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Control of F Plasmid Replication by a Host Gene: Evidence for Interaction of the *mafA* Gene Product of *Escherichia coli* with the Mini-F *incC* Region

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Replication of F (including mini-F) and some related plasmids is known to be specifically inhibited in *mafA* mutants of *Escherichia coli* K-12. We have now isolated and characterized mini-F mutants that can overcome the replication inhibition. Such plasmids, designated *pom* (permissive on *maf*), were obtained spontaneously or after mutagenesis with hydroxylamine or by transposon (Tn*3*) insertion. In addition to their ability to replicate in *mafA* mutant bacteria, the *pom* mutant plasmids exhibit an increased copy number and resistance to "curing" by acridine dye in the *mafA*$^+$ host. In agreement with these results, Tn*3*-induced *pom* mutants were found to carry Tn*3* inserted at the *incC* region of mini-F DNA, known to be involved in incompatibility, control of copy number, and sensitivity to acridine dye. Furthermore, three of the seven mini-F plasmids tested that carry Tn*3* within the tandem repeat sequences of the *incC* region (previously isolated by other workers) exhibit all the phenotypes of *pom* plasmids, the ability to replicate in the *mafA* strain, and high copy number and acridine resistance in the *mafA*$^+$ strain. The rest of the plasmids that contain Tn*3* just outside the tandem repeats remain wild type in all these properties. These results strongly suggest that the putative *mafA* gene product of host bacteria controls mini-F replication through interaction with the *incC* region.

The fertility (F) factor of *Escherichia coli* K-12 is a typical stringent plasmid. The strict control seems to be exerted on the replication and partitioning of the plasmid into daughter cells so as to maintain basically a single plasmid copy per host chromosome (9). Mini-F plasmid consists of an *Eco*RI fragment (f5) of 9.1 kilobases (kb) joined to a drug resistance DNA fragment and exhibits all the essential characteristics of F DNA replication (15, 28). Extensive studies in several laboratories led to the identification and localization of the mini-F genes or regions that are involved in autonomous replication, incompatibility, control of copy number, sensitivity to curing by acridine dye, and partitioning into daughter cells (see references 14 and 23). The assignment of these functions to a specific DNA region has been facilitated by structural and functional analyses of various DNA segments incorporated into multicopy plasmid vectors. The nucleotide sequence for a substantial portion of mini-F DNA has been determined (20, 21, 29), and at least seven proteins encoded by the distinct DNA segments have been detected (3, 12, 40).

Most of the genes or regions directly involved in mini-F DNA replication have been localized in a short segment of ca. 2 kb. This segment contains an origin of replication (*ori-2*); *repA*, whose product (29-kilodalton protein) is essential for DNA replication (16, 38); *copA*, which controls copy number (11); and nine tandem repeat sequences of 19 to 22 bases present in two clusters of opposite orientations (*incB* and *incC*) flanking the *repA* gene (see Fig. 1). The tandem repeat sequences have been shown to be specifically involved in incompatibility and control of plasmid DNA replication (11, 21, 29, 30). In addition, the *incC* region, which contains five repeat sequences, is involved in acridine sensitivity (39).

In contrast to the extensive studies of mini-F plasmid outlined above, little is known about specific host functions involved in F replication, other than the presumptive roles of DNA polymerase III (27), RNA polymerase (6), and the *dnaC* gene product (32). We previously showed that several *mafA* mutations located at 0.8 min on the *E. coli* chromosome specifically affect DNA replication of F-like plasmids such as F, ColV, and ColVB *trp* (34–37). Kinetic studies on mini-F replication in temperature-sensitive *mafA* mutants suggest that the initial step(s) of replication is blocked at high temperature (36). To further examine the mechanism of inhibition of plasmid replication in *mafA* mutants and the mode of involvement of the *mafA* gene product in F replication, we isolated and characterized mutants of mini-F that can overcome the replication inhibition. The results reported here strongly suggest that the putative *mafA* gene product actively participates in the control of F DNA replication via specific interaction with the mini-F *incC* region.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used are all derivatives of *E. coli* K-12 and are listed in Table 1. Plasmids are also listed in Table 1, and their structures, with major restriction sites, are indicated in Fig. 1. The mini-F plasmid (pKP1013) mainly used in this study consists of a 5.3-kb segment of F (44.1 to 49.4 kb on the F coordinate map; 26) and a chloramphenicol-spectinomycin resistance (Cm^r Sp^r) fragment (16) and was kindly supplied by Takeyoshi Miki. Mini-F plasmids with Tn*3* insertion at the *incC* region (pPB006, pPB034, pPB035, pPB037, pPB038, and pPB043) were derivatives of pPB104 and were generous gifts of H. E. B. Lane. Plasmids pMF45 and pSC204 were kindly provided by B. C. Kline.

Media and chemicals. Medium E (33) supplemented with 0.2% glucose, 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.), L-tryptophan (20 μg/ml), and ampicillin (20 μg/ml) or chloramphenicol (10 μg/ml) was used as a standard synthetic medium. A polypeptone-glucose medium (PG

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Sex	Chromosomal marker or description[b]	Phenotype	Reference or derivation
KY9522	F⁻	*pro* Δ(*trp-tonB*) *ilv arg thi lac tna pea rpsL supE tsx recA1*		(34)
KY9524	F⁻	*leu pro* Δ(*trp-tonB*) *ilv arg thi lac tna pea rpsL supE tsx mafA1 recA1*		(34)
KY9738	F⁻	*thr leu proA argE thi lacY galK ara xyl mtl rpsL tsx recA1*		*his*⁺ *recA1* derivative of KY917
KY2109	F⁻	*mafA36 thr*⁺; other markers as in KY9738		(35)
pKP1013		*Pst*I (F44.1)-*Eco*RI(F49.4) + Sp fragment + Cm fragment	Cm^r Sp^r	(16)
pSC138		*Eco*RI fragment (f5; F40.3 to 49.4) + Ap fragment of pI258	Ap^r	(28)
pSC204		Tn*3*-containing derivative of a Ts mutant of pSC101	Ap^r Tc^r	(13)
pPB104		*Eco*RI (F40.3)-*Bam*HI (F40.45) + *Bam*HI (F43.05)-*Eco*RI (F49.4) + Km fragment	Km^r	(2)
pMF45		pPB104::Tn*3*	Ap^r Km^r	(18)
pKV506		*Hin*dIII (F40.65)-*Eco*RI (F49.4) + Ap fragment	Ap^r	Derived from pSC138 This study

[a] Genetic symbols for bacterial strains are as described in reference 1.
[b] The numbers in parentheses indicate the locations (in kb) on the F coordinate map (26).

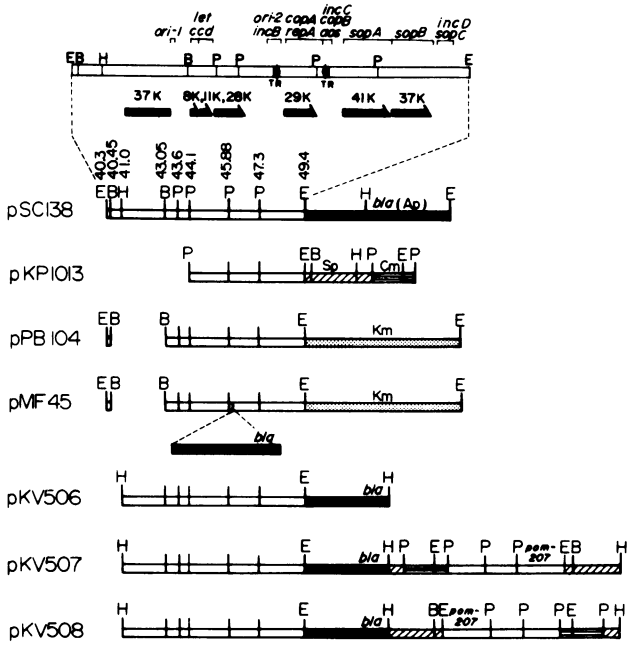

FIG. 1. Structure of some of the mini-F plasmids used (cf. Table 1). Only the major restriction sites are shown. The genes and sites on the mini-F f5 fragment (40.3 to 49.4 kb on the F coordinate map) are indicated at the top. The solid bars below indicate the proteins, with estimated molecular weights (in kb), encoded by the respective genes. *ori*, Replication origin; *inc*, incompatibility; *cop*, copy number control; *aos*, acridine orange sensitivity; *repA*, gene for a protein essential for replication; *let*, lethal; *ccd*, coupled cell division; *sop*, stability of plasmid. TR, Tandem repeat sequences. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Ap, ampicillin resistance; Sp, spectinomycin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; *bla*, β-lactamase gene. The structures of pKV506, pKV507, and pKV508 were confirmed by analysis of appropriate restriction fragments.

broth) was described previously (34). Antibiotic assay medium no. 3 (Difco) adjusted to pH 7.6 was used for curing of mini-F plasmid by acridine orange. [^{3}H]thymidine (20 Ci/mmol) and [^{14}C]thymidine (51.6 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. Lysozyme and ethidium bromide were supplied by Sigma Chemical Co., St. Louis, Mo., and acridine orange was supplied by Chroma Schmid & Co., Federal Republic of Germany. Ampicillin and chloramphenicol were obtained from Banyu Seiyaku Co., Tokyo, Japan, and Kyowa Hakko Co., Tokyo, Japan, respectively.

Determination of mini-F stability. An overnight culture grown in a synthetic medium with an antibiotic was inoculated into 5 ml of PG broth at 10^3 to 10^4 cells per ml. After shaking at an appropriate temperature for ca. 20 generations, samples were taken, diluted, and spread on polypeptone agar. Colonies that appeared after 2 days of incubation were replica plated onto a synthetic medium in the presence or absence of antibiotic to determine the frequency of plasmid-free segregants among the total population. Under these conditions, all mini-F plasmids used (except Pom$^-$ plasmids) were unstable in the *mafA* host bacteria, and plasmid-free segregants appeared at high frequency even when the plasmid contained the *Bam*HI-*Pst*I segment (43.05 to 43.6 kb on the F coordinate map) known to be involved in coupling host cell division with plasmid replication (10, 19, 24).

Curing of mini-F by acridine orange. Overnight cultures grown in a synthetic medium with an antibiotic were diluted to 10^4 cells per ml in 5 ml of antibiotic assay medium no. 3 containing 5 to 25 μg of acridine orange per ml and shaken at 30 or 37°C for 2 days, essentially as described (8). Appropriate dilutions were spread on polypeptone agar, and the resulting colonies were replica plated onto a synthetic medium with or without antibiotic to determine the frequency of plasmid-free segregants.

Preparation of mini-F DNA. Cell lysates were prepared essentially by the method of Cabello et al. (4). Cells were harvested from overnight cultures (250 ml), suspended in Tris-hydrochloride buffer (pH 8) containing sucrose, and treated with lysozyme-EDTA and with Triton X-100. The lysates were centrifuged to remove chromosomal DNA, and the supernatants were subjected to ethidium bromide-cesium chloride density centrifugation in a Spinco rotor no. 40 at 36,000 rpm for 45 h at 10°C. Fractions containing covalently closed circular (CCC) DNA were collected, extracted several times with isopropanol, saturated with CsCl, and dialyzed overnight against Tris-EDTA solution.

Determination of plasmid copy number. The plasmid copy number was determined by two methods. The ratio of radioactivity (counts per minute) incorporated into mini-F CCC DNA and total acid-insoluble material was calculated after ethidium bromide-cesium chloride centrifugation of cell lysates labeled with [^{3}H]thymidine or [^{14}C]thymidine. Alternatively, the copy number of ampicillin resistance plasmids

(pSC138 and its derivatives) was determined by measuring specific activity of β-lactamase by the published procedure (22) or by determining "single-cell resistance" to ampicillin, as defined by Uhlin and Nordström (31).

Recombinant DNA techniques. Analyses of plasmid DNA by restriction enzymes, gel electrophoresis, and ligation and transformation of DNA into bacterial host were carried out essentially as described by Maniatis et al. (17).

RESULTS

Isolation of mini-F mutants that can replicate in *mafA* host bacteria. The mini-F plasmid pKP1013, after mutagenization with hydroxylamine (5), was used to transform *mafA* mutant bacteria, selecting for resistance to chloramphenicol. Two *mafA* mutants were used: KY9524 (*mafA1*), which is defective in F DNA replication at all temperatures, and KY2109 (*mafA36*), which is defective only at high temperatures (40 to 42°C). Transformants were selected at 37°C with *mafA1* and at 40°C (or 42°C) with the *mafA36*(Ts) host. Plasmid DNA was extracted from several Cm^r transformants and was used to transform again the respective *mafA* bacteria. The final transformants contained a mutated mini-F plasmid; such plasmids selected in *mafA1* cells were designated *pom-101*, *pom-102*, etc., and those obtained in *mafA36*(Ts) cells were designated *pom-201*, *pom-202*, etc.

To examine the stability of these *pom* mutant plasmids, the strains carrying each plasmid were grown under nonselective conditions (without chloramphenicol) for ca. 20 generations, and the frequency of cells that had lost the plasmid was determined. If replication of the plasmid were inhibited in *mafA* hosts, plasmid-free cells would be expected to appear at high frequency. However, all *pom* plasmids isolated in the *mafA1* strain were stably maintained in *mafA1* as well as $mafA^+$ cells at 37°C. The *pom* plasmids selected in the *mafA36*(Ts) strain were more stable than the parental pom^+ plasmid at high temperatures, although some produced plasmid-free segregants at moderately high frequencies (Table 2). Some of the *pom* mutants isolated in the *mafA1* host (four of six mutants tested) were not stably maintained in *mafA36* cells at 40°C (data not shown), indicating that these *pom* mutations exhibit allele specificity with respect to the *mafA* mutations.

Replication and increased copy number of *pom* mutant plasmids. As expected from the stable maintenance of *pom* plasmids in *mafA* bacteria, [^{3}H]thymidine was actively incorporated into CCC DNA of *pom* mutant plasmids. [^{3}H]thymidine incorporation into the parental pom^+ plasmid in *mafA36*(Ts) cells was markedly inhibited at a high temperature (42°C), whereas incorporation into *pom* plasmids was little affected. When the *pom* plasmid was present in the $mafA^+$ strain, the radioactivity associated with CCC plasmid DNA was two to three times higher than that for the pom^+ plasmid (Table 3), suggesting that the copy number of *pom* plasmids may be higher.

To substantiate further the above observation, several *pom* mutants of the Ap^r mini-F plasmid (pSC138) were isolated in the *mafA1* host and were examined for the activity of β-lactamase. As expected, all strains ($mafA^+$) carrying each of the *pom* plasmids exhibited β-lactamase activities two to three times higher than those carrying the parental plasmid (pSC138) (data not shown). These results again indicate that the copy number of *pom* plasmids is higher when they replicate in $mafA^+$ bacteria and suggested that the *pom* mutations might affect the region of mini-F DNA which is involved in the control of plasmid copy number.

TABLE 2. Stability of mini-F *pom* plasmids in *mafA* host bacteria[a]

Plasmid	Plasmid-free segregants (%)			
	$mafA^+$ (37°C)	*mafA1* (37°C)	*mafA36* (30°C)	*mafA36* (40°C)
pom^+	<1.6	ND	<2.0	>98
pom-101	<1.3	<1.0		
pom-102	<1.3	<1.0		
pom-103	<1.2	<1.0		
pom-104	<1.0	<1.0		
pom-105	<1.4	<1.0		
pom-106	<1.1	<1.0		
pom-201[b]			<0.7	35
pom-202[b]			<0.6	<0.6
pom-203			<1.0	16
pom-204			<0.5	<1.0
pom-205			<0.6	30
pom-206			<0.6	13
pom-207			<3.0	<3.0

[a] Cultures of KY9522 ($mafA^+$) or KY9524 (*mafA1*) carrying pKP1013 or its derivative were grown at 37°C, and those of KY2109 (*mafA36*) carrying each plasmid were grown at 30 or 40°C in PG broth for ca. 20 generations. Appropriate dilutions were plated on polypeptone agar at 30°C, and plasmid-free segregants were scored as described in the text. The *pom* mutants 101 through 106 and 201 through 207 were originally isolated in strain KY9524 at 37°C or in strain KY2109 at 40°C. ND, Not determined.

[b] Spontaneous mutants. The rest of the mutants were obtained after hydroxylamine mutagenesis.

Altered sensitivity of *pom* plasmids to the "curing" effects of acridine dye. Mini-F plasmid pSC138, like the F factor, can be effectively cured when cells carrying the plasmid are grown in the presence of sublethal concentrations of acridine dyes (28). Similar sensitivity to acridine orange was observed with pKP1013, used in this study. In contrast to the parental pom^+ mini-F plasmid, which is sensitive to the dye, all the *pom* plasmids tested were resistant, provided that the host bacteria used were $mafA^+$ (Table 4). The same set of *pom* plasmids, when present in *mafA1* cells, showed normal sensitivity to acridine curing (Table 4). Similarly, *pom* plasmids present in *mafA36*(Ts) cells were resistant at 30°C (Maf^+ condition) and sensitive at 40°C (Maf^- condition) (Table 4). These results indicate that the *pom* mutations affect the sensitivity to acridine curing and that the altered sensitivity depends on the functioning of the *mafA* gene; the *pom* plasmids are sensitive to curing only when the *mafA* gene is mutated.

TABLE 3. Copy number of mini-F *pom* plasmids[a]

Plasmid	Radioactivity incorporated (cpm)			Relative copy number
	CCC	Total	CCC/total	
pom^+	910	8.8×10^5	0.0010	1.0
pom-101	1,690	6.0×10^5	0.0028	2.8
pom-102	1,150	5.0×10^5	0.0023	2.3
pom-103	1,600	6.5×10^5	0.0025	2.5
pom-104	1,330	6.3×10^5	0.0021	2.1
pom-105	1,560	8.2×10^5	0.0019	1.9
pom-106	1,580	8.8×10^5	0.0018	1.8

[a] Cultures (10 ml) of KY9522 ($mafA^+$) carrying pKP1013 or its derivative were grown at 37°C in a synthetic medium to 4×10^8 cells per ml and were labeled with [^{3}H]thymidine (4 μCi/ml) for 30 min. Lysates were prepared and analyzed by ethidium bromide-cesium chloride density centrifugation as described previously (36). The relative copy number was calculated on the basis of radioactivities specifically associated with CCC DNA and was normalized to the value for pom^+ (pKP1013). Plasmid-free segregants were less than 1% in all of the cultures used.

TABLE 4. Curing of mini-F *pom* plasmids by acridine orange[a]

Expt and host/ plasmid	Temp (°C)	Plasmid-free segregants (%) with acridine orange at (μg/ml):		
		0	10	25
I				
mafA$^+$/*pom*$^+$		0.18	37	75
mafA$^+$/*pom-101*		<0.3	<0.5	1.0
mafA$^+$/*pom-102*		0.26	<0.8	0.7
mafA$^+$/*pom-103*		<0.4	<1.4	1.2
II				
mafA1/*pom*$^+$		ND	ND	ND
mafA1/*pom-101*		0.37	25	*
mafA1/*pom-102*		0.16	50	*
mafA1/*pom-103*		0.16	31	*
III				
mafA$^+$/*pom*$^+$	30	<0.4	15	23
	40	5.0	12	49
mafA36/*pom*$^+$	30	<0.7	33	49
	40	>99.5	>99.5	>99.5
mafA36/*pom-201*	30	<0.4	0.6	*
	40	16	>99.8	*
mafA36/*pom-203*	30	<0.3	ND	<0.5
	40	<1.0	96	*
mafA36/*pom-204*	30	<0.5	ND	<0.5
	40	<1.0	98	>99.8
mafA36/*pom-205*	30	<0.3	<0.5	*
	40	4.0	>99.8	*
mafA36/*pom-206*	30	<0.7	ND	<0.5
	40	27	41	82
mafA36/*pom-207*	30	3.0	2.0	4.0
	40	3.0	92	*

[a] Cultures of KY9522 (experiment I) or KY9524 (experiment II) carrying pKP1013 or its derivative were grown at 37°C in the presence of acridine orange as indicated, and plasmid-free segregants (Cm^s) were determined as described in the text. In experiment III, KY9738 (*mafA*$^+$) or KY2109 (*mafA36*) carrying each plasmid was similarly examined at 30 or 40°C. ND, Not determined; *, no growth.

"Dominance" of the *pom-207* mutation in plasmid cointegrates. To circumvent the incompatibility barrier that precludes dominance and complementation tests of mini-F plasmids, mini-F Ap^r::mini-F Cm^r cointegrates that consist of a pair of *pom* and *pom*$^+$ plasmids were constructed in vitro. A *pom*$^+$ plasmid (pKV506; see Fig. 1) containing a unique *Hin*dIII site was constructed from pSC138 (mini-F Ap^r) and was ligated to the *Hin*dIII-digested *pom-207* mutant of pKP1013 (mini-F Cm^r). The ligated DNA mixture was used to transform KY2109 (*mafA36*) cells, selecting for Ap^r Cm^r clones at 30°C. Two types of plasmid cointegrates in which *pom* and *pom*$^+$ mini-F plasmids are joined to each other in opposite orientations were isolated (see Fig. 1). As shown in Table 5, both of these plasmids (pKV507 and pKV508) were found to be stably maintained in *mafA36*(Ts) bacteria at the restrictive temperature (40°C), as well as in *mafA*$^+$ bacteria. Moreover, the plasmid copy number of these cointegrates was comparable to that of *pom-207*, and the plasmids showed resistance to acridine curing when tested in the *mafA*$^+$ strain (Table 5). Thus, the *pom-207* mutation is dominant over *pom*$^+$ with respect to all the phenotypes tested under these conditions.

Pom$^-$ mutants induced by Tn*3* insertion. To examine further the nature of *pom* mutations, attempts were made to isolate *pom* mutants by inserting transposon Tn*3* into a mini-F plasmid (pKP1013). Ap^r Cm^r Tc^s clones were selected from a strain carrying both pKP1013 (Cm^r) and pSC204 (Ap^r Tc^r::Tn*3*) at 42°C, where replication of pSC204 is inhibited. The Ap^r Cm^r Tc^s colonies obtained were pooled, and plasmid DNA was prepared and used to transform *mafA1* bacteria at 37°C. Several *pom* mutants of pKP1013 that contain a Tn*3* insertion (as detected by the electrophoretic mobility) were thus isolated. Some of them (e.g., *pom-117*) were found to be stably maintained in *mafA1* bacteria, whereas others (e.g., *pom-119*) were stably maintained in *mafA36*(Ts) cells at high temperature, as well as in *mafA1* cells. The representative plasmids of each class (*pom-117* and *pom-119*) were picked and studied in detail. The plasmid copy number, as determined by the content of CCC DNA and ampicillin resistance with *mafA*$^+$ strains, is significantly higher than that of the *pom*$^+$ plasmid (Table 6). The sensitivity to curing by acridine orange was also lost in the *pom* mutants when present in the *mafA*$^+$ strain (Table 6). The acridine curing in *mafA1* cells could not be tested, because these cells carrying the plasmids failed to grow in the presence of even 5 μg of the dye per ml. These properties of Tn*3*-induced *pom* mutants are very similar to those of *pom* mutants obtained either spontaneously or by hydroxylamine mutagenesis.

Mapping of the Tn*3*-induced *pom* mutations. Plasmid DNA of *pom-117* and *pom-119* mutants was digested with appropriate restriction enzymes, and the resulting DNA fragments were analyzed by agarose or polyacrylamide gel electrophoresis to determine the sites and orientations of Tn*3* insertion. The results of these experiments clearly indicate that in both mutants, Tn*3* was inserted at or near the *incC* region but in opposite orientations (Fig. 2). A deletion of the *incC* region or a Tn*3* insertion into *incC* was previously shown to result in mutant plasmids with increased copy number and resistance to curing by acridine orange (2, 25, 39). The properties of *pom* mutants reported above therefore coincide well with these previous findings. It may be concluded that the *pom* mutations affect the *incC* region, which is known to contain five tandem repeat sequences of 19 to 22 bases and is

TABLE 5. Properties of plasmid cointegrates pKV507 and pKV508[a]

Plasmid	Plasmid-free segregants (%)[b]		Single-cell ampicillin resistance (μg/ml)[c]	Plasmid-free segregants (%) with acridine orange at (μg/ml)[c]:		
	30°C	40°C		0	10	25
pKV506 (*pom*$^+$)	<0.5	>99.5	100	1.0	88	ND
pom-207	<1.0	<1.0	ND	3.0	2.0	4.0
pKV507 (*pom*$^+$/*pom-207*)	<0.6	<0.4	150	1.0	2.0	4.8
pKV508 (*pom*$^+$/*pom-207*	<0.7	<1.1	190	0.8	<0.9	10

[a] Plasmid cointegrates were constructed as described in the text; the structures are shown in Fig. 1. ND, Not determined.
[b] Plasmid stability was determined with *mafA36* (KY2109) cells carrying each plasmid by the procedures described in Table 2, footnote *a*.
[c] Single-cell resistance to ampicillin that parallels plasmid copy number and the sensitivity to acridine curing were determined with *mafA*$^+$ (KY9738) cells harboring each plasmid grown at 30°C as described in the text.

involved in incompatibility, control of copy number, and acridine sensitivity.

Properties of previously isolated mini-F mutants that have Tn*3* at the *incC* region. Since a number of mini-F mutants with Tn*3* inserted at the precisely defined region of *incC* have been isolated previously (2, 11, 25), we examined some of these mutants to gain further insight into *pom* mutations. Three of the seven mutants tested exhibited the Pom$^-$ phenotype (ability to replicate in *mafA36* cells at 40°C) (Table 6). They also exhibited increased copy number (Cop$^-$) and resistance to acridine curing (Aos$^-$) when present in *mafA*$^+$ cells. In contrast, the remaining Pom$^+$ insertion mutants are normal with respect to these properties. The sites of Tn*3* insertion in these plasmids were determined by gel electrophoresis of *Pst*I (or *Pst*I plus *Eco*RV) restriction fragments. Three plasmids (pPB034, pPB043, and pMF45) carrying Tn*3* at the sites defined by base sequence analysis (11, 30) served as useful references in this study. As shown in Fig. 2, all Pom$^-$ plasmids (pPB043, pPB034, and pPB037), as well as *pom-117* and *pom-119*, seem to have Tn*3* in either orientation within the stretch of five tandem repeat sequences (46.01 to 46.26 kb on the F coordinate map). In contrast, the Pom$^+$ plasmids (pPB038, pPB035, and pPB006) appear to have Tn*3* outside of this region. Plasmid pMF45 contains Tn*3* within the region but not directly in any of the five repeat sequences (11). These results, taken together with other evidence, suggest the interesting possibility that the putative *mafA* gene product interacts directly with the tandem repeat sequences of the F *incC* region.

DISCUSSION

Mini-F *pom* mutants that can replicate in *mafA* mutant hosts have been isolated from pKP1013 or pSC138 either spontaneously or after hydroxylamine mutagenesis or by Tn*3* insertion. Some of the *pom* mutants isolated in the *mafA1* strain are stably maintained in the *mafA36*(Ts) strain at 40°C, as well as in *mafA1* strain, whereas others are stable only in *mafA1* strain, exhibiting allele specificity. Some *pom* plasmids isolated in the *mafA36*(Ts) strain have a limited capacity to replicate at 40°C (Table 2). In spite of these differences among *pom* mutants, they all share certain common properties: high copy number and resistance to curing by acridine orange.

The copy number of *pom* plasmids was found to be increased in the *mafA*$^+$ strain (Table 3) but perhaps not in the *mafA36*(Ts) strain at 30 or 42°C (data not shown). Similarly, the Tn*3*-induced *pom* mutants (*pom-117* and *pom-119*) appear to exhibit increased copy number only in the *mafA*$^+$ strains (Table 6). The copy number of the *pom*$^+$ plasmid does not differ significantly in the *mafA*$^+$ and *mafA36*(Ts) strains at 30°C (36). The resistance to acridine curing also depends on the normal functioning of the *mafA* gene product, because all hydroxylamine-induced *pom* plasmids replicating in the *mafA1* strain at 37°C or in the *mafA36*(Ts) strain at 40°C show normal sensitivity, in contrast to the marked resistance in the *mafA*$^+$ or *mafA36*(Ts) strain at 30°C (Table 4).

Maf$^-$ strains harboring a Tn*3*-containing mini-F plasmid are extremely sensitive to growth with acridine orange. Although molecular mechanisms underlying these observations remain unclear at present, the *mafA* gene product appears to be intimately involved in the control of replication of mini-F plasmids and in the curing of the plasmids by acridine dyes. Two representative *pom* mutants with *Tn3* insertions were mapped at or near the *incC* tandem repeat sequences. Though direct evidence is lacking, spontaneous or hydroxylamine-induced *pom* mutations probably affect the same *incC* region.

TABLE 6. Properties of mini-F plasmids that contain Tn*3* at the *incC* region[a]

Expt and plasmid	Plasmid-free segregants (%)				Pom phenotype in:		Copy number		Cop phenotype in *mafA*$^+$	Plasmid-free segregants (%) with acridine orange at (μg/ml):		Aos phenotype in *mafA*$^+$
	mafA$^+$ (37°C)	*mafA1* (37°C)	*mafA36* (30°C)	*mafA36* (40°C)	*mafA1*	*mafA36*	CCC DNA	SCR value		0	10	
I												
pKP1013	1.6	ND			+		1.0[b]	ND	+	0.18	37	+
pom-117	<2.0	<2.0			−		1.4[b]	180	−	<0.7	0.9	−
pom-119	<2.0	<2.0			−		2.1[b]	240	−	<1.0	<0.6	−
II												
pPB006			<0.5	89		+	0.5	130	+	<0.5	>99.5	+
pPB034			<0.4	1.7		−	1.5	190	−	<0.5	12	−
pPB035			<0.4	96		+	ND	140	+	<0.5	>99.5	+
pPB037			<0.3	<0.4		−	1.4	200	−	<0.5	6.0	−
pPB038			<0.5	>99.5		+	0.7	120	+	<0.5	>99.5	+
pPB043			<0.5	13		−	1.8	250	−	<0.4	21	−
pMF45			<0.5	>99.5		+	ND	100	+	2.3	>99.5	+
pom-117			<2.0	94		+	ND	360 (100)[c]	−	ND	ND	
pom-119			<2.0	<2.0		−	ND	360 (100)[c]	−	ND	ND	

[a] In experiment I, strain KY9522 (*mafA*$^+$) or KY9524 (*mafA1*) carrying the indicated plasmid was grown at 37°C in PG broth for ca. 20 generations, and plasmid-free segregants were scored as shown in Table 2. Plasmid copy number and acridine sensitivity were determined with KY9522 (*mafA*$^+$) as described in the text and Tables 3 and 4, footnotes *a*. In experiment II, strain KY2109 (*mafA36*) carrying each plasmid was grown in PG broth at 30 or 40°C for ca. 15 generations, and plasmid-free segregants were scored as in experiment I. Plasmid copy number and acridine sensitivity were examined with KY9738 (*mafA*$^+$) at 30°C, except that the copy numbers based on CCC DNA shown here were determined with strain PB1576 by Bergquist et al. (2). SCR value, value for single-cell resistance to ampicillin (μg/ml). ND, Not determined.

[b] These values have been normalized to that for pKP1013, taking into account the difference in DNA size. Plasmid-free segregants were less than 1% in all of the cultures used.

[c] The values in parentheses were determined with *mafA36* cells (KY2109), carrying each plasmid at 30°C.

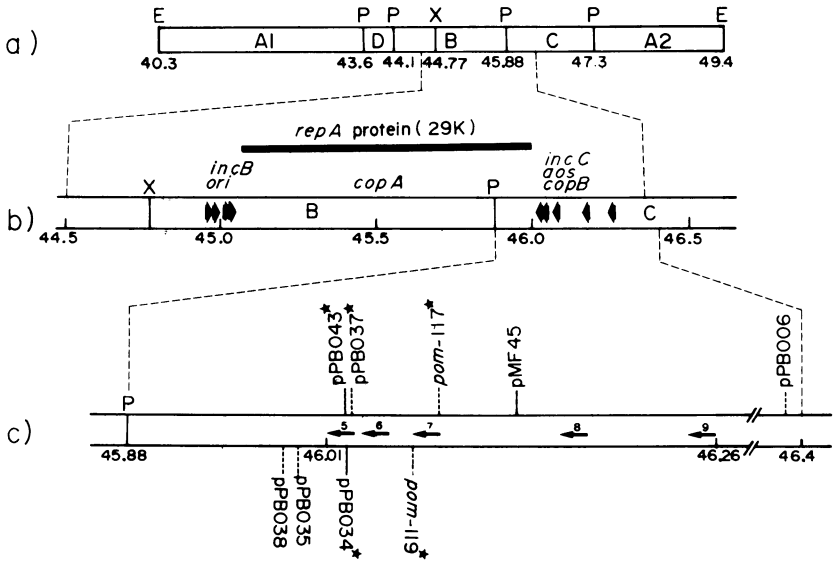

FIG. 2. The fine structure of mini-F replication region: Tn*3* insertion sites for Pom$^+$ and Pom$^-$ mutations. (a) *Eco*RI fragment (f5) of 9.1 kb (40.3 to 49.4 kb on the F coordinate map). Digestion with *Pst*I gives rise to five fragments designated A1, A2, B, C, and D (21). (b) The replication region showing genes and sites involved in DNA replication (2, 11, 16, 21, 29, 30, 38, 39). Arrows indicate tandem repeat sequences of 19 to 22 bases. (c) The *incC* region showing the sites for Tn*3* insertion in several mini-F mutants. The sites and orientations of Tn*3* insertion for *pom-117* and *pom-119* were determined by gel electrophoresis of restriction fragments digested with *Pst*I, *Eco*RI, *Bgl*II, *Bam*HI, and *Sma*I. Their locations relative to those for other plasmids were estimated from fragments obtained by *Pst*I (or *Pst*I plus *Eco*RV) digestion by referring to the sequence data of Tn*3* (7). The insertion sites and orientation for pPB034, pPB043, and pMF45 (solid lines) were determined at the base sequence level by Tsutsui et al. (30) and Kline and Trawick (11) and served as useful references for this study (dotted lines). In addition, the pPB series of plasmids were recently reexamined by the heteroduplex method, and the data coincide well with the present results (H. E. B. Lane, personal communication). The upward lines represent insertions in which the *bla* gene is located to the right, and the downward lines represent those of the opposite orientation. The insertion mutants marked with an asterisk exhibit the Pom$^-$ phenotype, whereas the rest exhibit the Pom$^+$ phenotype (see Table 6).

Tn*3* insertion into the *incC* region or deletion of *incC* was previously shown to result in reduced incompatibility, increased copy number, and resistance to acridine curing (2, 25, 39). Systematic studies with plasmids carrying different portions of the *incC* region revealed that the number of tandem repeats primarily determines the extent of inhibition of mini-F replication (11, 29, 30). It has been proposed that the tandem repeat sequences (or the putative RNA transcript thereof) exert their inhibitory effect by titrating out a certain factor(s) that is essential for mini-F replication or by acting as a repressor. The interaction of the putative *mafA* gene product with *incC* suggested by the present study raises the possibility that the active *mafA* gene product is required for normal control of F DNA replication; namely, the negative regulatory function of *incC* may be actively modulated by the host component, the *mafA* gene product. Such an interaction presumably affects the initial step(s) of plasmid replication, as suggested by our previous kinetic experiments (36).

The fact that the plasmid cointegrates (pKV507 and pKV508) that consist of *pom*$^+$ and *pom-207* plasmids can replicate in the *mafA36*(Ts) cells at high temperatures makes it unlikely that the interaction between *incC* and the mutated form (*mafA36*) of the *mafA* product fortuitously inhibits mini-F DNA replication. These cointegrates not only replicate in the *mafA* mutant host, but their modes of replication, including copy number and acridine sensitivity, resemble closely those of *pom* plasmids (Table 5). These results are also consistent with the notion that the *mafA*$^+$ gene product plays an active role in plasmid replication.

Tn*3* insertion (or possibly even point mutation) at one of five *incC* repeat sequences appears to confer upon the mini-F plasmid the ability to replicate in the *mafA* mutant hosts. This probably means that not only the number of repeat sequences, as suggested by other workers, but the secondary or tertiary structure around *incC* is important in determining the negative regulatory role of the *incC* region in plasmid replication. In the context of the "titration model" (30) for the *incC* activity, we may postulate that the *incC* repeat sequences control plasmid replication through binding to the *repA* protein. Then, a plausible working model that may explain all the present findings is that the *mafA*$^+$ gene product modulates the hypothetical interaction between *incC* and *repA* protein and that the *pom* mutations alter the secondary or tertiary structure of *incC* so as to reduce binding of *repA* protein to *incC* and to recover plasmid DNA replication. Cloning of the *mafA* gene and structural-functional analyses of its product would be required for understanding the detailed mechanisms of host-controlled plasmid DNA replication, as suggested by the present model.

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