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Mutants of the Mini-F Plasmid pML31 Thermosensitive in Replication

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Hydroxylamine mutagenesis was used for the induction of thermosensitive replication mutants of the mini-F plasmid pML31. Replication mutants were characterized by studying the segregation kinetics and the incorporation of [^{3}H]-thymidine into plasmid deoxyribonucleic acid at the nonpermissive temperature. Based on these experiments two types of mutants could be distinguished. Mutants of type I are fast segregating with the kinetics expected if plasmid replication was blocked immediately. Double-label experiments showed a rapid shut-off of replication in these mutants at 42°C. Mutants of type II segregate slower, showing only a partial inhibition of plasmid deoxyribonucleic acid synthesis at the nonpermissive temperature. The label incorporated at 42°C was predominantly found in open circular plasmid molecules.

The mini-F plasmid pML31 is a hybrid plasmid consisting of the *Eco*RI-generated, 9-kilobase (kb) fragment V of the F factor and a nonreplicating *Eco*RI fragment of pSC105 (6.75 kb) carrying kanamycin resistance (22). pML31 contains the replication region of F between coordinates 40.3 and 49.3 kb (14) and behaves like the F factor with respect to low copy number, F1-specific incompatibility, curing by acridine orange (22), and integrative suppression (27). In plasmid pML31 the origin of replication is located at F coordinate 42.6 kb and replication is bidirectional (9). When a restriction endonuclease (*Bam*HI)-generated fragment with 2.4-kb length containing this origin is deleted, a second origin located at F coordinate 44.4 kb is activated (10). Using the restriction endonuclease *Kpn*I, the region of pML31 essential for autonomous replication has been narrowed down to a 3-kb fragment between F coordinates 43.8 and 46.8 kb (10).

However, other DNA fragments of mini-F generated by restriction endonuclease *Bam*HI or *Pst*I, each carrying one of the two origins of replication, were not able to replicate (Eichenlaub, unpublished data). This can be interpreted to mean that the mini-F plasmid, in addition to the origin of replication, has to provide a function necessary for its replication. This function may not be present on either of these fragments. The isolation of a thermosensitive F'*lac* factor by Cuzin and Jacob (5) suggests that, indeed, plasmid-coded functions are involved in the maintenance of the F factor in a bacterial population. The isolation of conditional plasmid mutants affecting plasmid DNA replication may thus contribute to the understanding of the mechanism and control of this process.

In this report I describe the isolation of thermosensitive replication mutants of mini-F plasmid pML31, using hydroxylamine mutagenesis in vitro. These plasmid mutants were studied with respect to their segregation kinetics and DNA replication at the nonpermissive temperature. Two types of plasmid mutants were isolated. How these mutations possibly interfere with plasmid replication is discussed.

MATERIALS AND METHODS

Media and bacterial strains. *Escherichia coli* strains C600(pML31) and *E. coli* C600(ColE1) were obtained by transformation with plasmid DNA. C600 has the genotype *thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ^-. *E. coli* MA1091 Hfr *inc leu thi recA* was obtained from W. K. Maas. Bacterial cultures were grown in liquid L-broth medium or on L-broth agar plates containing 15 g of agar per liter. Selective medium contained 50 μg of kanamycin per ml. M9 medium was supplemented with 0.5% Casamino Acids.

Hydroxylamine mutagenesis and isolation of plasmid mutants. pML31 DNA isolated as described (lysozyme-Triton lysates) (20) was treated with hydroxylamine as reported by Humphreys et al. (17) with minor modifications: 10 μl of purified pML31 DNA (1 to 2 μg) in TES (Tris-hydrochloride, pH 7.5-50 mM NaCl-5 mM EDTA) buffer was added to 50 μl of 0.1 M sodium phosphate buffer, pH 6.0; then 40 μl of 1 M hydroxylamine, pH 6.0, was added, and the mixture was incubated for 30 min at 75°C and then dialyzed against 2 liters of TES buffer with two changes. In the control, hydroxylamine was omitted and an equal volume of 0.1 M sodium phosphate buffer was added. DNA samples, 50-μl, were used for transformation of *E. coli* C600 as described by Cohen et al.

(2), except that the $CaCl_2$ concentration was 100 mM and the heat treatment was for 3 min at 42°C. The transformed culture was aerated for 1.5 h at 25°C to allow expression of plasmid genes and then plated on L-broth-kanamycin plates and incubated at 25°C for 2 days.

Kanamycin-resistant (Km^r) clones were picked, transferred to fresh L-broth-kanamycin plates, and incubated at 25 and 42°C, respectively. Clones not growing at 42°C were tested for plasmid segregation.

Test for plasmid segregation. The segregation kinetics were followed by growing cells containing a mutant plasmid at 42°C in L-broth medium and plating dilutions in parallel on L-broth and L-broth-kanamycin plates at 30-min intervals.

Plasmid DNA replication at 25 and 42°C. *E. coli* C600 harboring plasmids pML31 and the thermosensitive mutants of pML31 were grown in M9 medium supplemented with 0.5% Casamino Acids and 250 μg of 2-deoxyadenosine per ml. The DNA was prelabeled by the addition of 1 μCi of [^{14}C]thymine (50 mCi/mmol) per ml and grown for 6 h (five to six generations) at 25°C in a shaking water bath. Bacteria were sedimented by low-speed centrifugation at room temperature, resuspended in 20 ml of fresh M9 medium, and shifted to 42°C. After 5 min of preincubation at 42°C, 200 μCi of [^{3}H]thymidine (5 Ci/mmol) was added, and the cultures were incubated for another 40 min at 42°C. Incorporation was stopped by the addition of 5 M sodium azide to a final concentration of 0.1 M, and cells were chilled on ice and harvested by centrifugation. The cells were then lysed using the lysozyme-dodecyl sulfate-salt procedure (12). Cells were resuspended in 1.5 ml of 25% sucrose in TES to give a cell concentration of 4×10^9 per ml. Then 0.25 ml of lysozyme (Sigma Chemical Co.; 5 mg/ml in TES) and 0.1 ml of 0.25 M EDTA, pH 7.5, were added, and the cell suspension was placed in an ice bath for 20 min. Cells were lysed at room temperature by the addition of 10% sodium dodecyl sulfate in TES to a final concentration of 1%. After complete lysis, 5 M NaCl was added to the viscous lysate to a final concentration of 1 M. The mixing of the cell suspension with sodium dodecyl sulfate and the final mixing of the lysate with NaCl has to be done very gently to avoid shearing forces. The lysates were stored in an ice bath overnight and then centrifuged at 40,000 × *g* for 30 min at 4°C. Plasmid DNA in the supernatant was analyzed on CsCl-ethidium bromide density gradients. Gradient tubes were punctured at the bottom with a needle, and fractions were collected. Aliquots of these fractions were spotted on Whatman 3M filter paper. Filters were dried, washed twice with 10% trichloroacetic acid and ethanol, and dried. Radioactivity was determined in a scintillation counter. Assuming that at the permissive temperature (25°C) in exponentially growing cultures the rate of incorporation of thymidine into wild-type and mutant plasmid DNA is the same, the gradients were normalized to the total [^{14}C]thymine counts recovered on the gradient of pML31 wild type. This calculation accounts for differences in the efficiency of lysis, cell titer, and other factors affecting the recovery of labeled plasmid DNA.

Electron microscopy. Plasmid DNA was directly spread from the fractions of CsCl-ethidium bromide gradients on Parlodion-coated grids, using the method described by Davis et al. (6). The grids were examined in a Siemens Elmiskop 101, and electron micrographs were taken on 6- by 9-cm sheet film. Open circular (OC) ColE1 DNA (2.15 μm) was used as an internal length standard.

RESULTS

Hydroxylamine mutagenesis and isolation of thermosensitive plasmid mutants. Supercoiled DNA of plasmid pML31 purified by two successive CsCl-ethidium bromide gradient centrifugations was treated with hydroxylamine as described in Materials and Methods. After dialysis the DNA was used for transformation of strain *E. coli* C600, and selection was for Km^r clones at 25°C. Hydroxylamine-treated plasmid DNA usually gave only 5 to 10% the transformation frequency of comparable untreated plasmid DNA. In a representative experiment, of 126 Km^r clones growing at 25°C, 17 did not grow at 42°C on L-broth-kanamycin plates. To distinguish between plasmid mutants thermosensitive in kanamycin resistance and mutants thermosensitive in replication, the following experiment was performed. Cultures of the thermosensitive clones were grown without selection for 4 h (8 to 10 generations) at 42°C and then shifted to 25°C and grown for another hour. Cells were diluted, plated on L-broth and L-broth-kanamycin plates, and incubated at 25°C. Cells harboring a mutant plasmid thermosensitive in kanamycin resistance give rise to the same number of colonies in both platings, whereas the thermosensitive replication mutants of pML31 segregate at 42°C, resulting in a reduction of Km^r clones. Among the 17 clones tested, 12 were found to be thermosensitive for kanamycin resistance and 5 were found to be thermosensitive for maintenance of the plasmid at 42°C. To verify that the mutations were located on the plasmid, plasmid DNA was prepared from these strains and retransformed into *E. coli* C600. The transformants again showed segregation of the plasmid at 42°C.

Segregation of plasmid mutants at 42°C. Figure 1 illustrates the segregation kinetics of the five mutants of plasmid pML31. Based on these data two types of plasmid mutants can be distinguished. Type I mutants as represented by Ts2, Ts9, and Ts10 segregate rapidly after a lag phase of about 45 min (1.5 generations). The rate of segregation is consistent with an immediate stop of plasmid replication and conservation of the number of Km^r cells in the growing bacterial population at 42°C. Rapid cessation of plasmid synthesis at 42°C in these mutants is also supported by the good fit of the points and the expected segregation curve for such an event starting with two copies per cell.

Type II plasmid mutants Ts13 and Ts17 seg-

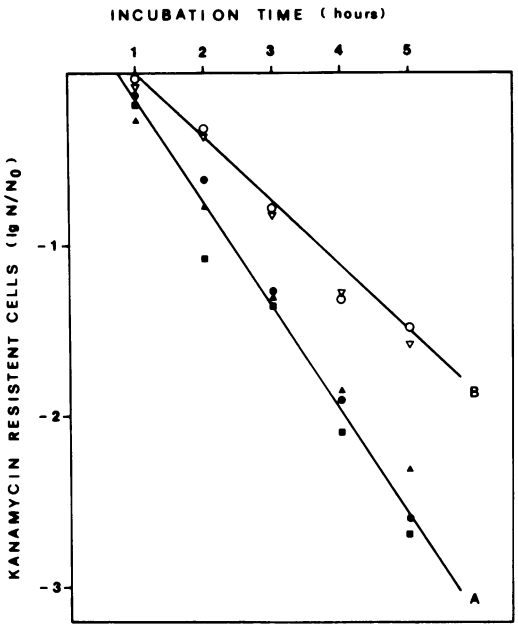

FIG. 1. *Segregation kinetics of thermosensitive mutants of plasmid pML31. Cultures of E. coli C600 harboring pML31 mutants Ts2, Ts9, Ts10, Ts13, and Ts17 were grown in L-broth medium at 42°C as described in the text. At the indicated times samples were removed, diluted, and plated on L-broth and L-broth-kanamycin plates. After incubation at 25°C, plates were scored and the survival (log N/N_0) of Km^r cells was calculated. Line A is the calculated segregation curve for two plasmid copies per cell and a complete stop of plasmid replication upon temperature shift. Line B is a calculated segregation curve when plasmid synthesis was reduced to 30% at the nonpermissive temperature. Symbols (●) pML31 Ts2; (▲) pML31 Ts9; (■) pML31 Ts10; (○) pML31 Ts13; (▽) pML31 Ts17.*

regate at a slower rate. In these mutants plasmid DNA may still replicate at 42°C, but at a reduced rate. Therefore, the number of Km^r cells in the bacterial population is increasing. By a simple equation one can calculate the rate of plasmid synthesis from the segregation kinetics (15).

$$P_n = P_0 \left[\frac{(1+A)}{2}\right]^n \quad (1)$$

$$A = \exp\left[\ln 2 - \frac{\ln \frac{P_n}{P_0}}{n}\right] - 1 \quad (2)$$

In these equations A is the rate of plasmid synthesis per generation, P_0 is the fraction of cells carrying the plasmid at generation 0 (time of temperature shift), P_n is the fraction of cells carrying the plasmid at generation n (after temperature shift), and n is the number of generations. Using the second equation and the data from the segregation experiment, a residual rate of 0.3 for plasmid DNA synthesis was obtained for mutants Ts13 and Ts17.

Plasmid replication at permissive and nonpermissive temperatures. To confirm that plasmid replication indeed is blocked or reduced at 42°C, the synthesis of plasmid DNA was studied with radioactively labeled DNA. Cultures of *E. coli* carrying the mutant plasmids were prelabeled with [^{14}C]thymine for five to six generations (6 h) at 25°C and then shifted to 42°C and pulse-labeled with [^{3}H]thymidine for 40 min (about 1.2 generations). Cleared lysates were prepared according to the lysozyme-sodium dodecyl sulfate-salt procedure (12). The plasmid DNA from cleared lysates was analyzed by buoyant density centrifugation in CsCl-ethidium bromide gradients. Profiles of such gradients are shown in Fig. 2. Fast segregating type I mutants Ts9 and Ts10 show virtually no incorporation of [^{3}H]thymidine into plasmid DNA at the nonpermissive temperature, which is consistent with the segregation kinetics. Type II mutants Ts13 and Ts17, which segregate at a slower rate supposedly due to some residual DNA replication at 42°C, in fact incorporated label into plasmid DNA at the high temperature, but predominantly into OC DNA. Only very little of the pulse-label is recovered in supercoiled plasmid DNA in these mutants. Surprisingly, the ^{14}C-prelabeled supercoiled plasmid DNA was not completely converted into the OC form, suggesting that only a few molecules of the plasmid pool do replicate. In mutant Ts2, which according to the segregation kinetics belongs to the fast segregating type I mutants, [^{3}H]thymidine incorporation did not stop at the nonpermissive temperature, and a similar gradient profile as for type II mutants is observed. Again the pulse-label was found almost exclusively in OC DNA.

To examine to what extent DNA banding in the second light peak of dye-CsCl gradients contains chromosomal DNA, this material from a gradient of Ts17 was analyzed on neutral sucrose gradients. The gradient profile obtained is shown in Fig. 3, demonstrating that there was only slight contamination by chromosomal DNA. More than 70% of the ^{3}H-labeled DNA loaded on the gradient sedimented with an $s_{20,w}$ value of 26, common to OC pML31 DNA. Examination of OC molecules of type II mutants in alkaline sucrose gradients, in addition, showed that most of the molecules carry only one single-strand break (Eichenlaub, unpublished data). DNA syntheses of the mutant and wild-type

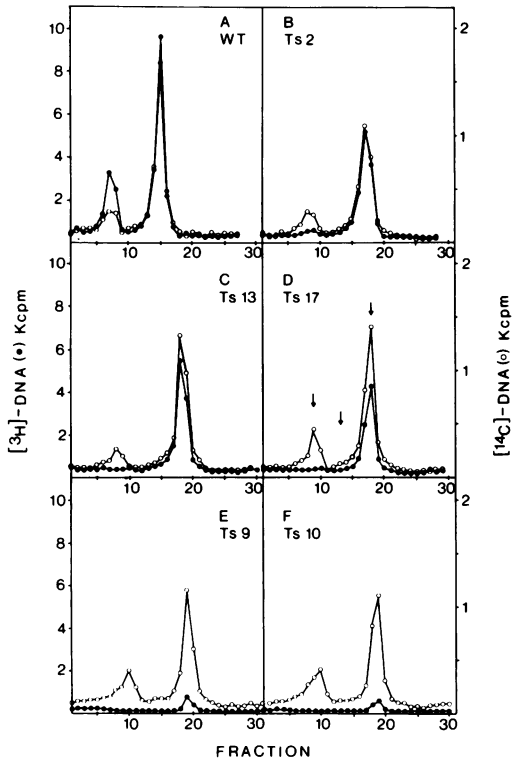

FIG. 2. *Plasmid replication at the permissive and the nonpermissive temperature. Cultures of E. coli C600 harboring plasmid pML31 (wild type) or a mutant plasmid were grown in supplemented M9 medium and prelabeled with [^{14}C]thymine at 25°C. Cells were then shifted to 42°C and pulse-labeled with [^{3}H]thymidine for 40 min. Cleared lysates were prepared as described in the text, and plasmid DNA was analyzed in dye-CsCl gradients (40 h at 40,000 rpm and 15°C in a Beckman 65 rotor). Gradients were normalized to the ^{14}C label recovered from the gradients. (A) pML31, (B) pML31 Ts2, (C) pML31 Ts13, (D) pML31 Ts17, (E) pML31 Ts9, and (F) pML31 Ts10. Symbols: (○) DNA prelabeled with ^{14}C at 25°C; (●) DNA labeled with ^{3}H at 42°C. Arrows in (D) indicate fractions which were examined in the electron microscope.*

plasmids at 25 and 42°C were calculated from normalized gradient profiles (Table 1). The rates of plasmid DNA synthesis of wild-type and mutant plasmids at 25°C did not differ significantly. Mutants Ts9, Ts17, and Ts10 seem to accumulate some covalently closed circular DNA. In contrast, mutant Ts13 has less supercoiled DNA at 25°C, but total plasmid DNA synthesis (OC plus covalently closed circular) again is very similar to that for the wild-type plasmid.

Plasmid DNA synthesis at 42°C, however, is reduced in all mutants. In mutants Ts2, Ts13, and Ts17 60 to 80% of the label is found in OC plasmid DNA as compared to pML31 wild type. Incorporation of label into supercoils is only 20 to 30%; this is in good agreement with the data obtained from the segregation experiments which predicted that plasmid DNA synthesis should be reduced to this level at 42°C.

Electron microscopic examination of

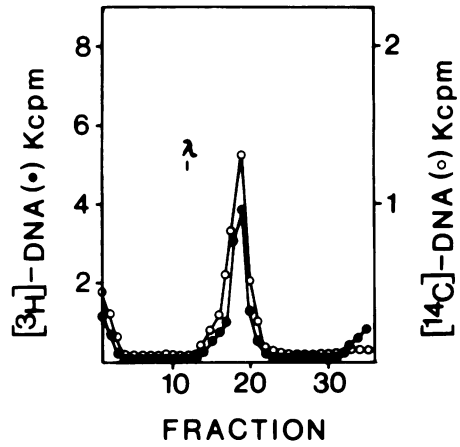

FIG. 3. *Sedimentation analysis of OC DNA from density gradients. Fractions covering the second, light density peak of a CsCl-ethidium bromide gradient of pML31 Ts17 were pooled, and ethidium bromide was extracted with n-butanol followed by dialysis versus TES. ^{32}P-labeled λ DNA was added as an internal sedimentation standard. The DNA was loaded on a 5 to 20% sucrose gradient and run for 120 min at 45,000 rpm and 15°C in a Beckman SW56 rotor. Gradient tubes were punctured at the bottom, and fractions were directly dropped onto Whatman 3M filter paper. Filters were prepared for determination of radioactivity as described for the density gradients.*

TABLE 1. *Incorporation of radioactive label at 25 and 42°C*[a]

Plasmid	[^{14}C]thymine incorporation at 25°C			[^{3}H]thymidine incorporation at 42°C		
	CCC	OC	CCC + OC	CCC	OC	CCC + OC
pML31	1	1	1	1	1	1
pML31 Ts2	1	0.9	0.9	0.3	0.8	0.6
pML31 Ts13	0.6	1	0.9	0.2	0.8	0.6
pML31 Ts17	1.2	1	1	0.2	0.6	0.5
pML31 Ts9	1.4	0.8	0.9	0.1	0.1	0.1
pML31 Ts10	1.4	0.8	0.9	0.05	0.1	0.1

[a] The rate of [^{14}C]thymine and [^{3}H]thymidine incorporation into covalently closed circular (CCC) and OC DNA of wild-type and mutant plasmids at 25 and 42°C was calculated from normalized gradients shown in Fig. 2. Incorporation of label into covalently closed and open circles of plasmid DNA was normalized to 1 for pML31 wild type.

plasmid replication products at the nonpermissive temperature. We examined a representative type II mutant (pML31 Ts17) when shifted to 42°C to test whether any specific replicative intermediates are accumulated under nonpermissive conditions.

Samples from the fractions of dye-CsCl gradients indicated by arrows in Fig. 2D were examined in the electron microscope (Fig. 4). Material from the covalently closed circular peak contained more than 80% supercoiled plasmid molecules. In the region between the supercoil and OC peak, supercoiled and relaxed plasmid molecules were found together with very few replicative intermediates (Fig. 4C). The second peak contained mostly OC unit-length molecules of pML31 and some linear chromosomal DNA (less than 20%) of random size. Also, numerous OC molecules with replication loops were found in this region of the gradient (Fig. 4D).

This examination supports the notion made already in the centrifugation experiments that in mutant pML31 Ts17 replication only continues to the stage of newly replicated OC molecules. No indication was found that plasmid replication is blocked at another step which should be accompanied by a accumulation of this specific intermediate.

Growth of replication mutants of pML31 in an *inc* Hfr host. The fact that plasmid mutants thermosensitive in replication can be isolated indicates that a plasmid-coded protein is involved in plasmid replication. Therefore, experiments were performed in which we tested whether an integrated F factor could complement the thermosensitive mutations of pML31. A prerequisite for such a complementation is that F replication functions are not permanently switched off in an Hfr host. Integrative suppression of a *dnaA* mutation at the nonpermissive temperature (23) and incompatibility between an integrated and an autonomous F (7) seems to indicate that an integrated F is expressing functions involved in F replication. Strain *E. coli* MA1091 Hfr *inc* (7) was transformed with DNA of plasmids pML31, pML31 Ts10, and pML31 Ts17. Transformants were then plated on selective medium and incubated at 25 and 42°C. Table 2 shows that none of the experiments showed complementation of the thermosensitive defect. A similar negative result has been obtained by Jamieson and Bergquist (19) with transient heterozygotes of F'*gal*/F' Ts114 *lac*. Possible explanations for these observations are that the specific protein needed for complementation is not available in sufficient quantities or that it is not synthesized from the integrated plasmid.

DISCUSSION

Generally, the replication of a plasmid element in a bacterial cell depends largely on host functions. It was reported that gene products essential for chromosomal replication are also needed for F-factor replication, e.g., *dnaB* (11), *dnaC* (28, 30), and *dnaE* (26). Cessation of F replication in a *dnaA* mutant after 1 h at the nonpermissive temperature has also been observed (16, 30). However, this observation in context with the integrative suppression of a *dnaA* mutation by F remains to be explained. Since an autonomous F is associated with the folded chromosome (21), it is possible that cessation of chromosomal replication indirectly affects F replication. In addition, chromosomal mutations have been described which affect only F-factor replication (4, 18). The stringent mode of F-factor replication allowing only the presence of one to two copies of the plasmid per chromosome implies a regulation of plasmid DNA replication. Two different models for the regulation of DNA replication have been proposed by Jacob et al. (18) and Pritchard et al. (25) postulating positive or negative control of this process. Cabello et al. (1) discuss a negative control of plasmid replication based on experiments with a pSC101-ColE1 hybrid plasmid. The action of a replication repressor could also in part explain the phenomenon of plasmid incompatibility. The question remains whether any functions involved in plasmid DNA synthesis or its regulation are plasmid encoded. An answer may come from specific plasmid mutants. Cuzin and Jacob (5) were the first to describe a thermosensitive mutant of F'*lac* which segregated at 42°C. In the meantime conditional replication mutants of various other plasmids have been isolated (3, 13, 15, 24, 29) suggesting that plasmid gene products are involved in plasmid replication.

In the present study I have described the isolation of conditional mutants of mini-F plasmid pML31 thermosensitive in replication using in vitro mutagenesis with hydroxylamine. Based on the segregation kinetics and on studies of plasmid DNA replication in double-label experiments, two types of mutants could be distinguished.

Type I mutants are completely blocked in plasmid replication when shifted to the nonpermissive temperature. Presumably they are defective in a function involved in the initiation of plasmid DNA synthesis. Whether this plasmid function exerts a positive or negative control on initiation remains open at present. To decide this question pML31-coded proteins have to be

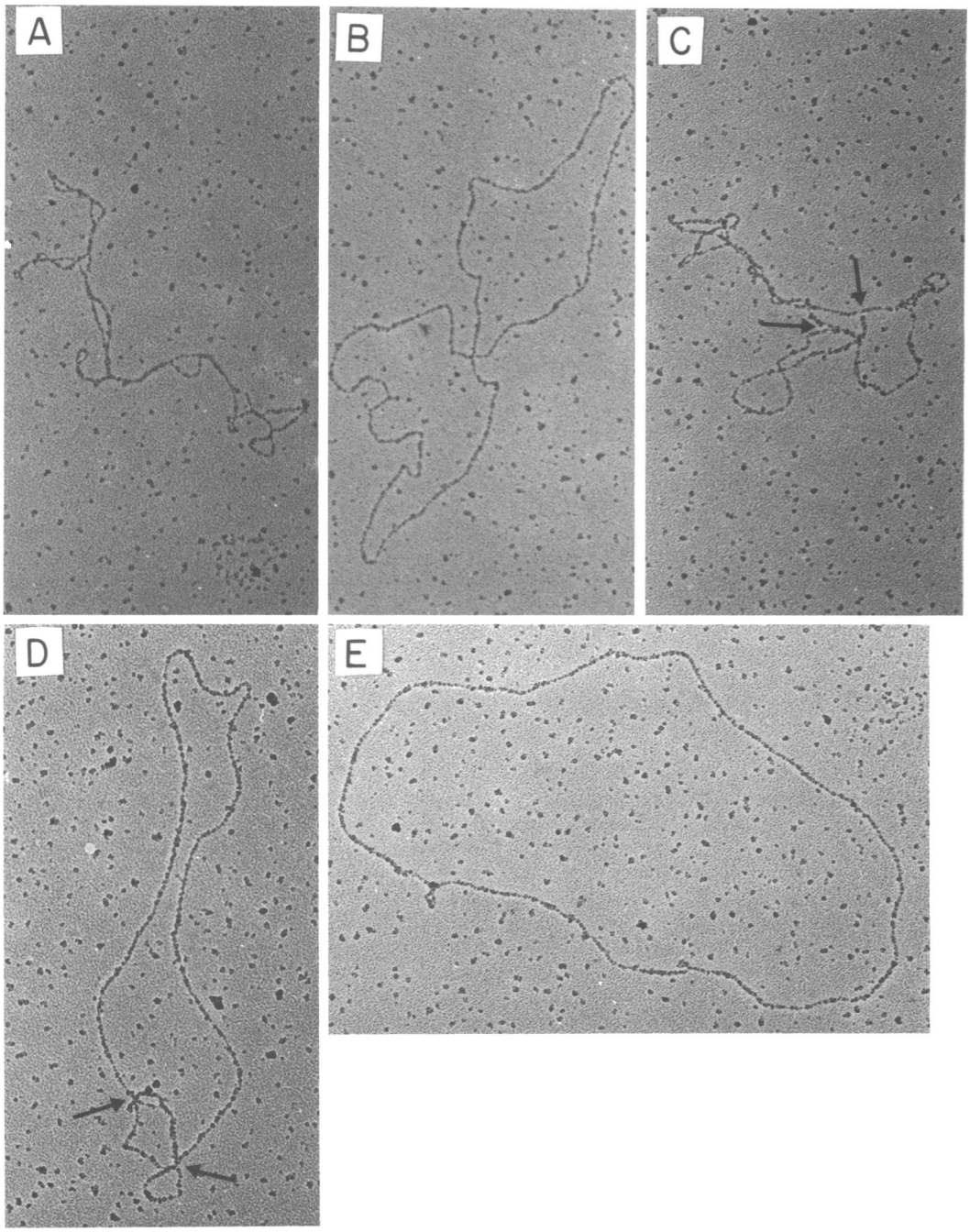

FIG. 4. *Electron micrographs of plasmid pML31 Ts17. Plasmid pML31 Ts17 DNA from the CsCl-ethidium bromide gradient shown in Fig. 2D. (A) Supercoiled molecules (48/61 molecules) and (B) OC molecule (13/61 molecules) from fraction 9; (C) replicative intermediate (5/84 molecules) from fraction 13; (D) relaxed replicative intermediate (3/46 molecules) and (E) OC molecule from fraction 18. Arrows indicate the replication forks.*

TABLE 2. *Test for complementation of thermosensitive pML31 in E. coli MA1091*[a]

Plasmid	Host	Growth on kanamycin plates	
		25°C	42°C
pML31	C600	+	+
	MA1091	+	+
pML31 Ts10	C600	+	−
	MA1091	+	−
pML31 Ts17	C600	+	−
	MA1091	+	−

[a] Strains C600 and MA1091 were transformed with purified plasmid DNA of pML31, pML31 Ts10, and pML31 Ts17. Clones harboring the plasmid were tested for growth on L-broth-kanamycin plates at 25 and 42°C.

characterized and their direct action on in vitro replication has to be demonstrated. The mutants of plasmid mini-F described in this report could be useful for this purpose.

Mutants of type II continue plasmid DNA synthesis at 42°C, although at a reduced rate. One possible explanation for the nature of type II mutants is that they represent leaky mutants of type I. Although we cannot rule out this possibility completely, it seems more likely that they are defective in a different function than type I mutants. Since radioactive label is incorporated predominantly into OC plasmid DNA, it seems that the sealing of nicks, introduced into the plasmid molecules during replication, is delayed in these mutants. This causes a retardation of plasmid DNA synthesis and concomitantly leads to plasmid segregation, since both of these processes may require supercoiled DNA. In this context a recent report on the replication of the thermosensitive R factor Rts1 describes a similar situation (29). Like type II mini-F mutants, Rts1 replication at the nonpermissive temperature yields very little supercoiled DNA. Rts1 replication at high temperature does not require a covalently closed DNA template but may involve linear replicative intermediates (29). From our examinations we have no evidence that mini-F can replicate in a similar mode.

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