apparent copy numbers of less than 1. This reflects a change in the percentage of cells harboring the plasmid and not in copy number (13). pUCD473, which lacks *parA* but contains *repA*, was assigned a value of 1 for comparative purposes in Table 1. pUCD481, identical to pUCD473 as well as containing the origin of pBR325, remained at a copy number equivalent to that of pUCD473. Moreover, the copy number remains unaffected by the deletions represented by pUCD482 and pUCD483. These derivatives still possess the region containing the palindromic repeats upstream of *repA*

TABLE 1. Copy number mutants of pTAR

Plasmid	Copy number
pUCD473	1
pUCD470	0.8
pUCD481	1
pUCD482	1
pUCD483	1
pUCD484	5
pUCD485	4
pUCD486	12

and likely contain a locus that controls pTAR copy number. As the deletions are progressively increased as in pUCD484, pUCD485, and pUCD486, the copy numbers increased to 5, 4, and 12, respectively. Since this region probably contains the *repA* promoter and may also serve as the regulatory site for the gene, deletions which alter these functions will alter RepA production; i.e., the loss of *repA* results in the loss of RepA protein, whose function likely regulates pTAR copy number. The efficiency of RepA in maintaining low copy number is decreased by progressive truncation of *repA*.

Plasmid stability. We have shown previously that the *par* gene of pTAR completely stabilizes pTAR and its derivatives (12, 13). Derivatives of pTAR containing only the pTAR *oriV* and not the *par* gene are relatively unstable in the cell population under nonselective conditions for the plasmid. In addition, there are other plasmid-related factors, such as alterations in copy number under the control of the *cop* locus and mutations within the region controlling replication, that will affect the rate of loss of *par*-deficient plasmids. Figure 5 illustrates the effect on plasmid stability caused by mutations in the *ori* region. For instance, pUCD473 containing *oriV* but not *par* shows a typical rate of loss for a *par* mutant derivative as compared with that of pTAR itself (Fig. 5A), and pUCD470 exhibiting low copy number and altered RepA is lost more rapidly than pUCD473 from the cell population. Although the pBR325 *oriV* is nonfunctional and does not alter plasmid copy number in *A. tumefaciens*, its presence results in a substantial rate of loss for pTAR *par* mutant derivatives. This is demonstrated by pUCD481 (Fig. 5B), which is equivalent to pUCD473 but contains *oriV* of pBR325. When pTAR *par* is present, there is no detectable loss of the plasmid (pUCD401 in Fig. 5B). Deletion derivatives of pUCD481 exhibiting unaltered copy number (e.g., pUCD482 and pUCD483) showed rates of loss similar to that of pUCD481. For those plasmids which exhibited increased copy number (e.g., pUCD484, pUCD485, and pUCD486), the rate of loss decreased significantly, in accordance with the general observation that a high-copy-number *par* mutant plasmid is lost at a slower rate than a low-copy-number *par* mutant plasmid.

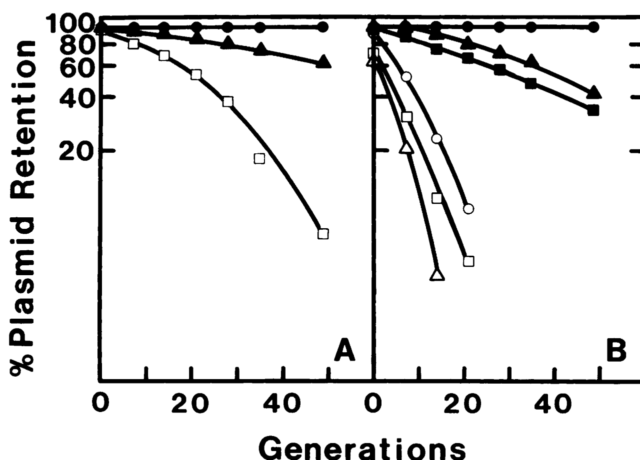


FIG. 5. pTAR *ori* mutations which affect plasmid stability. The assay is described in Materials and Methods. Percent plasmid retention equals the percentage of the cell population which still harbors the plasmid. The *x* axis represents the number of generations of growth under nonselective conditions. Panel A: ●, pTAR; ▲, pUCD473; □, pUCD470. Panel B: ●, pUCD401; ▲, pUCD486; ■, pUCD484 and pUCD485; ○, pUCD483; □, pUCD482; △, pUCD481.

DISCUSSION

In our present study, we have defined a 1-kb region of pTAR that is required for the replication. Nucleotide sequence analyses have revealed an ORF in this region that has a coding capacity for a protein of 28,000 Da. Deletion analyses of pTAR derivatives by using a coupled transcription-translation system suggested that a protein of 34,000 Da (as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is synthesized from this ORF region; its loss results in the concomitant loss in plasmid replication. On the basis of the high basic amino acid content of this protein, which we designate as RepA, it is possible that the electrophoretic size determinations were an overestimate of RepA. Indeed, deletion mutants delineate a 823-bp region, encompassing the 753-bp ORF for the 28,000-Da protein, which produces a single protein that migrates at 34,000 Da in sodium dodecyl sulfate-polyacrylamide gels. Because the host range of pTAR is limited to members of the *Rhizobiaceae*, studies of RepA, *oriV*, and a putative copy control locus may provide a means for elucidation of the intriguing feature of host range limitation of bacterial plasmids.

The repeated sequences directly upstream of *repA* are of special interest since they may serve as binding sites for proteins needed for the regulation of *repA*. Directly upstream from *repA* are four 8-bp palindromic repeats in a region where the *repA* promoter is presumably located. Deletion of these repeats elevates the copy number of pTAR, and, therefore, this region likely contains a locus for controlling plasmid copy number. A second set of repeats, unrelated to those upstream of *repA*, were found 120 bp downstream from *repA* in a 125-bp region that is essential for replication and may comprise *oriV*. As this region does not appear to have any protein-coding potential, we can only speculate on the role of this region in replication.

Interestingly, comparisons of published repeated nucleotide sequences in phage lambda and plasmids RK2, mini-F, P1, and R6K (10) with the repeats in the origin region of pTAR revealed no similarities. Also, there is a lack of sequence homology between *repA* and the *rep* genes of these plasmids. Nonetheless, the RepA protein of pTAR is similar in size to the Rep proteins of the above plasmids and to those of pSa (38), RSF1010 (31), and pT181 (26). Whether or not the repeated sequences play an important role in host range specificity remains to be elucidated.

ACKNOWLEDGMENTS

We thank Jeff Hall and Paul Hara for capable technical assistance. This work was supported by Department of Health and Human Services grant CA-11526 from the National Cancer Institute.

LITERATURE CITED

1. **Abeles, A., K. M. Snyder, and D. Chattoraj.** 1984. P1 plasmid replication: replicon structure. J. Mol. Biol. **173**:307–324.
2. **Armstrong, K. A., R. Acosta, E. Ledner, Y. Machida, M. Pancotto, M. McCormick, H. Ohtsubo, and E. Ohtsubo.** 1984. A 37×10^3 molecular weight plasmid-encoded protein is required for replication and copy number control in the plasmid pSC101 and its temperature-sensitive derivative pHS1. J. Mol. Biol. **175**:331–347.
3. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618–630.
4. **Chattoraj, D. K., K. M. Snyder, and A. L. Abeles.** 1985. P1 plasmid replication: multiple functions of RepA protein at the origin. Proc. Natl. Acad. Sci. USA **82**:2588–2592.
5. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**: 165–170.
6. **Churchward, G., P. Linder, and L. Caro.** 1983. The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101. Nucleic Acids Res. **11**:5645–5659.
7. **Datta, N., and R. W. Hedges.** 1972. Host ranges of R-factors. J. Gen. Microbiol. **70**:453–460.
8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
9. **Donoghue, D. J., and P. A. Sharp.** 1978. Replication of colicin E1 plasmid DNA in vivo requires no plasmid-encoded proteins. J. Bacteriol. **133**:1287–1294.
10. **Filutowicz, M., M. McEachern, A. Greener, P. Mukhopdhyay, E. Uhlenhopp, R. Durland, and D. Helinski.** 1985. Role of the initiation protein and direct nucleotide sequence repeats in the regulation of plasmid R6K replication, p. 125–140. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
11. **Gallie, D. R., M. Hagiya, and C. I. Kado.** 1985. Analysis of *Agrobacterium tumefaciens* plasmid pTiC58 replication region with a novel high-copy-number derivative. J. Bacteriol. **161**: 1034–1041.
12. **Gallie, D. R., and C. I. Kado.** 1987. *Agrobacterium tumefaciens* pTAR *parA* promoter region involved in autoregulation, incompatibility, and partitioning. J. Mol. Biol. **193**:465–478.
13. **Gallie, D. R., S. Novak, and C. I. Kado.** 1985. Novel high and low copy stable cosmids for use in *Agrobacterium* and *Rhizobium*. Plasmid **14**:171–175.
14. **Gallie, D. R., D. Zaitlin, K. L. Perry, and C. I. Kado.** 1984. Characterization of the replication and stability regions of *Agrobacterium tumefaciens* plasmid pTAR. J. Bacteriol. **157**: 739–745.
15. **Imber, R., R. L. Low, and D. S. Ray.** 1983. Identification of a primosome assembly site in the region of the *ori2* replication origin of the *Escherichia coli* mini-F plasmid. Proc. Natl. Acad. Sci. USA **80**:7132–7136.
16. **Kado, C. I., M. G. Heskett, and R. A. Langley.** 1972. Studies on *Agrobacterium tumefaciens*: characterization of strains 1D135 and B6, and analysis of the bacterial chromosome, transfer RNA and ribosomes for tumor-inducing ability. Physiol. Plant Pathol. **2**:47–57.
17. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
18. **Kaguni, J. M., L. L. Bertsch, D. Bramhill, J. E. Flynn, R. S. Fuller, B. Funnell, S. Maki, T. Ogawa, K. Ogawa, A. van der Ende, and A. Kornberg.** 1985. Initiation of replication of the *Escherichia coli* chromosomal origin reconstituted with purified enzymes, p. 141–150. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
19. **Kamio, Y., A. Tabuchi, Y. Itoh, H. Katagiri, and Y. Terawaki.** 1984. Complete nucleotide sequence of mini-Rts1 and its copy mutant. J. Bacteriol. **158**:307–312.
20. **Kao, J. C., K. L. Perry, and C. I. Kado.** 1982. Indoleacetic acid complementation and its relation to host range specifying genes on the Ti plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. **188**:425–432.
21. **Khan, S., S. Carleton, and R. P. Novick.** 1981. Replication of plasmid pT181 DNA in vitro: requirement for a plasmid coded product. Proc. Natl. Acad. Sci. USA **78**:4902–4906.
22. **Lacatena, R. M., and G. Cesareni.** 1981. Base pairing of RNA I with its complementary sequence in the primer transcript inhibits ColE1 replication. Nature (London) **294**:623–626.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Morrison, D. A.** 1979. Transformation and preservation of competent bacterial cells by freezing. Methods Enzymol. **68**: 326–331.
25. **Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami.** 1981. Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene **15**: 257–271.
26. **Novick, R. P., G. K. Adler, S. Majumder, S. A. Khan, S. Carleton, W. Rosenblum, and S. Iordanescu.** 1977. Coding sequence for the pT181 *repC* product: a plasmid-encoded protein uniquely required for replication. Proc. Natl. Acad. Sci. USA **79**:4108–4112.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Scherer, G.** 1978. Nucleotide sequence of the O gene and of the origin of replication in bacteriophage λ DNA. Nucleic Acids Res. **5**:3141–3156.
29. **Scherzinger, E., M. M. Bagdasarian, P. Scholz, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1984. Replication of the broad host range plasmid RSF1010: requirements of three plasmid-encoded proteins. Proc. Natl. Acad. Sci. USA **81**:654–658.
30. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Host range replication and maintenance determinants of *Inc*P group plasmid RK2, p. 874. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
31. **Scholz, P., V. Haring, E. Scheringer, R. Lurz, M. M. Bagdasarian, H. Schuster, and M. Bagdasarian.** 1985. Replication determinants of the broad host-range plasmid RSF1010, p. 243–259. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
32. **Sheikholeslam, S., B. C. Lin, and C. I. Kado.** 1979. Multiple-size plasmids in *Agrobacterium radiobacter* and *A. tumefaciens*. Phytopathology **69**:54–58.
33. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254**:34–38.
34. **Smith, C. A., and C. M. Thomas.** 1984. Nucleotide sequence of the *trf*A gene of broad host range plasmid RK2. J. Mol. Biol. **175**:251–262.
35. **Stalker, D. M., R. Kolter, and D. R. Helinski.** 1982. Plasmid R6K DNA replication. I. Complete nucleotide sequence analysis of an autonomously replicating segment. J. Mol. Biol. **161**: 33–43.
36. **Stalker, D. M., C. M. Thomas, and D. R. Helinski.** 1981. Nucleotide sequence of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181**:8–12.
37. **Tait, R. C., T. J. Close, R. C. Lundquist, M. Hagiya, R. L. Rodriguez, and C. I. Kado.** 1983. Construction and characterization of a versatile broad host range DNA cloning system for Gram-negative bacteria. Bio/Technology **1**:269–275.
38. **Tait, R. C., T. J. Close, R. L. Rodriguez, and C. I. Kado.** 1982. Isolation of the origin of replication of the *Inc*W-group plasmid pSa. Gene **20**:39–49.
39. **Tait, R. C., R. C. Lundquist, and C. I. Kado.** 1982. Genetic map of the crown gall suppressive *Inc*W plasmid pSa. Mol. Gen. Genet. **186**:10–15.
40. **Tolun, A., and D. R. Helinski.** 1982. Separation of the minimal replication region of the F plasmid into a replication origin

segment and a trans-acting segment. Mol. Gen. Genet. **186**:372–377.
41. **Tomizawa, J., T. Itoh, G. Selzer, and T. Som.** 1981. Inhibition of ColE1 RNA primer formation by a plasmid-specific small RNA. Proc. Natl. Acad. Sci. USA **78**:1421–1425.
42. **Tsurimoto, T., and K. Matsubara.** 1981. Purified bacteriophage O protein binds to four repeating sequences at the λ replication origin. Nucleic Acids Res. **9**:1798–1799.
43. **Veltkamp, E., and A. R. Stuitje.** 1981. Replication and structure of the bacteriocinogenic plasmids Clo DF13 and Col E1. Plasmid **5**:76–99.
44. **Williams, M., and F. A. Loewus.** 1978. Biosynthesis of (+)-tartaric acid from L-[4-^{14}C]ascorbic acid in grape and geranium. Plant Physiol. **61**:672–674.