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Second *Eco*RI Fragment of F Capable of Self-Replication

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The cloning of fragments of F' plasmid deoxyribonucleic acid produced by restriction endonuclease *Eco*RI has revealed that fragment f7, not previously suspected to have replicative properties, is able to replicate autonomously. The ability of f7 to replicate was observed when it was cloned with fragments coding for resistance to either kanamycin or streptomycin and sulfonamide. Such f7 miniplasmids have been obtained from an F'*lac*$^+$ and two F'*gal*$^+$ temperature-sensitive mutant plasmids and from the unmutated F plasmid. Plasmids containing both f5 and f7 fragments were also obtained. Expression of resistance to "female-specific" bacteriophages requires that f5 and f7 be present in the same plasmid since cells containing separate f5 and f7 plasmids are not resistant to bacteriophage φII. f7 plasmids were less stable than miniplasmids containing f5, particularly at fast growth rates. The bearing of these results on the isolation and behavior of temperature-sensitive F mutants is discussed.

For certain plasmids, the genetic information that is absolutely required for replication is clustered in a relatively small region of the plasmid. For example, all the commonly observed characteristics of the replication of the 94.5-kilobase pair (kb) F plasmid, such as copy number, acridine orange curing, incompatibility, and *seg* function dependence, are manifested by a DNA fragment only 9 kb in length (mini-F). Recent reports indicate that still smaller derivatives of this fragment exhibit the same behavior (8, 9, 17, 30). Similar findings have been made for certain R-factors (10, 20; D. Lane and L. Caro, unpublished data) and for ColE1 (9, 12). Whereas such self-replicating fragments bear the only essential information for plasmid replication, it is possible that other genes outside these regions normally participate in replication of the large parent in some nonessential manner or lie quiescent and only become activated in the absence of the primary replication system or upon transfer to another host cell. A case in point may be the ability of the r-determinant region of plasmid R100.1 to replicate in *Proteus mirabilis* (22, 23) and its failure to do so in *Escherichia coli* (15). In this paper we report the discovery of what appears to be a quiescent replicating system of the F plasmid in *E. coli*.

Several temperature-sensitive mutants of F'*gal*$^+$ (F8 and its deleted derivative, F8-4) have been isolated in this laboratory (11). As part of the characterization of each mutant we have cloned the *Eco*RI fragment known to be capable of self-replication, f5 (i.e., the fifth largest of the 19 fragments produced by *Eco*RI digestion of F DNA). In the course of these cloning experiments we obtained a plasmid which consists of the fragment used for selection (which is incapable of replicating alone) joined to another *Eco*RI fragment of F, f7. The f7 fragment lies adjacent to f5 in F (24). We describe here the characterization and some of the properties of this novel self-replicating fragment.

MATERIALS AND METHODS

Bacteria, bacteriophage, and media. The *E. coli* K-12 strains used are listed in Table 1. Bacteriophage φII, from P. L. Bergquist's collection, was prepared by infection and lysis of AB1157 in L-broth and was stored in L-broth over chloroform.

L-broth and 56/2 minimal salts were described by Bergquist and Adelberg (1) and M9 was described by Lane and Denhardt (16). Solid media consisted of L-broth or 56/2 with 2% Davis (NZ) agar. Media were supplemented with carbohydrates at 0.4% (wt/vol) and, where required, with vitamin B_1 (1 μg/ml), Casamino Acids (vitamin-free; Difco) at 0.4% (wt/vol), thymine at 20 μg/ml, kanamycin sulfate (Km) at 50 μg/ml, streptomycin sulfate (Sm) at 10 μg/ml, and sulfathiazole (Su) at 100 μg/ml.

Cultures were grown at 32 or 37°C in Erlenmeyer flasks equipped with side arms, and growth was monitored with a Klett-Summerson photoelectric colorimeter. Growth of Km^r transformants was on L-agar with kanamycin, whereas growth of Sm^r Su^r transformants was on 56/2-glucose agar with Casamino Acids, streptomycin, and sulfathiazole.

Enzymes. Restriction endonuclease *Eco*RI, prepared by the method of Tanaka and Weisblum (29), was generously provided by P. L. Bergquist. *Bam*HI, *Hae*III, and T4 DNA ligase were purchased from New England Biolabs. Lysozyme was purchased from

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
C600	F⁻ *thi-1 thr-1 leu-6 supE44 lacY1 tonA21*	L. Caro
PB1576	F⁻ *ΔgalKTE* λ⁻	P. Bergquist
AB1157	F⁻ *thi-1 argE3 proA2 leu-8 thr-4 his lacY mtl xyl-5 ara-14 gal-2 tsx rpsL31*	P. Bergquist
CR34	F⁻ *thi-1 thr-1 leu-6 supE44 lacY1 tonA21 thyA6 drm*	

Sigma, and *E. coli* DNA polymerase I was from Boehringer.

Plasmid DNA preparation. For the preparation of purified plasmid DNA, stationary-phase cultures were harvested, washed with 0.05 M Tris (pH 8.0)–0.05 M NaCl–0.005 M EDTA (TES), and resuspended in 0.01 to 0.03 volume of 25% sucrose–0.05 M Tris (pH 8.0). After addition of lysozyme and EDTA (4) the cells were lysed by slowly adding 0.1% Triton X-100 to a final concentration of 0.05%. The lysates were cleared by centrifugation at 18,000 rpm for 15 min in a Sorvall SS-34 rotor, and the supernatants were added to solid NaCl and polyethylene glycol 6000 to give concentrations of 0.5 M and 10%, respectively. The solutions were left overnight at 4°C, and the precipitates were collected by centrifuging at 5,000 rpm for 5 min at 4°C in the SS-34 rotor. The precipitates were redissolved in 4 or 8 ml of TES, kept on ice for 30 min, and then centrifuged as before. The supernatants were mixed with 1 g of CsCl (Varlacoid) and 0.1 ml of a 5-mg/ml amount of ethidium bromide per ml of solution. The solutions were centrifuged at 40,000 rpm for 40 to 48 h in a Ti50 rotor (Beckman) at 15°C, and the plasmid bands were collected through a needle inserted through the tops of the tubes. Ethidium bromide was removed by two or three extractions with isopentenyl alcohol, and the DNAs were dialyzed extensively against 10 mM Tris (pH 8.0)–1 mM EDTA. DNA concentrations were measured by the diphenylamine reaction (2).

For partial purification of plasmid DNA from transformants the same lysis procedure was used, as described by Lane and Chandler (14).

Enzyme reactions. For cleavage of DNA with restriction endonucleases, 0.2 to 1 μg of DNA in 20 μl of 10 mM Tris–1 mM EDTA, pH 8.0, was added to: (i) 2 μl of 0.9 M Tris (pH 7.4), 0.1 M $MgCl_2$, and 1 μl of *Eco*RI (1 U/μl); (ii) 2 μl of 0.5 M NaCl, 0.06 M Tris (pH 7.4), 0.06 M $MgCl_2$, 0.06 M 2-mercaptoethanol, 1 mg of bovine serum albumin per ml, and 1 μl of *Bam*HI (2 U/μl); (iii) 2 μl of 0.06 M Tris (pH 7.4), 0.06 M NaCl, 0.06 M $MgCl_2$, 0.06 M 2-mercaptoethanol, 1 mg of bovine serum albumin per ml, and 1 μl of *Hae*III (1 U/μl). Reactions were carried out for 1 h at 37°C.

Before ligation, *Eco*RI-digested preparations were incubated at 65°C for 10 min to inactivate the endonuclease and then chilled. Purified drug resistance restriction fragments (about 0.5 μg) were added, together with Tris, pH 8.1 (to 30 mM), $MgCl_2$ (4 mM), $(NH_4)_2SO_4$ (10 mM), EDTA (1.2 mM), dithiothreitol (10 mM), ATP (0.1 mM), bovine serum albumin (50 μg/ml), and 0.1 to 0.2 U of T4 DNA ligase per ml. Ligation was carried out at 0°C for 18 to 24 h, and the mixture was used directly for transformation of $CaCl_2$-treated C600 cells according to the procedure of Cohen et al. (5).

Gel electrophoresis. Electrophoresis of DNA was carried out on horizontal agarose slab gels (18) in Tris-borate buffer (19). DNA solutions were mixed with agarose (0.25% final concentration), pipetted into the wells, allowed to set, and overlaid with 0.8% agarose. Electrophoresis was at 135 V (about 20 mA) for 2.5 to 3 h. After electrophoresis the slab was cut out, soaked in 1 μg of ethidium bromide per ml, placed on a Blak Ray UV transilluminator screen, and photographed with Agfapan 400 film. The film was developed by using a reversal process.

Preparation of purified restriction fragments. To prepare drug resistance restriction fragments for cloning, 50 to 100 μg of pLC36 (containing the G fragment, see Table 2) or pML21 (containing the Kan fragment) DNA was digested with *Eco*RI. The reaction was terminated by adding EDTA, and the reaction mixture was layered on a 5 to 20% gradient of sucrose in 1 M NaCl–0.05 M Tris (pH 8.0)–0.005 M EDTA–20 μg of ethidium bromide per ml and centrifuged in a Beckman SW27 rotor at 26,000 rpm for 26 h at 50°C. The appropriate band was drawn off through a needle connected to a peristaltic pump, briefly dialyzed, and recentrifuged as above. The band was removed as before, and the DNA was precipitated by adding 0.05 volume of 4 M sodium acetate (pH 5.5) and 2 volumes of isopropanol. The precipitation was repeated, and the DNA was finally dissolved in 0.3 ml of 10 mM Tris–1 mM EDTA. DNA prepared in this way was free of the ColE1-derived vector fragments as judged by its failure to give rise to transformants after incubation with ligase.

Labeling of DNA. For use as probes in hybridization experiments, plasmid DNAs were labeled by incorporation of labeled triphosphates during the nick-translation reaction of *E. coli* DNA polymerase I. Our method follows that of Jeppesen et al. (13). [^{3}H]dTTP (47 Ci/mmol; Amersham) was dried in a desiccator and redissolved at 17 μM in reaction buffer, triphosphate mixture, and the DNA to be labeled (1 to 2 μg). To the 25-μl reaction mixture at room temperature (19 to 21°C) were added DNase I (1 μl of 0.1 μg/ml in activating solution) and, 1 min later, 1 μl (3 U) of *E. coli* DNA polymerase I. Samples of 1 μl were taken to monitor incorporation, and when maximum incorporation was obtained (usually 60 to 75 min) the reaction was terminated by addition of EDTA (20 mM). The mixture was passed over a Sephadex G150 column (0.5 by 5 cm) and eluted with 10 mM Tris (pH 8.0)–1 mM EDTA–0.01% diethylpyrocarbonate. Fractions, 0.2 ml, were collected, and samples were assayed for radioactivity on Whatman GFC filters in toluene-Omnifluor (New England Nuclear Corp.) scintillation fluid. The excluded peak fractions were pooled and stored at −20°C. Usually 50 to 60% of the [^{3}H]dTTP was incorporated into high-molecular-weight material, to give a specific activity of about 1×10^7 to 2×10^7 cpm/μg.

DNA transfer and hybridization. Restriction endonuclease fragments in agarose gels were transferred to nitrocellulose sheets (AAWG; Millipore) according to the method of Southern (26). After transfer the sheets were baked under vacuum at 80°C for 2 h, then

treated with 70% ethanol for 2 to 3 min, and dried. The radioactive "probe" DNA was made up in 1.0 to 1.5 ml of 2× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide, denatured at 100°C for 2 min, and chilled. The nitrocellulose sheet was saturated with DNA solution, wrapped in a double envelope of Saran-wrap, and incubated at 42°C for 18 to 24 h. After hybridization the sheet was washed twice in 100 ml of 2× SSC–50% formamide (20 min per wash at room temperature) and then twice in 2× SSC. The sheet was dried, soaked in 7% diphenyloxazole in ether for 5 to 10 s, and autoradiographed with Kodak X-Omat R X-ray film for 4 to 7 days at −70°C.

RESULTS

Presence of f7 in mini-F plasmids. Our cloning procedure follows that of previous workers (17, 30) and consists of incubating *Eco*RI-treated F'_{ts} *gal*$^+$ DNA in the presence of T4 DNA ligase and another *Eco*RI-generated fragment (G fragment) which encodes resistance to Sm and Su but is incapable of self-replication. Transformation of competent *E. coli* cells with the ligated DNAs yields Sm^r Su^r transformants. These transformants contain plasmids consisting of the G fragment joined to a self-replicating fragment of the F'_{ts} *gal*$^+$ DNA.

During an analysis of such plasmids derived from one F'_{ts} *gal*$^+$ (F8-4-11), we observed that one of the plasmids (pNZ310) contained not the usual f5, but a fragment whose mobility is identical to that of f7 (Fig. 1B). The obvious conclusion from this observation was that f7 is capable of self-replication, but other explanations seemed possible. The apparent f7 could have been a contaminant plasmid, a deletion derivative of f5, or enabled to assume replicative properties solely by virtue of its attachment to the G fragment. Moreover, it was possible that while the fragment itself was f7 DNA, it had undergone an internal rearrangement or had been mutated during the original nitrosoguanidine treatment of F8-4 to become a replicon and that wild-type f7 DNA would be incapable of replication.

To demonstrate that the apparent f7 DNA is derived from F, we hybridized ^{3}H-labeled pNZ310 DNA to *Eco*RI fragments of F DNA that had been separated by agarose gel electrophoresis and transferred to a nitrocellulose sheet (Fig. 1B). After hybridization the sheet was exposed to X-ray film to produce the autoradiograph shown in Fig. 1A. The ^{3}H-labeled pNZ310 DNA had hybridized to the f7 fragment of both F (lane a) and F8-4 (lane b). An identical set of fragments (electrophoresed on the same slab gel) was hybridized with ^{3}H-labeled pNZ001 DNA. The resulting autoradiograph (Fig. 1C) shows that the [^{3}H]DNA hybridized with the f5 fragment of F (lane a), F8-4 (lane b), and pML31 (lane c) but not with the f7 portion of pNZ310 and pNZ311 (lanes d and e). Hence, pNZ310 is neither a contaminant plasmid nor derived from f5 DNA.

It is very unlikely that attachment to the G

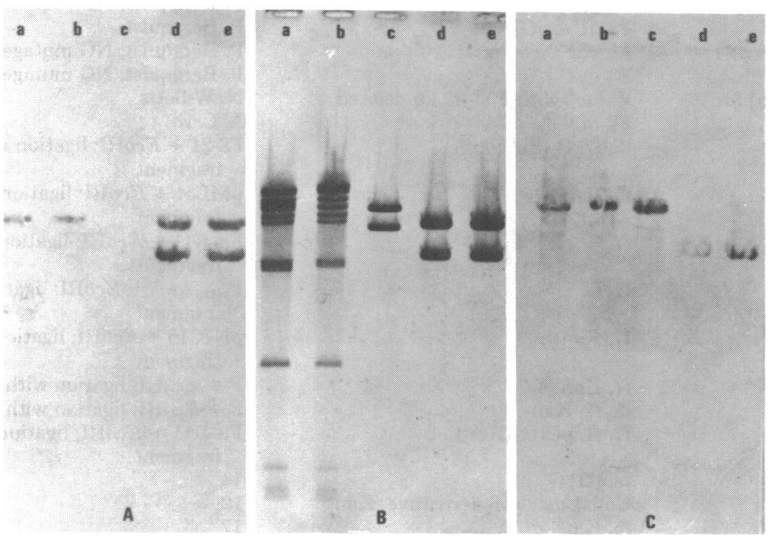


FIG. 1. *Absence of f5 DNA from presumptive f7 plasmids. DNA of the plasmids pNZ310 and pNZ311 was purified by CsCl-ethidium bromide centrifugation of cleared lysates and treated with EcoRI. Duplicate samples were electrophoresed on 0.8% agarose gels together with EcoRI-digested DNA of related plasmids (B). After staining and photography, the fragments were transferred to a nitrocellulose filter and hybridized with ^{3}H-labeled pNZ310 (A) or ^{3}H-labeled pNZ001 (C) DNA. a, F; b, F8-4; c, pML31; d, pNZ310; e, pNZ311.*

fragment enables f7 to replicate. *Eco*RI-treated pNZ310 DNA was ligated with an *Eco*RI-derived fragment that encodes Km resistance. The subsequent transformation yielded Km^r clones whose plasmids (e.g., pNZ320, Table 2) consisted solely of f7 DNA joined to the Kan fragment (gel not shown). The possibility that both G and Kan fragments can confer replicative ability on an attached nonreplicating fragment is remote.

To determine the ability of wild-type f7 DNA to replicate, we digested F DNA with *Eco*RI and ligated the fragments with Kan fragment DNA. C600 cells were transformed with this mixture and spread on L-agar containing kanamycin. The Km^r colonies formed two distinct size classes, with about two-thirds large and one-third small (Table 3). The plasmids of eight transformants from each size class were extracted, and the DNA was treated with *Eco*RI and electrophoresed in agarose gels. The results of this analysis are shown in Table 3 and Fig. 2. All the plasmids extracted from small colony formers were of the f7-Kan type (Fig. 2B), whereas most of those extracted from the large colony formers contained f5 (Fig. 2D). Possible reasons for the difference in colony size are discussed below. The molecular weights of the f7 and Kan fragments are similar (5.0×10^6 and 4.7×10^6, respectively), and they were not well resolved in these agarose gels (Fig. 2B, lanes a–d, g–j). For this reason we showed that the presumptive f7-Kan plasmids contained the same piece of F DNA as pNZ310 by hybridizing ^{3}H-labeled pNZ310 DNA with the DNA transferred from these gels to nitrocellulose sheets (Fig. 2A and D). Note that pNZ310 DNA hybridizes in the region of the Kan fragment (Fig. 2A, lanes a–d, g–j; 2D, lanes b, g, h, j) but not to the Kan fragment itself (lane f). This hybridization revealed that one of the plasmids (pNZ280) consisted of both f5 and f7 in addition to the Kan fragment (Fig. 2D, lane b, and 2F, lane b).

Our conclusion from this experiment is that the ability to replicate is a property of the f7 fragment in unmutated F DNA. We have also obtained f7 plasmids from F'_{ts113} *lac*$^+$ (6) (see Fig. 1) and F8-16 (Table 2). Although we have three such plasmids obtained from different temperature-sensitive F-primes, none show detectable temperature sensitivity.

Arrangements of DNA sequences in f7 plasmids. To confirm the conclusions we had reached, and to demonstrate that no gross rear-

TABLE 2. *Plasmids used or constructed*[a]

Plasmid	Description	Source or reference
F	Wild type	P. Bergquist
F8-4	F′ *gal*$^+$	P. Bergquist
F8-4-11	F'_{ts} *gal*$^+$	P. Bergquist; NG mutagenesis of F8-4[b]
F8	F′*gal*$^+$*aroG*$^+$*nad*$^+$	P. Bergquist
F8-16	F'_{ts} *gal*$^+$	P. Bergquist; NG mutagenesis of F8
F8-21	F'_{ts} *gal*$^+$	P. Bergquist; NG mutagenesis of F8
F′Δ[33–43 kb] *lac*$^+$	F′ *lac*$^+$ with F33–43 kb deleted	N. Willetts
F'_{ts113} *lac*$^+$	F'_{ts} *lac*$^+$	L. Caro
pNZ200	f5, f7, G (Su^r Sm^r)	F8-21 + *Eco*RI; ligation with G fragment
pNZ001	f5, G	pML31 + *Eco*RI; ligation with G fragment
pNZ310	f7, G	F8-4-11 + *Eco*RI; ligation with G fragment
pNZ311	f7, G	F'_{ts113} *lac*$^+$ + *Eco*RI; ligation with G fragment
pNZ320	f7, Kan	pNZ310 + *Eco*RI; ligation with Kan fragment
pNZ322	f7, Kan	F + *Eco*RI; ligation with Kan fragment
pNZ280	f5, f7, Kan	F + *Eco*RI; ligation with Kan fragment
pNZ300	f7, G, pCR1 (Km^s)	F8-4-11 + *Eco*RI; ligation with G fragment
pLC36	pCR1, G	14
pML21	ColE1 deletion derivative, Kan	12
pML31	f5, Kan	17

[a] The G fragment, which carries genes for resistance to streptomycin and sulfonamide, was derived ultimately from R100.1. The Kan fragment, which carries a gene for kanamycin resistance, was derived originally from R6-5. pCR1 Km^s is a fortuitously obtained deletion derivative of pCR1 (Km^r) which was present as a contaminant in a G fragment preparation used for cloning. f5 and f7 are the fifth and seventh largest *Eco*RI fragments of F.

[b] NG, Nitrosoguanidine.

rangement of DNA sequences within f7 had occurred, we made use of the observation of Childs et al. (3) and Skurray et al. (25) that at the f5-distal end of f7 there is a characteristic cluster of sites recognized by restriction endonuclease *Bam*HI.

We first incubated the DNAs of pNZ310 and F with *Bam*HI and separated the fragments by electrophoresis on a 1% agarose gel (Fig. 3A). Four of the fragments, 7, 8, 9, and 11, were present in the digests of both plasmids as expected from the results of the above authors. One of the small F fragments, 10, was not present in the pNZ310 pattern. This result is not in accord with the restriction map published by Childs et al. (3) but does agree with the results of Skurray et al. (25). This point is discussed later.

TABLE 3. *Plasmids present in cells transformed with cloned F EcoRI fragments*[a]

Colony size	No. of transformants	Type of plasmid			Total analyzed
		f5-Kan	f7-Kan	f5-f7-Kan	
Large	29	4	3	1	8
Small	15	0	8	0	8

[a] *Eco*RI-treated F DNA was incubated with ligase and the Kan fragment, and C600 cells were transformed with the ligation mixture. The partially purified plasmids from cleared lysates of 16 transformants were identified by *Eco*RI digestion and hybridization with ^{3}H-labeled pNZ310 DNA (Fig. 2).

To demonstrate the source of the fragments shown in Fig. 3A, we performed a series of restriction enzyme digestions. The DNAs of pNZ310, G fragment, pNZ001, pNZ200, and pNZ322 were digested with *Bam*HI alone and with both *Bam*HI and *Eco*RI. The digested DNAs were electrophoresed on 1.2% (Fig. 3B) and 0.7% (not shown) agarose gels, together with restriction fragments of known molecular weight. G fragment is cleaved by *Bam*HI to yield four fragments and hence has three *Bam* sites (Fig. 3B, lanes c and g). Because *Bam*HI digestion of pNZ310 (lane i) gives eight fragments, f7 must have five *Bam* sites, as found by Skurray et al. (25). Treatment of *Bam*-digested pNZ310 DNA with *Eco*RI yielded a total of 10 fragments as expected; a comparison of this fragment pattern (lane e) with those of G (lane c and g) and the *Bam* digest of pNZ310 (lane i) allows a ready identification of the bands arising from f7. In addition to 7, 8, 9, and 11, the largest and second smallest (Δ10) fragments seen in lane e are derived from f7. Note that these six fragments are also seen in the combined digests of pNZ322 (lane a) (the Kan fragment in this plasmid has no *Bam* sites) and pNZ200 (lane b). These results confirm the identity of the f7 component of pNZ310 and pNZ322 and demonstrate that any internal rearrangement of sequences in f7 has not been of sufficient extent to alter the relative positions of the *Bam* and *Eco* sites.

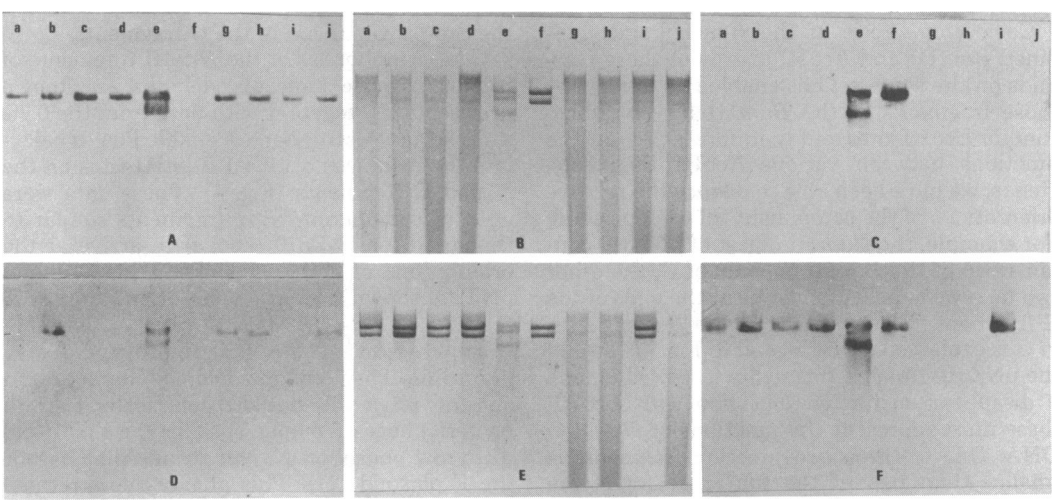


FIG. 2. *Ability of wild-type f7 DNA to form an autonomous plasmid. EcoRI-treated F DNA was ligated with the Kan fragment, and C600 was transformed with the ligated DNAs. Eight small and eight normal sized transformant colonies were picked into L-broth + kanamycin and grown to saturation. Plasmid DNA was extracted, treated with EcoRI, and electrophoresed on 0.8% agarose gels. (B) Lanes a–d, g–j, small colonies; (E) lanes a–d, g–j, normal colonies; (B and E) lane e, pNZ200, and lane f, pML31. The fragments were transferred to a nitrocellulose sheet until transfer was estimated to be about half complete. The DNA remaining in the gel was then transferred to a second sheet. One sheet was hybridized with ^{3}H-labeled pNZ310 DNA (A and D); the other was hybridized with ^{3}H-labeled pNZ001 DNA (C and F).*

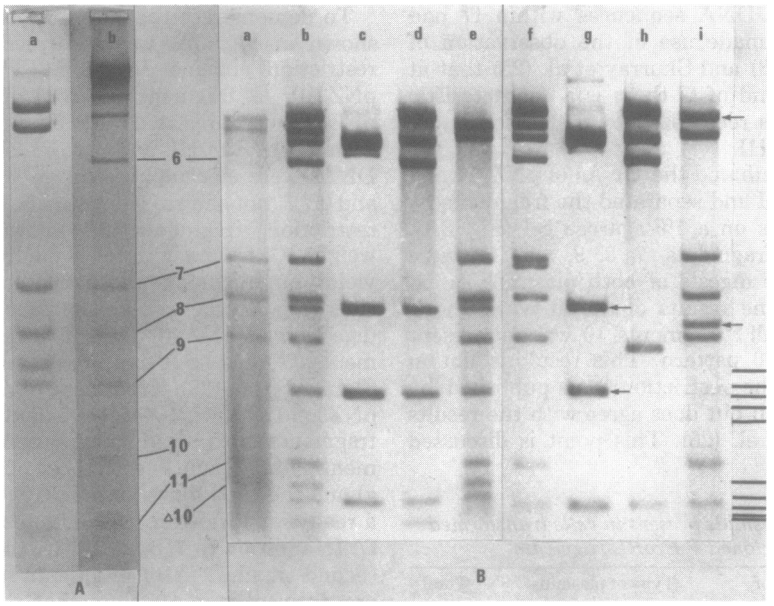


FIG. 3. *Agarose gel electrophoresis of BamHI and BamHI + EcoRI fragments of pNZ310 and related plasmids. (A) pNZ310 and F DNAs were incubated with BamHI and electrophoresed on a 1% agarose gel, as described in the text. a, pNZ310; b, F. (B) pNZ310 and related plasmid DNAs were incubated with BamHI. Half of each digest was then treated with EcoRI. All samples were electrophoresed on a 1.2% agarose gel together with a HaeIII digest of pBR322 DNA. a–e, BamHI + EcoRI; f–i, BamHI. a, pNZ322; b and f, pNZ200; c and g, G fragment; d and h, pNZ001; e and i, pNZ310. The bars on the right-hand side show the positions of the larger pBR322 fragments; their lengths (in base pairs) are: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184 (27). The numbers between (A) and (B) refer to the smaller BamHI fragments of F. Δ10 is the name we have given to the smallest fragment obtained by combined digestion of f7 on the assumption that it is part of the BamHI 10 fragment of F (see text). The arrows denote fragments containing termini of G and f7 (see text).*

Coelectrophoresis of the *Bam*HI and combined *Bam*HI and *Eco*RI digests of these plasmids on the same gel has enabled us to identify those fragments in the *Bam*HI digests which contain *Eco*RI sites and therefore represent the junctions between various *Eco*RI fragments. Hence, we have been able to deduce the relative orientations of the components of each plasmid. For example, the *Bam*HI digest of pNZ310 contains two of the *Bam* fragments of G; the other two (arrows in lane g) must be at the ends of the G fragment. (The ordering of the *Bam*HI sites in G is explained in the legend to Fig. 4.) Two of the pNZ310 *Bam*HI fragments (arrowed in lane i) disappear on further digestion with *Eco*RI; these must represent the junctions of f7 and G DNA. One of these two junction fragments is smaller than two of the four new fragments produced by *Eco*RI treatment of *Bam*-digested pNZ310 (lane e). Hence, it must give rise to the two smallest of these new fragments, that called Δ10 and the smaller of the G end fragments. We can conclude that the orientation of the f7 and G fragments in pNZ310 is as shown in Fig. 4, with the cluster of *Bam*HI sites in f7 adjacent to the Sm-proximal end of the G fragment.

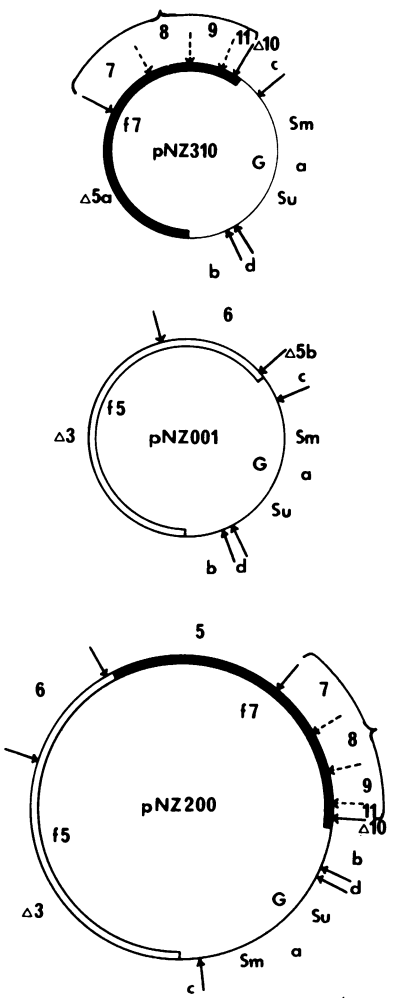
Coelectrophoresis of the *Hae*III fragments of pBR322, whose molecular lengths are known precisely (27), together with data from the 0.7% agarose gel electrophoresis enabled us to calculate the distances between *Bam*HI sites on the f7 and G fragments (Fig. 4). These data were used in conjunction with arguments similar to that used for pNZ310 (above) to arrive at the orientations of the fragments in pNZ001 and pNZ200 shown in Fig. 4. The orientation of f5 relative to f7 in pNZ200 is the same as that in F.

Expression of the Pif phenotype by f7 plasmids. The f7 fragment can be characterized not only physically but also genetically. Certain bacteriophages ("female-specific"), e.g., T7 and φII, grow poorly on *E. coli* strains that harbor the F plasmid (21). This phenotype is termed Pif (or Fex), and hence F is *pif*$^+$. From cloning experiments, Skurray et al. (24) were able to deduce that f7 bears at least some of the information required for expression of Pif. Subsequently Skurray et al. (25) showed that DNA sequences in both f7 and f5 were required. The question remains as to whether two separate *pif*

genes exist on f7 and f5, or whether a gene that spans the *Eco*RI site between these fragments is involved.

We transformed strain AB1157 with various plasmids, singly or in combination, and tested the ability of the transformants to inhibit growth of bacteriophage φII (Table 4). Strains that contained f5 plasmids (e.g, pML31) or f7 plasmids (e.g., pNZ310), or both (e.g., pML31 and pNZ310), showed little inhibition of φII growth compared with AB1157 containing no plasmid; the efficiency of plaque formation was high and the plaques were large. In contrast, strains containing plasmids in which f5 and f7 are linked, as in F, severely inhibited φII growth; plaque-forming efficiency was 10- to 50-fold lower than for the other strains, and the plaques were minute. Therefore, expression of Pif depends on the f5–f7 junction remaining intact. Presumably this junction lies within a *pif* gene or connects such a gene with its promoter.

Instability of f7 plasmids at fast growth rates. When a strain harboring an f7 plasmid is grown in L-broth without antibiotics, the pro-



Fragment	Length (kb)
Ga	3.4
Gb	0.91
Gc	0.54
Gd	0.23
f5	9.2
f7	7.5
Δ3	6.5
5	4.2
Δ5a	4.0
Δ5b	0.18
6	2.5
7	1.24
8	0.97
9	0.74
10	0.49
Δ10	0.28
11	0.33

FIG. 4. *Orientation of fragments within plasmids. The orientations were deduced as described in the text. The numbers outside the perimeters are those of the BamHI fragments. Preliminary results from this laboratory suggest the order shown for the bracketed sites within f7. The BamHI sites in the G fragment were mapped as follows. pLC36 (Table 2) contains two SalI sites, one in G and one in pCR1. SalI-digested pLC36 was treated with ligase to form a plasmid, pLC80, which contains one SalI site and still encodes resistance to streptomycin and sulfonamide. The SalI site in G is known to be at the Su-proximal end (28). Digestion of pLC80 with BamHI and EcoRI yielded fragments Ga and Gc. Hence, Gc is the Sm-proximal Bam fragment in G. Gb is the other end fragment (Fig. 3B, lanes g and i). As Gd was not seen in the pLC80 digest it must lie between Gb and Ga.*

TABLE 4. *Inhibition of plaque formation by phage φII in plasmid-bearing strains*[a]

Plasmid	Relevant *Eco*RI fragments	Plating efficiency of φII (relative to AB1157 pNZ001)	Pif
pNZ001	f5	1.0	−
pML31	f5	0.75	−
F'Δ[33–43 kb] *lac*$^+$	f5	0.50	−
pNZ310	f7	0.90	−
pNZ322	f7	0.90	−
pNZ300	f7	0.73	−
F'Δ[33–43 kb] *lac*$^+$ + pNZ310	f5, f7	0.80	−
F'Δ[33–43 kb] *lac*$^+$ + pNZ300	f5, f7	0.40	−
pML31 + pNZ310	f5, f7	0.80	−
pML31 + pNZ300	f5, f7	0.80	−
pNZ001 + pNZ322	f5, f7	0.70	−
F'*gal* (F8-4)	f5-f7	0.013 (tiny plaques)	+
pNZ280	f5-f7	0.047 (tiny plaques)	+
pNZ200	f5-f7	0.10 (tiny plaques)	+
None		2.5	−

[a] Various plasmids were introduced into strain AB1157. The strains were grown in the presence of drugs appropriate for maintenance of f7 plasmids (streptomycin or kanamycin) before plating on L + Sm or L + Km with samples of diluted phage φII. The titer of phage plated on each strain is presented relative to the titer on AB1157 containing pNZ001.

portion of plasmid-carrying cells in the culture steadily declines. This instability appears to be less severe in cultures with slower growth rates.

To measure this apparent dependence of plasmid stability on growth rate, strains harboring different f7 plasmids were inoculated into media which supported growth rates between 0.6 and 3 generations per hour. Samples were removed from each culture during 8 to 14 generations of exponential balanced growth. Each sample was plated to determine the total cell titer and the drug-resistant cell titer. From these data, the growth rates of the total population and the drug-resistant subpopulation were measured, and the ratio of growth rates was computed. In Fig. 5 we show the change in this ratio with respect to total cell growth rate. Although these experiments provided results too variable for the derivation of precise relationships, it is clear that as growth rate increases, the segregation of plasmid-negative cells increases proportionately. At growth rates of less than one generation per hour segregation still occurs, but at a relatively low frequency. The three f7 plasmids tested show the same response to growth rate, and the response is similar in both host strains used, CR34 and PB1576.

It is reasonable to conclude that although f7 plasmids are readily obtained by the type of cloning experiment we have used, the maintenance of such plasmids in *E. coli* is partially defective.

DISCUSSION

We have found that F contains an *Eco*RI fragment, in addition to f5, capable of autonomous replication. f7 is the only such fragment found, and it is unlikely in view of the cloning experiment described in Results (Table 2) that

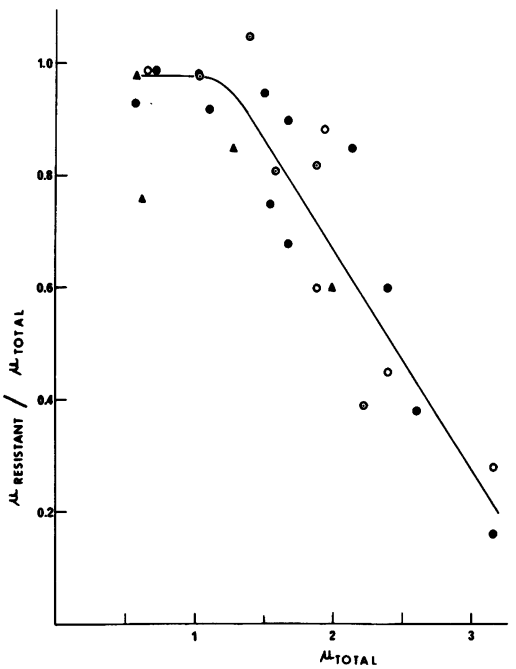


FIG. 5. *Segregation of f7 plasmids at different growth rates. Cells harboring f7 plasmids were inoculated from washed M9-glycerol or M9-glucose-Casamino Acids cultures into the following media (without drugs): M9-glycerol, M9-glucose, M9-Casamino Acids, M9-glucose-Casamino Acids, L-broth, L-broth + glucose. Samples were taken from the resulting exponential cultures (at 37°C) over 8 to 14 generations and plated on 56/2-glucose-Casamino Acids agar with and without drugs. The colonies were counted after incubation at 37°C. The data were used to calculate growth rates of the drug-resistant and total populations. Each point represents a single culture, and each symbol represents a different strain: (●) pNZ310/CR34; (○) pNZ320/CR34; (◎) pNZ322/CR34; (▲) pNZ310/PB1576. μ = generations per hour.*

any other self-replicating *Eco*RI fragment exists. However, other fragments may be capable of complementing defective replication of adjoined replicons as shown by Timmis et al. (31) for R6-5. Alternatively, further regions of F, isolated by cleavage with enzymes other than *Eco*RI, may prove able to replicate. We regard as unlikely the possibility that both G and Kan fragments are responsible for the ability of f7 to replicate. There is no reason to suppose that f7 replication is in any way connected with conjugal transfer, which is initiated within f6 at least 20 kb away (24).

The isolation of f7 plasmids enabled us to investigate two features of f7 DNA. Childs et al. (3) published a *Bam*HI restriction map of F showing six *Bam*HI sites in the f7 fragment. Skurray et al. (25) cloned f7 with the pSC101 vector and found that *Bam*HI cut f7 at only five sites; they suggested that the *Bam*HI fragment 10 may be derived from cleavage by *Bam*HI in both f7 and the adjacent f8 *Eco*RI fragments. Our results confirm those of the latter authors for the following reasons. First, pNZ310 has eight *Bam* sites, of which three are in G and the remaining five are in f7 (Fig. 3). Second, the 0.49-kb fragment 10 obtained by *Bam*HI digestion of F DNA is not present in similar digests of f7 plasmids. Combined *Bam*HI and *Eco*RI digestion of f7 plasmids generates a 0.28-kb fragment which comes from that end of f7 which is adjacent to f8 in F. It is possible that this 0.28-kb fragment is part of *Bam*HI fragment 10 and that the other part (0.21 kb) comes from the *Eco*RI f8 fragment.

The second feature of f7 DNA is its involvement in the expression of Pif. Only strains in which both the f5 and f7 fragments were present in the same plasmid significantly inhibited growth of φII. It is possible that some strains containing distinct f5 and f7 plasmids failed to inhibit phage growth because the unstable f7 plasmid was lost from a large proportion of the cells. Although this might have occurred for a strain containing pML31 and the unstable pNZ310, it is not possible for a strain which contained pML31 together with the stable, high-copy-number plasmid pNZ300. Moreover, the φII platings were made in the presence of the antibiotic appropriate for the enforced maintenance of the f7 plasmids. This reinforces our conclusion that the integrity of the f5-f7 junction is required for the inhibition of φII growth. We have demonstrated that one cloned derivative (pNZ200) that expressed the Pif$^+$ phenotype retains the relative orientation of f5 and f7 found in F.

In view of the similarity of our cloning experiments to those of previous workers (17, 30), it is surprising that the ability of f7 to replicate has not been reported previously. Possibly the small colonies formed by cells containing f7 plasmids militated against such isolates being found. We presume that the small colony size on L-plates containing antibiotics reflects the instability of f7 plasmids at fast growth rates, since on minimal plates cells containing f7 plasmids give rise to normal sized colonies. The instability of f7-Kan plasmids probably also accounts for the high background of DNA seen after agarose gel electrophoresis (Fig. 2). This background is likely to result from the degradation of DNA in plasmid-negative segregants which are killed by the kanamycin in the medium.

We have not yet investigated the reason for the instability of f7 plasmids. Possibly DNA from the adjacent f5 or f8 fragments contributes to the formation of a stable supplementary replication system in F. The f7 replication system might be stable in other bacterial taxa. Alternatively, f7 replication may always be a relatively low-frequency event. The degree of segregation of f7 plasmids at different growth rates (Fig. 5) suggests that they may have a maximum replication rate of about once per hour. Plasmid incompatibility has proved difficult to test owing to the instability of these plasmids. To date we have failed to detect an increased level of segregation, indicative of incompatibility, in strains selected with antibiotics to contain both pNZ310 (f7-G) and pNZ320 (f7-Kan).

The existence of the f7 replication system may explain certain features of the behavior of the parent F-primes. The isolation of F mutants with a temperature-sensitive defect in plasmid maintenance has proved an exceptionally laborious task. This difficulty may have arisen from the possibility that inactivation of the primary (f5) replication system at the restrictive temperature was masked by replication from within f7. Indeed, we have observed that replication of f5 miniplasmids derived from temperature-sensitive F-primes is more severely inhibited at the restrictive temperature than parent F-prime replication. Moreover, at the restrictive temperature most of the F-prime mutants are more stable at low growth rates (unpublished observations), a phenomenon also evident in the data of Cuzin and Jacob (6). This behavior parallels that of the f7 plasmids.

Secondary sources of replicative ability have been observed in other plasmids. R100.1 appears to possess both a primary (*repA*) and a secondary (*repB*) replication system (33). The secondary system can exist as a separate, autonomous, partially stable plasmid. However, it is not yet

known how much of the parent plasmid is present in the *repB* derivatives, and a direct comparison with the f7 plasmids would be premature. Secondary replicative systems have also been detected in R6-5 (31) by the ability of fragments cloned in a ColE1 vector to promote replication in a *polA* mutant strain. The *E. coli* chromosome also has been demonstrated to contain two regions of DNA, each capable of autonomous replication as a miniplasmid (7, 32). It may prove that most complex genomes include two or more regions of DNA with at least some ability to promote replication. An interesting possibility is that this reflects the evolution of these genomes by partial or complete fusion of at least two smaller autonomous elements.

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