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Escherichia coli Mutants Incapable of Supporting Replication of F-like Plasmids at High Temperature: Isolation and Characterization of *mafA* and *mafB* Mutants

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Mutants of *Escherichia coli* K-12 defective in replication of F-like plasmids at a high temperature (42°C) were found among threonine-independent (Thr^+) revertants of a threonine-requiring F′ strain after localized mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Transduction experiments with phage P1 permitted us to divide these mutations into two classes with respect to map location; some mutations were located between *thr* and *ara* at about 0.8 min, very close to *maf-1* reported previously (Wada et al., J. Mol. Biol. **108**:25-41, 1976 and the others probably were located between *leu* and *azi* at about 1.8 min. The former class of mutants designated *mafA* exhibited the same plasmid specificity as *maf-1*; replication of plasmids F and ColVB *trp*, but not R386 or R222, were affected at a high temperature. By contrast, the latter mutants designated *mafB* were defective in replication of any of these plasmids at a high temperature. When a culture of *mafA* mutants carrying an F′ plasmid was transferred from 30 to 42°C, the plasmid replication as determined by incorporation of [^{3}H]thymidine into covalently closed circular F DNA was markedly inhibited. Under certain conditions, the temperature shift-up caused severe growth inhibition of the mutant cells. Examination of merodiploids (*mafA*/*FmafA*$^+$) for plasmid maintenance suggested that the two *mafA* mutations tested (*mafA23* and *mafA36*) were both dominant, at least partially, over the wild-type *mafA*$^+$ allele. These properties of the *mafA* mutants, manifested at the restrictive temperature, are similar to those previously reported for the *maf-1* mutant. Taken together with other evidence, it is likely that these mutations affect either the same gene (*mafA*) or a set of closely linked genes, playing a specific role in autonomous plasmid replication in *E. coli*.

The fertility (F) factor of *Escherichia coli* K-12 is a typical stringent-type plasmid, and its replication and subsequent distribution into daughter cells appear to be strictly controlled to maintain basically a single copy of the plasmid per host chromosome. As one approach to the mechanism of regulating replication of such plasmids, we previously studied a host bacterial mutant, *maf-1*, which is incapable of supporting vegetative replication of F and certain other F-like plasmids (20, 21). It has been shown that F DNA can be transferred into the mutant cells at normal frequencies and converted to covalently closed circular (CCC) duplex molecules, but it cannot be replicated further during subsequent cell division. Other F-like plasmids, ColV and ColVB *trp*, also failed to replicate in this mutant. In contrast, F-like drug resistance plasmids such as R386 and R222 replicated normally in the *maf-1* cells as in the wild-type bacteria. In addition, a special class of F′ plasmids, called F*poh*, that is supposed to contain the chromosomal replication origin (7), was found to replicate in the *maf-1* mutant unlike the ordinary F′ plasmids (22).

In contrast to similar mutants studied by other workers (2, 6, 9, 10, 17, 24), the *maf-1* mutation has been mapped between the genes *thr* and *ara* and shown to be dominant over its wild-type allele (*maf*$^+$). Available evidence suggested that *maf-1* represents an ordinary point mutation of the host gene *maf*; however, this has not been established unequivocally (21). To examine the mechanism of participation of the *maf* gene(s) in F plasmid replication as well as to obtain additional mutants independent of *maf-1*, we attempted the isolation of temperature-sensitive mutants that exhibit properties similar to those of *maf-1* at a restrictive temperature. Taking advantage of the close linkage between the genes *maf* and *thr* and of the ability of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine to in-

duce closely linked simultaneous mutations (4), we plated mutagenized cells of a threonine auxotroph carrying F13-1 (F*lac*) on a minimal medium lacking threonine to first select for Thr$^+$ revertants; then we examined them for the Maf$^-$ phenotype at high and low temperatures. A number of temperature-sensitive *maf* mutants were indeed obtained among those Thr$^+$ revertants, and some of their properties will be reported in this paper.

MATERIALS AND METHODS

Bacterial and phage strains. Bacterial strains used in this study are all derivatives of *E. coli* K-12 and are listed in Table 1. Those F-like plasmids used were described previously (21), except for KLF1, which contains the *thr-leu* region of the *E. coli* chromosome (12). Bacteriophages T4 (T4D) and λ *vir* were from our laboratory stock, and P1 *vir* was used in all transduction experiments.

Media. Polypeptone-glucose medium, synthetic minimal medium E (19), minimal medium A, and

TABLE 1. *E. coli K-12 strains*

Strain	Sex or plasmid	Chromosomal marker[a]	Derivation or reference
KY917	F$^-$	*thr leu proA his argE thi lacY galK ara xyl mtl rpsL tsx*	Thy$^+$ derivative of AB1157 (21)
KY9317	F13-1 (F*lac*$^+$)	*argE ilvC his proA lacY galK thi*	(21)
KY9510	F13-1 (F*lac*$^+$)	The same as KY917	KY917 mated with KY9317
HR9	F$^-$	*thr car proA his mtl xyl thi gal lacY rpsL supE44*	AB1133 (15)
CSH41	F*lacI P proA*$^+$*B*$^+$	Δ(*lac-pro*) *galE thi*	(14)
J53 (R386)	R386 (Tc)	*proA met*	(3)
CSH-2 (R222)	R222 (Su Sm Cm Tc Fa Hg)	*met*	(23)
KY113	ColVB *trp*$^+$	Δ(*trp-tonB*) (λ)	(20)
KY2103	F$^-$	The same as KY917 except Δ(*trp-tonB*)	Δ(*trp-tonB*) derivative of KY917
KY2104	F$^-$	The same as KY2103 except Thr$^+$ *mafA23*	F$^-$ Δ(*trp-tonB*) derivative of Thr$^+$ *mafA23* mutant of KY9510
KY2105	F$^-$	The same as KY2103 except Thr$^+$ *mafB41*	F$^-$ Δ(*trp-tonB*) derivative of Thr$^+$ *mafB41* mutant of KY9510
KY2117	F$^-$	The same as KY917 except Thr$^+$ *mafA36*	F$^-$ derivative of Thr$^+$ *mafA36* mutant of KY9510
KY2466	F$^-$	The same as KY2103 except *ilv recA1 his*$^+$	*ilv recA1 his*$^+$ derivative of KY2103
KY2470	F$^-$	The same as KY2104 except *ilv recA1 his*$^+$	*ilv recA1 his*$^+$ derivative KY2104
KY2108	F$^-$	The same as KY2105 except *mafB41 ilv recA1 his*$^+$	*ilv recA his*$^+$ derivative of KY2105
KY2109	F$^-$	The same as KY2117 except *recA1 his*$^+$	*recA his*$^+$ derivative of KY2117
KY2110	F*lacI P proA*$^+$*B*$^+$	The same as KY2466	KY2466 mated with CSH41
KY2111	F*lacI P proA*$^+$*B*$^+$	The same as KY2470	KY2470 mated with CSH41
KY2112	F*lacI P proA*$^+$*B*$^+$	The same as KY2108	KY2108 mated with CSH41
KY2113	F*lacI P proA*$^+$*B*$^+$	The same as KY2109	KY2109 mated with CSH41
KY306	F$^-$	*maf-1* Δ(*trp-tonB*) *pro lacY tna rpsL azi supE*	Derivative of C600.1 (20)
AB2463 (KLF1)	KLF1 (F*thr*$^+$ *leu*$^+$)	*thr leu argE his pro thi recA lacY galK mtl xyl ara rpsL tsx supE*	B. Bachmann; CGSC4250
KY2118	KLF1 (F*thr*$^+$ *leu*$^+$)	*thr leu thi lacY tonA*	CR34 mated with AB2463 (KLF1)
KY9550	KLF1 (F*thr*$^+$ *leu*$^+$)	*thr dapE9 ilv recA rif*	Derivative of KN2193 (16) mated with AB2463 (KLF1)
KY2120	KLF1 (F*thr*$^+$ *leu*$^+$)	The same as KY2470	KY2470 mated with AB2463 (KLF1)

[a] Symbols for chromosomal genes are as described by Bachmann et al. (1). Δ denotes a deletion; Tc, tetracycline; Su, sulfanilamide; Sm, streptomycin; Cm, chloramphenicol; Fa, fusidic acid; Hg, mercuric ion.

peptone-triphenyltetrazolium-lactose medium were described previously (20, 21). Penassay broth (Difco), L-broth, and tryptone broth with 0.2% maltose were used for experiments involving mating, transduction, and growth of λ *vir* phage, respectively.

Chemicals. [6-^{3}H]thymidine (29.4 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Lysozyme was a product of Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Streptomycin sulfate and tretracycline hydrochloride were obtained from Meiji Seika Kaisha, Ltd., Tokyo, and Lederle (Japan) Ltd., Tokyo, respectively.

Mating conditions. Overnight cultures of donor and recipient strains grown in Penassay broth (antibiotic medium no. 3, Difco) were diluted 20-fold in a fresh medium and shaken at 30°C to midlog phase (2×10^8 to 3×10^8 cells/ml), unless otherwise indicated. Donor and recipient cultures were mixed at a ratio specified for each experiment in a 300-ml flask and incubated with gentle shaking. Mating was interrupted by blending with a Thermo-mixer (Thermonics Co., Ltd., Tokyo) at the maximum speed for 10 s, and appropriate dilutions were plated on selective media.

Quantitative determination of F DNA. Cells labeled with [^{3}H]thymidine were lysed by sequential additions of lysozyme, ethylenediaminetetraacetic acid, and sodium dodecyl sulfate in that order, and the lysates were treated with 1 M NaCl to remove the host chromosomal DNA by the method of Guerry et al. (5). The supernatant fluids obtained were immediately analyzed by alkaline sucrose gradient centrifugation as described previously (21).

RESULTS

Isolation of temperature-sensitive *maf* mutants. Strain KY9510 that carries an autonomous F*lac* plasmid (F13-1) and requires threonine for growth was grown in minimal glucose medium containing 0.2% Casamino Acids and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine essentially as described by Guerola et al. (4), and the cells were plated at 30°C on a minimal lactose medium with appropriate supplements but lacking threonine. Thr$^+$ Lac$^+$ colonies appeared at a frequency of about 10^{-6}, which is at least 100-fold higher than the spontaneous level of Thr$^+$ reversion. These colonies were picked and tested for their capacity to maintain F*lac* at 30 and 42°C by using minimal lactose and peptone-triphenyltetrazolium-lactose agar. About 5% of the Thr$^+$ Lac$^+$ clones tested were found to yield Lac$^-$(F$^-$) segregants at higher frequencies when grown at 42°C than when grown at 30°C. Fifteen independent mutants thus obtained were used for further study. When the F$^-$ segregant clones derived from each mutant were mated with strain KY9317 carrying F*lac* at 30°C, the resulting transconjugants were Lac$^+$ at 30°C but segregated F$^-$(Lac$^-$) clones at high frequencies upon incubation at 42°C. This indicated that the mutants were defective in the maintenance of an autonomous F plasmid (thus called *maf*) at high temperatures, and that a mutation(s) on the host chromosome was responsible for the temperature-sensitive Maf$^-$ phenotype.

Mapping of the mutations affecting F*lac* maintenance. Since *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is known to induce a number of closely linked mutations (4), it was expected that many of the mutations obtained above were localized around the *thr* gene where most Thr$^+$ reversions should occur. Thus each of the 15 mutants with the genotype *maf ara leu* was infected with phage P1 *vir* grown on strain HR9 (*maf*$^+$ *ara*$^+$ *leu*$^+$), and the infected cells were plated on an appropriate agar medium to select for Leu$^+$ transductants at 30°C. Examination of unselected markers *maf* and *ara* of these transductants immediately suggested that the 15 mutants fall into two groups (Table 2). One class of mutants designated *mafA* yielded *maf*$^+$ transductants at 3 to 14%, depending on the strain, and most *maf*$^+$ transductants were also *ara*$^+$, suggesting that *mafA* lies distal to *ara* from *leu*, the gene order being *thr-mafA-ara-leu* (Fig. 1). The other class of mutants designated *mafB* yielded *maf*$^+$ at very high frequencies (91 to 93%), and the distribution of *ara* markers was random among both *maf*$^+$ and *maf* transductants, indicating that *mafB* maps very close to *leu* but probably on the opposite side of *leu* from *ara*, namely *thr-ara-leu-mafB* (Fig. 1).

Mating between F$^-$ *maf* mutants and donor strains carrying various F-like plasmids. To obtain preliminary data on the plasmid specificity of the mutations, an F$^-$ derivative of each mutant was mated with several donor strains carrying an F-like plasmid at 30°C, and apparent transfer frequencies were determined by scoring the number of transconjugant colonies obtained at 30 and 42°C on appropriate selective media. For this and all subsequent experiments, F*lac-pro* was used as an F' plasmid. It was found that the apparent transfer frequency of F*lac-pro* was significantly reduced, particularly at 42°C, with all the mutants tested as compared with the *maf*$^+$ strain (10^{-4} to 10^{-5} for *mafA*, and 3×10^{-2} to 10×10^{-2} for *mafB*). Similar results were obtained for another F-like plasmid, ColVB *trp*, but the effects of *mafA* mutations seemed to be generally less marked than with F*lac-pro*. On the other hand, the number of transconjugants that received a drug resistance plasmid, either R386 (type FI) or R222 (type FII), was significantly affected by the *mafB* mutations but not by the *mafA* mutations (data not shown).

Growth of mutant cells that have received an F-like plasmid by conjugation. To further examine whether the decrease in appar-

TABLE 2. *Classification of* maf *mutants on the basis of transductional mapping data*[a]

Mutant no.	No. of transductants tested	Transductants (%) with markers				Designation
		maf[+b] *ara*[+c]	*maf*[+] *ara*	*maf ara*[+]	*maf ara*	
2	100	6	0	58	36	*mafA*
11	100	6	0	49	45	*mafA*
22	98	54	39	5	2	*mafB*
23	100	5	0	69	26	*mafA*
25	61	8	0	92[d]		*mafA*
28	100	4	0	96[d]		*mafA*
32	100	12	2	55	31	*mafA*
33	35	9	0	91[d]		*mafA*
35	100	4	1	95[d]		*mafA*
36	100	3	0	97[d]		*mafA*
38	49	8	2	90[d]		*mafA*
39	99	8	1	54	37	*mafA*
41	100	53	40	4	3	*mafB*
42	100	3	0	65	32	*mafA*
44	100	51	40	6	3	*mafB*

[a] Donor (HR9): F⁻ *thr car ara*[+] *leu*[+] *maf*[+]; recipient (Maf⁻ mutant): *thr*[+] *car*[+] *ara leu maf*/F13-1. Cells of *maf* mutants harboring F13-1 were infected with phage P1 grown on HR9 and plated on a selective medium to obtain Leu[+] transductants. Colonies were picked, purified, and examined for unselected markers (*maf* and *ara*).

[b] The *maf* marker was first scored by replica plating the patched cells of mutants onto minimal lactose agar at 42°C; it was further confirmed by spreading cell suspensions of representative transductants on peptone-triphenyltetrazolium-lactose agar to estimate the frequency of Lac⁻ segregants at 42°C.

[c] The *ara* marker was scored by replica plating the patched cells of mutants onto eosin-methylene blue-arabinose agar at 30°C.

[d] Total number of transductants that retained the *maf* mutant allele, including both *ara*[+] and *ara*.

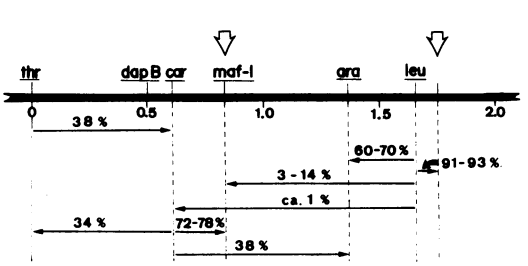

FIG. 1. *Genetic map of the thr-leu region of the E. coli chromosome, indicating the locations of the mafA and mafB mutations. Numbers below the solid bar (chromosome) represent the time scale (minutes), and the values with arrows indicate cotransduction frequencies (%), calculated from the data of Tables 2 and 4. The ranges of values reflect variations among experiments with different maf mutants.*

ent transfer frequency of F-like plasmids observed above reflects plasmid instability in the mutant, the fate of mutant cells that have received an F-like plasmid by conjugation was observed in a series of kinetic experiments. Thus F⁻ streptomycin-resistant strains carrying *maf*[+] or *maf* were mated with an F′ streptomycin-sensitive donor harboring the F*lac-pro* plasmid at 30°C, interrupted after 90 min, diluted in prewarmed broth containing streptomycin, and shaken further at 42°C. Samples were taken at intervals and plated on appropriate media to score the numbers of Pro[+] (plasmid-containing) and total recipient (Str[r]) cells. In the *maf*[+] strain, the numbers of Pro[+] and total recipient cells increased coordinately, keeping a constant proportion of Pro[+] cells among total recipients (Fig. 2a). In the *mafA* and *mafB* mutants, however, the number of Pro[+] cells increased at rates of 25 to 35% that of total recipients, resulting in a decrease in the relative frequency of Pro[+] cells. The rate of decrease was 30 to 40% per generation at the initial stage but gradually slowed down due to retransfer of the plasmid and overgrowth of the cells in which the plasmid had been integrated into the host chromosome (the integration was verified by their inability to transfer the plasmid into F⁻ *pro recA* recipients; these Hfr cells grow normally at 42°C). The number of Pro[+] transconjugants obtained at zero time was also lower in the *maf* mutants, suggesting that F*lac-pro* maintenance is partially defective even at 30°C. These results suggest that the transfer of F*lac-pro* into *mafA* or *mafB* mutant cells occurs normally but that further replication or subsequent distribution of the plasmid into daughter cells is affected in the mutants at high temperatures.

Similar results were obtained in the experiments using a donor strain carrying ColVB *trp*

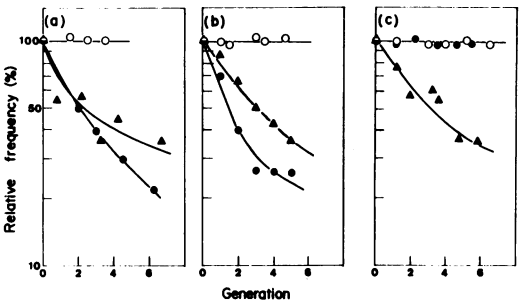

FIG. 2. *Growth of maf$^+$, mafA23, and mafB41 cells that have received an F-like plasmid by conjugation with donor cells. (a) Cells of an F′ donor carrying Fpro-lac (CSH41) and an F$^-$ recipient (maf$^+$, mafA23, or mafB41) were mated at a ratio of 1:2 (in 3 ml) in a 300-ml flask. Mating was carried out for 90 min at 30°C. After interruption of mating, samples (0.1 or 0.3 ml) were diluted into 10 ml of prewarmed Penassay broth containing 100 μg of streptomycin per ml and shaken further at 42°C. Samples were taken at intervals, and appropriate dilutions were plated on two media; medium E-glucose agar, supplemented with nutrients required by the recipients except for proline, and peptone agar, both containing 100 μg of streptomycin per ml, were used to score the numbers of Pro$^+$ [Str^r] cells and of total recipient cells, respectively. Plates were incubated at 30°C for 4 days. Frequency of Pro$^+$ [Str^r] cells per donor was normalized to the zero-time value and plotted against the number of generations of growth of the recipient population. (○) maf$^+$ recipients (KY2103); (●) mafA23 recipients (KY2104); (▲) mafB41 recipients (KY2105). The 100% values were 4×10^{-2}, 4.1×10^{-3}, and 2.3×10^{-3} per donor for the maf$^+$, mafA23, and mafB41 recipients, respectively. (b) Crosses between KY113 carrying ColVB trp (donor) and the same recipient strains used in (a) were carried out under the same conditions. The selective medium used for scoring Trp$^+$ [Str^r] cells was medium E supplemented with 0.2% Casamino Acids, 2 μg of thiamine per ml, and 100 μg of streptomycin per ml. Frequency of Trp$^+$ [Str^r] cells was plotted as in (a) after correction for the differential growth rates found between Col$^+$ and Col$^-$ strains. The 100% values were 0.10, 0.052, and 0.014 per donor for the maf$^+$, mafA23, and mafB41 recipients, respectively. (c) Crosses between J53 (R386) and the same recipients used in (a) were carried out under the same conditions except that the selective medium used to score Tc^r[Str^r] cells was peptone agar containing 100 μg of streptomycin per ml and 25 μg of tetracycline per ml. The 100% values were 5.1×10^{-5}, 5.8×10^{-5}, and 1.0×10^{-4} per donor for the maf$^+$ (○), mafA23 (●), and mafB41 (▲) recipients, respectively.*

(Fig. 2b). In contrast, experiments with a donor strain carrying R386 revealed that the *mafB* but not *mafA* mutations affected plasmid replication or distribution into daughter cells (Fig. 2c). The plasmid specificities of the *mafA* mutations revealed by these experiments are in good agreement with those previously reported for the *maf-1* mutation (20, 21).

Effect of high temperature on plasmid maintenance in the mutant strains. Cultures of the representative *mafA* and *mafB* mutants carrying F*lac-pro* were grown in broth for about two generations at 30°C and transferred to 42°C. Samples were taken at intervals, and the numbers of cells with and without F*lac-pro* were determined. In the case of the *mafA23* mutant, the proportion of cells that had lost the plasmid increased significantly after the temperature shift-up, whereas it increased only slightly with the *mafB41* mutant (data not shown). No appreciable number of F$^-$ segregants appeared in the *maf*$^+$ control culture.

In another series of experiments, the two *mafA* mutants (*mafA23* and *mafA36*) were grown in a minimal medium lacking proline at 30°C and shifted to 42°C immediately after addition of proline to the medium. The cell density was kept low throughout the experiment to avoid conjugal transfer of the plasmid that can occur normally even at 42°C. Under these conditions, F$^-$ segregants appeared at low frequencies (<1 or 4% after 4 h of growth at 42°C for *mafA23* and *mafA36* mutants, respectively), and the number of viable cells gradually stopped increasing within a few hours in both mutants (Fig. 3b and c). Upon prolonged incubation, cells in which the plasmid had been integrated into the host chromosome accumulated in the mutant population. In sharp contrast, the *mafB41* or *maf*$^+$ cells carrying F*lac-pro* as well as any of the F$^-$ *mafA* mutants tested continued to grow normally under the same conditions. It is apparent from these results that growth of the *mafA* mutants carrying an autonomous F*lac-pro* is severely inhibited at high temperatures. Thus in the case of *mafA* mutants, the apparent increase in F$^-$ segregants observed above at 42°C may be due mostly to overgrowth of F$^-$ cells that were present in the culture before the temperature shift-up.

Effect of high temperature on [^{3}H]thymidine incorporation into CCC F DNA. Cultures of the representative *mafA* and *mafB* mutants carrying F*lac-pro* were grown in a minimal medium at 30°C and shifted to 42°C. Portions of the cultures were taken and labeled with [^{3}H]thymidine before and after the temperature shift. Cells were lysed, and supernatant fluids containing F DNA were examined by alkaline sucrose gradient centrifugation. As shown in Fig. 4 and Table 3, the proportion of radioactivities associated with CCC F DNA is about the same at 30 and 42°C in the *maf*$^+$ strain, whereas it is greatly reduced at 42°C in the *mafA23* and *mafA36* mutants. A significant but less marked

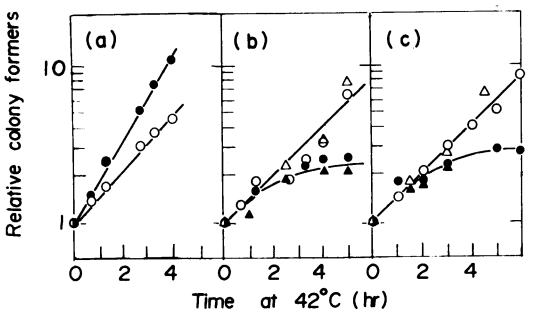

FIG. 3. *Effect of temperature shift-up on growth of mafA mutants carrying Flac-pro. (a) maf⁺ (KY2110); (b) mafA23 (KY2111); (c) mafA36 (KY2113). Overnight cultures grown at 30°C in medium E containing thiamine (2 μg/ml) and all amino acids except proline were diluted to 10^4 to 10^5 cells per ml in the same medium and shaken at 30°C for 3 h. After addition of proline into the medium, the cultures were immediately divided into two parts and shaken at 30 and 42°C. After 2.5 h of incubation at 42°C, the cultures of strains (a) and (b) were diluted 10-fold into prewarmed medium. Samples were taken at intervals and plated on medium E containing 0.2% Casamino Acids, L-tryptophan (20 μg/ml), and thiamine (2 μg/ml), and the plates were incubated at 30°C for 3 days. The number of colony formers obtained has been normalized to the respective zero-time value (cells/milliliter): (a) 2.4 × 10^6 (○, ●); (b) 1.3 × 10^6 (○, ●) and 4.8 × 10^5 (△, ▲); (c) 1.1 × 10^3 (○, ●) and 1.3 × 10^4 (△, ▲). Open symbols represent values for 30°C, whereas closed symbols represent those for 42°C. Circles and triangles represent two independent experiments.*

effect of high temperature was observed in the *mafB41* mutant (Table 3). These results indicate that vegetative replication of CCC F DNA is severely inhibited at high temperatures in the *mafA* mutants but only slightly inhibited in the *mafB41* mutant.

To test whether the observed decrease of [^{3}H]thymidine incorporation into CCC F DNA in the *mafA* mutants might be due to rapid degradation of CCC DNA molecules at high temperatures, cells of *mafA23* that had been labeled with [^{3}H]thymidine at 30°C were further incubated at 42°C in the presence of excess unlabeled thymidine. Samples were taken after 60 and 120 min, and cell extracts were examined for radioactivities associated with the CCC plasmid DNA. The percent of radioactivity found in the CCC fraction increased rather than decreased during the chase period in the *mafA23* mutant as well as in the *mafA*⁺ strain (data not shown). Since the total radioactivities found in the acid-insoluble fraction did not increase during this period, the observed increase in the percent CCC may be due to completion of replicating F DNA molecules or stabilization of CCC F DNA or both. In any event, there was no indication that CCC F DNA formed in the *mafA23* mutant degraded rapidly at 42°C. These results therefore support the conclusion that a certain step(s) of vegetative replication of F DNA is inhibited in the *mafA* mutants, and possibly in the *mafB* mutants as well, when exposed to high temperature. In addition, the anomaly in F DNA replication of the *mafA* mutants observed at 42°C apparently precedes that in host growth and viability (cf. Fig. 3 and 5). The apparent decrease of F DNA replication in the *maf*⁺ strain (Fig. 5a) presumably reflects

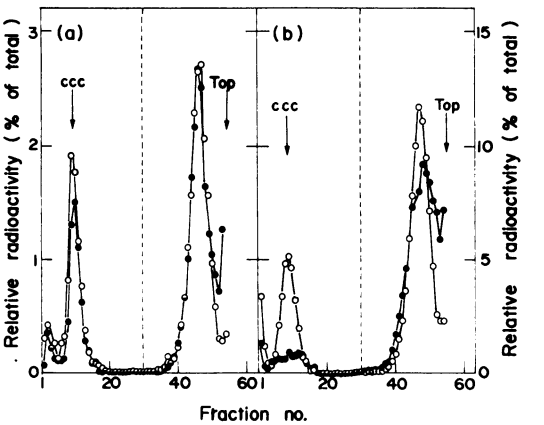

FIG. 4. *Alkaline sucrose gradient centrifugation of F DNA synthesized in maf⁺ and mafA23 cells at 30 or 42°C. Cells of (a) KY2110 (maf⁺ recA) and (b) KY2111 (mafA23 recA), both carrying Flac-pro, were grown in medium E containing thiamine and all amino acids except proline (20 μg each per ml) overnight at 30°C. The cultures were diluted with the same medium but containing proline (20 μg/ml) and deoxyadenosine (250 μg/ml), grown to a midlog phase (1 × 10^8 to 2 × 10^8 cells per ml), and divided into two portions. One portion was shaken at 30°C (○) and the other was shaken at 42°C (●). After incubation for 60 min, [6-^{3}H]thymidine (29.4 Ci/mmol) was added to each culture at a final concentration of 25 μCi/ml, and the labeling continued for 30 min. The labeled cells were collected by centrifugation and lysed, and the supernatant fluids containing F DNA were analyzed by alkaline sucrose gradient centrifugation as described in Materials and Methods and in the previous paper (21). The supernatant fluid used contained 1 to 2% of total acid-insoluble radioactivity incorporated into the cells. Total acid-insoluble radioactivity applied for each gradient was within the range of 5 × 10^4 and 1 × 10^5 cpm. Radioactivity is presented as the percentage of total acid-insoluble radioactivity recovered from each gradient. CCC indicates the locations expected for covalently closed circular DNA. Scales on the righthand ordinate of each figure (right of the dotted line) are five times higher than those on the left-hand ordinate (left of the dotted line).*

TABLE 3. *Incorporation of [³H]thymidine into CCC F DNA in cells carrying* Flac-pro[a]

Strain	Radioactivity in CCC F DNA (% of total) at:	
	30°C	42°C
KY2110 (*maf*⁺)	0.26	0.18
KY2111 (*mafA23*)	0.18	0.03
KY2113 (*mafA36*)	0.31	0.02
KY2112 (*mafB41*)	0.20	0.08

[a] Cells were grown and pulse-labeled with [³H]thymidine (total incorporation 2×10^6 to 2×10^7 cpm) essentially as described in the legend to Fig. 4, except that the labeling at 42°C was carried out for 30 min after prior incubation at that temperature for 180 min (in the case of *mafA36*, cells were incubated for 120 min and then labeled for 30 min). Incorporation of [³H]thymidine into CCC F DNA was determined as described in Materials and Methods.

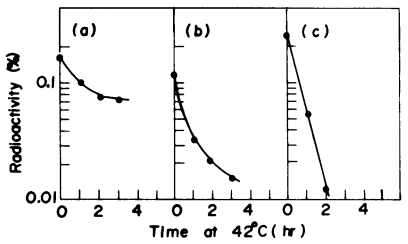

FIG. 5. *Effect of temperature shift-up on F DNA synthesis in maf⁺ and mafA strains. Cells of (a) maf⁺ (KY2110), (b) mafA23 (KY2111), and (c) mafA36 (KY2113) carrying Flac-pro were grown and labeled with [³H]thymidine as in Fig. 4, except that the culture was divided into four portions and the labeling was done at 42°C for 30 min after prior exposure to 42°C for the indicated periods. Samples were taken and analyzed for radioactivity associated with CCC F DNA as in Fig. 4. The values represent percent of total radioactivity found in CCC F DNA.*

an increased rate of chromosomal DNA replication upon temperature shift-up. These results are consistent with the notion that the defect in plasmid DNA replication somehow causes the inhibition of host cell multiplication.

Capacity of *maf* mutants to support growth of bacteriophages. Some of the *mafA* and *mafB* mutants were tested for their ability to grow phages T4 and λ *vir*. No appreciable differences were found among the bacterial strains tested in efficiency of plating for either phage at any of the temperatures tested (33, 37, and 42°C) (data not shown). In a separate experiment, burst size of λ *vir* was compared among *mafA*, *mafB*, and *maf*⁺ strains. The values obtained were significantly lower at 40°C than at 30°C, but no great differences were found among the various strains used (data not shown).

Further genetic characterization of the *mafA* mutants. Transduction experiments were performed using strain HR9 (*thr car maf*⁺ *ara*⁺) as recipient and each of three *maf* mutants, *mafA23*, *mafA36*, and *maf-1* (*thr*⁺ *car*⁺ *maf ara*) as donor. The results obtained with these *maf* mutants were similar; they all seemed to map between *car* and *ara*, with the cotransduction frequencies between *car* and *maf* being 75, 78, and 72% for *mafA23*, *mafA36*, and *maf-1*, respectively (Table 4). This strongly suggests that these mutations occurred in one gene or in genes very closely linked to each other on the *E. coli* chromosome. It may tentatively be concluded that these mutations represent different alleles of the same gene designated *mafA*.

To study the dominance relationship between a temperature-sensitive *mafA* mutation and its wild-type *mafA*⁺ allele, an F′ plasmid carrying *mafA*⁺ (KLF1) was transferred into cells of the *mafA23* mutant. The resulting F′ transconjugants were grown at 30°C in a selective medium (lacking threonine), diluted 1,000-fold into broth, and shaken further at 30 or 42°C. During incubation under these nonselective conditions, samples were taken at intervals to determine the frequency of F⁻ (Thr⁻) segregants. That the KLF1 plasmid carries the *mafA*⁺ allele was shown by the finding that phage P1 grown on KY2118 (*thr mafA*⁺ *leu*/KLF1 *thr*⁺ *mafA*⁺ *leu*⁺) can transduce *maf*⁺ together with *leu*⁺ into a recipient strain KY2104 (*mafA23 leu*) at an expected frequency (4.4%). The frequency of Thr⁺ (plasmid-containing) cells decreased with time at a rate significantly higher at 42°C than at 30°C, as in the case of the same mutant carrying F*lac-pro* (Fig. 6). In a separate experiment, growth of the merodiploid strain was shown to be markedly inhibited when cells grown on a selective medium at 30°C were directly transferred to peptone-glucose medium at 42°C. These results strongly suggest that *mafA23* is at least partially dominant over *mafA*⁺. In similar experiments with another strain carrying *mafA36*, the merodiploid strain (*mafA36*/KLF1 *mafA*⁺) exhibited no appreciable increase in F⁻ segregants at 42°C as compared with those at 30°C. However, severe inhibition of host cell growth was observed just as for the same mutant strain carrying F*lac-pro* (Fig. 3c). The latter observation again suggests at least partial dominance of *mafA36* over *mafA*⁺. Thus both *mafA23* and *mafA36* appear to be dominant to *maf*⁺ with respect to plasmid maintenance or host growth, or both.

DISCUSSION

Mutants defective in autonomous replication of F and ColVB *trp* plasmids at high tempera-

TABLE 4. *Transductional mapping of the* mafA *mutations by phage P1* vir[a]

Characteristics of donor and recipient cells	Selection	Unselected marker				No. obtained
		thr	*maf*	*ara*	*leu*	
Donor: KY2470 *thr*$^+$ *car*$^+$ *mafA23 leu*	Car$^+$	1	1	–	1	11
Recipient: HR9 *thr car maf*$^+$ *leu*$^+$		1	1	–	0	21
(48 scored)		1	0	–	0	8
		0	1	–	0	4
		0	0	–	1	1
		0	0	–	0	3
Donor: KY2109 *car*$^+$ *mafA36 ara leu*	Car$^+$	–	1	1	1	14
Recipient: HR9 *car maf*$^+$ *ara*$^+$ *leu*$^+$		–	1	1	0	5
(50 scored)		–	1	0	0	18
		–	1	0	1	2
		–	0	0	1	1
		–	0	0	0	10
Donor: KY306 *thr*$^+$ *car*$^+$ *mafA1*	Car$^+$	1	1	–	–	10
Recipient: HR9 *thr car maf*$^+$		1	0	–	–	7
(50 scored)		0	1	–	–	26
		0	0	–	–	7

[a] Cells of strain HR9 were infected with phage P1 *vir* grown on the donor strain and plated on a selective medium to obtain Car$^+$ (pyrimidine-nonrequiring) transductants at 30°C. The *maf* marker was scored by transferring F*lac-pro* to each transductant and examining the resulting transconjugant clones for the capacity to maintain the plasmid at 42°C. This was done by spreading transconjugant clones on minimal agar containing a limited amount of proline (1 μg/ml) together with other supplements; F$^-$ (Pro$^-$) segregants formed small flat colonies that were easily distinguishable from normal colonies formed by F*lac-pro*–containing clones. The donor and recipient markers are represented by 1 and 0, respectively.

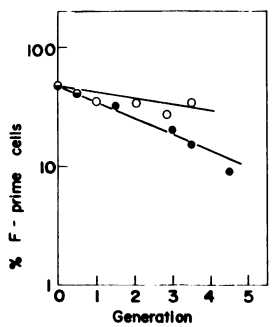

FIG. 6. *Segregation of F$^-$ clones from strain KY2120 (mafA23 recA) carrying plasmid KLF1 (mafA$^+$). Cells were grown in a selective medium containing all amino acids except leucine to about 10^7 cells per ml, diluted 1,000-fold into a peptone-glucose medium, and shaken for 30°C for 2 h. The culture was divided into two portions and shaken further at 30 (○) and 42°C (●). Samples were taken at intervals, and appropriate dilutions were plated on peptone-glucose agar. After incubation at 30°C for 3 days, colonies were replicated onto a pair of supplemented medium E plates with and without leucine. Frequency of Leu$^+$ (KLF1-containing) cells among the total population was plotted against the number of generations of growth at each temperature.*

tures were found among Thr$^+$ revertants after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It seems remarkable that each of all the 15 mutants selected on the basis of temperature-sensitive Maf$^-$ phenotype carries a mutation located either at the *mafA* (0.8 min) or *mafB* (1.8 min) region, both closely linked to *thr* (0/100 min). Since the *seg* mutations that lead to a phenotype similar to *maf* were reported to map between 99.5 and 0/100 min (9), we might expect to find some temperature-sensitive *seg* mutants among the new mutants tested. We can offer no explanation for our failure to find *seg* mutants in the present screening.

All the 12 *mafA* mutations obtained seem to share certain distinct properties: they have similar chromosomal location and plasmid specificity, among other features. In addition, two mutants studied most extensively (*mafA23* and *mafA36*) both exhibited inhibition of host cell growth at high temperatures, as well as inhibition of plasmid DNA replication. Furthermore, both mutant alleles appear to be at least partially dominant over *maf*$^+$. These properties are quite similar to those reported previously for the *maf-1* mutation (20, 21), although the *maf-1* mutant expresses the Maf$^-$ phenotype even at low temperatures. It may be noted in this connection that *maf-1* mutant cells that have received F13-1 by conjugation formed small and flat Lac$^+$ colonies which contained many elongated cells (C. Wada, unpublished data). This is at least consistent with the idea that the Lac$^+$ transconjugant clones of the *maf-1* mutant are not only unable to support F DNA replication

but are also unable to grow and divide normally. In view of these findings and considerations, we have tentatively concluded that all the *mafA* mutants as well as *maf-1* carry a mutation in the same gene which plays a specific role in the maintenance of certain autonomous F-like plasmids, although the possible involvement of a set of closely linked genes is by no means excluded.

As to the mode of participation of the *maf* genes in plasmid maintenance, evidence suggests that at least the *mafA* gene is specifically involved in, and possibly essential for, plasmid DNA replication, because incorporation of [^{3}H]thymidine into CCC F DNA is inhibited under restrictive conditions. However, the possibility that F DNA replicates in a form other than CCC in the mutant cells, as recently reported in the Rtsl plasmid (25), cannot be ruled out at present. It is interesting that the *mafA* mutations, including *maf-1*, affect the maintenance of F and ColVB *trp*, but not of R386 and R222 which are closely related to F in their incompatibility properties. Such a high plasmid specificity of the mutations as well as the lack of vegetative replication of CCC F DNA in *maf-1* recipient cells that have received the plasmid by mating (21) suggests strongly that replication initiation rather than DNA chain elongation is affected by the *mafA* mutations.

The finding that all the three *mafA* mutations examined are dominant at least partially over *mafA*$^+$ was rather unexpected and may require explanation. These mutants are supposed to make altered forms of the *mafA* gene product (protein), which may somehow exert deleterious effects on F DNA replication, even when the wild-type *mafA* protein is also produced. (It seems quite unlikely that the *mafA*$^+$ gene located on the plasmid KLF1 is not expressed.) Such situations may be obtained by several alternative mechanisms. For example, the *mafA* protein may form an active complex containing several *mafA* polypeptides, and the complex containing one or more mutant polypeptide is inactive. Alternatively, a number of *mafA* proteins may be functionally organized in such a way that one mutant (inactive) polypeptide inhibits functioning of the entire set of proteins or complexes thereof (e.g., polyribosomes). Isolation and characterization of the *mafA* gene products would be required to discriminate among these various possibilities.

The observed inhibition of host cell growth accompanying inhibition of plasmid DNA replication in the *mafA* mutants is reminiscent of the mutant plasmids that affect growth of the host bacteria at high temperatures (11, 13, 18). In the latter cases, a mutation on the plasmid (Rtsl, F*lac*, or ColVB *trp*) is responsible for temperature sensitivities in both host growth and plasmid replication. The present finding that a host mutation affecting plasmid replication can also affect host growth at the restrictive temperature suggests the presence of some mechanisms that can coordinate between plasmid DNA replication and host cell growth. This problem will be examined more closely in a separate paper.

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