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Inhibition of Replication of an F'*lac* Episome in Hfr Cells of *Escherichia coli*

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Hfr strains of *Escherichia coli* K-12 were found capable of accepting a F'*lac* episome during mating, with a frequency approximating that of F^- strains. However, the F'*lac* episome was unable to replicate in the Hfr cells, and was diluted out during the growth of the culture. The lac^+ gene of the episome can be "rescued" by recombination into the host chromosome, as shown by the appearance of variegated recombinant colonies on a lactose-fermentation indicator medium. In *recA* Hfr strains, however, no lac^+ offspring were obtained in crosses with F'*lac* donors. The induced synthesis of β-galactosidase in F'lac^+ × Hfr zygotes was studied. Rates of enzyme synthesis were approximately constant with respect to time as expected from unilinear inheritance of the F'*lac* episome. However, the rate of synthesis eventually increased, presumably due to integration of the lac^+ gene in some of the zygotes. In F'lac^+ × *recA* Hfr zygotes the rate of β-galactosidase synthesis remained constant with respect to time, as expected.

In Hfr strains of *Escherichia coli* K-12 the sex factor is integrated into the chromosome, whereas in F^+ strains it replicates independently of the chromosome. The fact that one does not usually find strains carrying the sex factor in both states simultaneously suggests that the two states are incompatible with each other. Further evidence in support of such an incompatibility has been obtained in several laboratories. Maas and Maas (18, 19) found that the lac^+ marker was very rarely recovered in chromosomal recombinants in crosses between an F'*lac* donor and an Hfr recipient. Scaife and Gross (26) infected Hfr, F^+ and F^- recipients which were otherwise isogenic, with F'*lac* and found that, when the recipient was a male, lac^+ offspring formed variegated colonies on EMB lactose agar, indicating that the superinfecting F'*lac* was not inherited by all progeny cells. Those lac^+ offspring which were still Hfr carried no free F'*lac;* instead, the lac^+ gene was integrated in the chromosome. They concluded that multiplication of free F'*lac* is inhibited in Hfr cells.

The work to be described deals with the fate of a superinfecting F factor in Hfr strains. The results of our experiments confirm that the F'*lac* episome enters Hfr recipients, but in the great majority of cells, if not all, its replication is inhibited. This inhibition of replication does not prevent the expression of the $lacZ^+$ gene on the episome, leading to the production of β-galactosidase.

MATERIALS AND METHODS

Media and strains. The bacterial strains used are listed in Table 1. Strain designations and symbols for genotypes follow the recommendations of Demerec et al. (8). Strains were kept on nutrient or neopeptone agar slants at 4 C. The nutrient growth medium was prepared from a digest of beef heart to which was added Neopeptone (10 g per liter) and NaCl (5 g per liter). Minimal medium used was medium A (6) supplemented with the required growth factors and, unless otherwise noted, with 0.2% glucose as carbon source. Amino acids and streptomycin were added at final concentration of 100 μg/ml, and sugars at 0.2%. When solid media were required, Difco agar was added at a final concentration of 2%. All bacterial dilutions were made in sterile 0.85% NaCl. Ability to ferment sugars was tested on MacConkey agar base with the test sugar added at a concentration of 2%. "Conditioned broth" was prepared by adding 4 ml of an overnight culture to 400 ml of broth, to give an optical density (OD) of about 0.01; the cells were grown until an OD of 0.1 was reached. This corresponds to about 10^8 cells per ml. All OD measurements were made with a Lumetron colorimeter at 580 mμ. The culture was then passed through a membrane filter (HA, 0.45 μ; Millipore Corp., Bedford, Mass.). The sterile filtrate was used as a medium for experiments involving β-galactosidase induction. Revel (23) reported that there is a shorter lag in β-galactosidase induction in "conditioned medium" than in a medium that has not been conditioned.

Mating methods. F'*lac* transfer to Hfr recipients was performed as follows. One loopful of an overnight broth culture of the Hfr recipient was inoculated into 5 ml of broth in a 125-ml flask and incubated with

TABLE 1. *Bacterial strains used* [a]

Strain	Nutritional requirements									Response to		Recombination genotype	Lactose fermentation	Lac genotype	Mating type
	Thy	Thi	Pro	His	Arg	Thr	Leu	Met	Ade	Str	Spc				
AB1353	+	−	−	−	−	+	+	+	+	s	s	*rec*$^+$	−	*lacY*	F$^-$
MA3020	+	−	−	−	−	+	+	+	+	s	s	*rec*$^+$	+	*lacY*/*lac*$^+$	F′*lac*
AB253	+	−	+	+	+	−	−	+	+	r	s	*rec*$^+$	−	*lacZ*	F$^-$
MA27	−	−	+	+	+	−	−	+	+	r	s	*rec*$^+$	−	*lacZ*	F$^-$
AB284	+	−	+	+	+	−	−	+	+	r	s	*rec*$^+$	−	*lacZ*	F$^+$
AB311	+	−	+	+	+	−	−	+	+	r	s	*rec*$^+$	−	*lacZ*	Hfr[b]
MA1025	−	−	+	+	+	−	−	+	+	r	s	*rec*$^+$	−	*lacZ*	Hfr[b]
JC1553	+	+	+	−	−	+	−	−	+	r	s	*recA*	−	*lacY*	F$^-$
MA1048	+	+	+	−	−	+	−	−	+	r	s	*recA*	−	*lacY*	Hfr[c]
MA28	+	−	+	+	+	−	−	+	+	r	s	*recA*	−	*lacZ*	F$^-$
MA1026	+	−	+	+	+	−	−	+	+	r	s	*recA*	−	*lacZ*	Hfr[b]
MA1049	+	−	+	+	+	+	+	+	−	s	r	*rec*$^+$	+	*lac*$^+$	Hfr[d]
MA1044	+	−	+	+	+	+	+	+	−	s	r	*rec*$^+$	−		Hfr[d]

[a] Strains AB1353, AB253, AB284, and AB311 are from E. Adelberg. Strain JC1553 is from A. J. Clark. Abbreviations used for nutritional requirements are: Thi = thiamine; Pro = proline; His = histidine; Arg = arginine; Thr = threonine; Leu = leucine; Met = methionine; Thy = thymine; Ade = adenine. Streptomycin is abbreviated Str and spectinomycin, Spc. Resistance to these drugs is abbreviated r and sensitivity is abbreviated s. Recombination genotype is abbreviated *rec*$^+$, or *recA* for the recombination-deficient strains used. The structural gene for β-galactosidase is abbreviated *lacZ* and for β-galactosidase permease, *lacY*.

[b] The F factor is integrated between *his* and *metG*, and the strain transfers the chromosome in the order *his*, *try*, *lac*, etc.

[c] The F factor is integrated between *xyl* and *ilv*, and the strain transfers the chromosome in the order *ilv*, *thr*, *lac*, etc.

[d] The strain has two integrated factors: one between *argG* and *serA*, which causes transfer of the chromosome in the order *argG*, *metE*, *thr*, etc., and the other factor integrated between *purA* and *thr*, which causes transfer of the chromosome in the order *thr*, *proA*, *lac*, etc.

rapid shaking for 20 to 25 hr at 37 C. After growth under these conditions, male strains behave phenotypically like females (15). The phenocopy culture was plated out for single colonies, and the colonies were tested for maleness by replica plating. They were all males. Whenever female strains were used as recipients in control crosses, they were also grown under the same conditions as the Hfr strain. The donor strain was grown to the exponential phase and was then mixed with the recipient in a ratio of one donor per recipient cell. The mating mixture was incubated for 30 min with slow shaking at 37 C.

F′*lac* transfer to F$^-$ recipients was performed as follows. Donor and recipient cultures were mated when they reached the exponential phase of growth, unless the F$^-$ recipient was used as a control for a mating involving an Hfr recipient (*see above*). The donor and recipient were mixed in a ratio of 1:1, and the mating mixture was incubated for 30 min with slow shaking.

For interrupted-mating experiments with F$^-$ recipients, exponentially growing broth cultures of the donor and recipient were mixed in a ratio of 1:1. Samples were removed at various times, diluted 100-fold, and interrupted with a vibratory blendor (16) for 5 sec. They were then diluted and plated on the appropriate selective media to assay for the number of recombinants.

Induction and assay of β-galactosidase in F′lac zygotes. A 1-ml amount of mating mixture was diluted into 28.4 ml of prewarmed conditioned broth containing 0.3 ml of 1% streptomycin and 0.3 ml of 0.5 M thio-methyl-β-D-galactopyranoside (TMG). TMG was made up in medium A (*p*H 7). This mixture was incubated with rapid shaking at 37 C and, at timed intervals, OD readings were taken, and samples were removed for β-galactosidase assay. The cells were maintained in the exponential phase of growth by dilution with prewarmed medium whenever the OD reached about 0.3, which corresponded to about 3×10^8 cells per ml. Samples (2 ml) were filtered through a membrane filter (HA, 0.45 μ), and the cells were resuspended in 2 ml of ice-cold Revel buffer (23) on a Vortex mixer for 30 sec. Three drops of toluene were added to the samples, and they were incubated with shaking at 37 C for 30 min.

A 1-ml amount of toluene-treated cells was incubated at 37 C with 0.6 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), for a measured time period, until visible yellow color developed. After that, the reaction was stopped by the addition of 0.4 ml of 10% Na_2CO_3. The ONPG solution was prepared in medium A (*p*H 7), and contained 66.7 mg/100 ml. The samples were read at 420 mμ in a Gilford spectrophotometer to measure the production of *o*-nitrophenol (ONP). This reading was corrected for light scattering by subtraction of the OD at 550 mμ times 1.65. One unit of enzyme is defined as the production

of 1 mμmole of ONP per min. The molar extinction of ONP at 420 mμ is 4×10^6. The TMG and ONPG were obtained from Calbiochem, Los Angeles, Calif.

Selection of thy mutants. The method used was given to us by George Hitchings of Burroughs Wellcome Co. (*personal communication*), who kindly supplied the sample of trimethoprim. An overnight broth culture (0.1 ml) was inoculated into 5 ml of medium A containing Casamino Acids (0.25%), thiamine (1 μg/ml), thymine (50 μg/ml), and trimethoprim (10 μg/ml). This culture was grown to visible turbidity (24 to 48 hr), and then 0.1 ml of it was inoculated into 5 ml of minimal medium enriched with Casamino Acids (0.25%), thiamine (1 μg/ml), thymine (1 μg/ml), and trimethoprim (20 μg/ml). This culture was again grown to visible turbidity (about 48 hr) and spread on minimal agar containing 50 μg of thymine per ml. The colonies were tested for thymine dependence. Nearly all were found to require thymine. Some required a high concentration (50 μg/ml) of thymine; others required a low concentration (1 μg/ml) of thymine.

Transfer of the recA mutation to AB311 and AB253. The *recA* gene in JC1553 is located near the *thy* gene (K. B. Low, *personal communication*). *Thy*$^+$ recombinants were selected in crosses between MA1048 (which carries the *recA* mutation of JC1553) and *thy* mutants of AB311 (MA1025) and of AB253 (MA27). These were purified and tested for the *recA* gene. With MA1025 as recipient, the usual mating procedure for Hfr recipients was used; mating was allowed to continue 2.5 hr, and then *thy*$^+$ recombinants were selected. The donor was counterselected by omission of arginine, leucine, methionine, and histidine from the selective medium.

The frequency of *thy*$^+$ recombinants was 0.01% per donor cell with MA27 as recipient and 0.02% per donor cell with MA1025 as recipient. Recombinants were purified and tested for the *recA* genotype as well as for all other recipient markers. Although MA1048 is *lac*, we have found that it makes β-galactosidase in the presence of inducer. MA27 and MA1035, however, are *lacZ*, for no detectable β-galactosidase is made after incubation with TMG. The *recA* recombinants were also found to be *lacZ*. MA1048 also differs from the recipient strains in that it is a nonfermenter of xylose, galactose, maltose, and melibiose, whereas MA27 and MA1025 ferment these sugars.

A *recA* recombinant was chosen from the cross with MA27, and was found to have the genotype *thi, thr, leu, lacZ, str, recA*. This recombinant, MA28, is isogenic with AB253 in all known markers except the *recA* gene. Another *recA* recombinant was chosen from the cross with MA1025, and was found to have the genotype *thi, thr, leu, str, lacZ, recA, xyl, gal*, Hfr. This recombinant, MA1026, has the same origin of chromosome transfer as does AB311, as tested by interrupted mating, and is isogenic with AB311 for all known markers except *recA, xyl*, and *gal*.

The *recA* gene was scored by the ultraviolet (UV)-sensitivity method of Clark and Margulies (3). A General Electric G15T8 15-w germicidal lamp was used at a distance of 37.5 cm.

Isolation of a lac$^-$ mutant of MA1049. An exponentially growing culture of MA1049 was treated with ethyl methane sulfonate (EMS; obtained from the Eastman Kodak Co., Rochester, N.Y.); about three drops of EMS were added to 1 ml of medium A (*p*H 7), and 0.1 ml culture was added after the EMS had dissolved. The final concentration of EMS was approximately 50 mg/ml. The cells were incubated for 20 min, diluted 100-fold into broth, grown overnight, and plated on MacConkey lactose-agar to score for lac$^-$ mutants.

RESULTS

F'lac transfer to rec$^+$ male strains. Crosses were made between MA3020, the standard F'*lac* donor, and the following recipients: AB253, AB284, and AB311, which are F$^-$, F$^+$, and Hfr, respectively, and are otherwise isogenic. We found AB311 to be a stable Hfr. *Lac*$^+$ offspring were scored on MacConkey lactose-streptomycin-agar. The frequencies of *lac*$^+$ offspring per recipient cell, after the 30-min mating period, were as follows: 60 to 80% with the F$^-$ recipients, 30 to 50% with the F$^+$ recipient, and 15 to 30% with the Hfr recipient. No *lac*$^+$ revertants of the recipients or *str* mutants of the F'*lac* donor were detected in cultures used in the crosses. Between 90 and 100% of the colonies of *lac*$^+$ offspring obtained from the male recipients, whether F$^+$ or Hfr, appeared highly variegated on MacConkey lactose-agar, and often appeared as tiny lac$^+$ papillae on a lac$^-$ colony. In contrast, only 1 to 10% of the colonies of *lac*$^+$ offspring obtained from female recipients were variegated. The variegated colonies observed with the female recipient were usually mainly lac$^+$, with small lac$^-$ sectors. Similar results have been reported by Scaife and Gross (26).

Analogous results were obtained when *lac*$^+$ offspring were selected on minimal medium supplemented with the growth requirements of the recipient and with lactose as a sole carbon source. The *lac*$^+$ recombinant colonies were all large when the F$^-$ was the recipient, but they varied from large to very small when F$^+$ or Hfr strains were used as recipients. This suggests that the *lac*$^+$ gene, although transferred at high frequency to male recipients, is not inherited by all the cells in a clone. Since the F factor and the *lac*$^+$ gene are very closely linked in the F'*lac* episome, we conclude that the newly introduced F factor is unstable in male recipients.

A double male strain, MA1044, a *lac spc* derivative of JC182, was also used as a recipient of F'*lac*. This strain has two integrated sex factors (2). The results were similar to those with the single Hfr strain.

F'lac transfer to recA Hfr recipients. The *recA* mutation, first described by Clark and Margulies

(3), results in loss of ability to undergo genetic recombination and in an increased sensitivity to UV light. These authors showed that F'*lac* can be transferred to F⁻ *recA* strains.

To test whether recombination is needed to recover *lac*⁺ progeny from matings between *F'lac* and Hfr recipients, we performed some F'*lac* crosses using various *recA* recipients. The F'*lac* donor MA3020 was mated with the *recA* female JC1553 and the *recA* Hfr MA1048. *Lac*⁺ was transferred to JC1553 at a frequency of 56% per recipient cell, and the proportion that were variegated was the same (1 to 10%) as with *rec*⁺ F⁻ recipients. No *lac*⁺ offspring were found among several thousand colonies of the MA1048 recipient. Both the *recA* F⁻ and the *recA* Hfr strains, when used as recipients with the F'*lac* donor, were capable of forming *leu*⁺ *str* chromosomal recombinants at the same low frequency (6×10^{-8} to 100×10^{-8} per recipient cell) characteristic of *recA* recipients. Therefore, the failure to detect *lac*⁺ offspring among Hfr *recA* recipients is presumably due not to the failure to form mating pairs, but to the inability to integrate the *lac*⁺ gene. The transfer of F'*lac* to *recA* Hfr strains can be demonstrated by β-galactosidase synthesis in the F*lac* × Hfr zygotes, as described below.

Three other *recA* Hfr strains, including MA1026, all derived as recombinants from a cross between AB311 and the *recA* Hfr MA1048, were used as recipients in crosses with the F*lac* donor MA3020; similar results were obtained. In these experiments, *lac*⁺ offspring were selected on minimal medium supplemented with the growth requirements of the recipient and with lactose as a sole carbon source. *Lac*⁺ offspring appeared at a frequency of about 10^{-8} per recipient cell. Six such presumptive recombinants were tested and found unable to transfer the *lac*⁺ gene at high frequency; therefore, they presumably did not carry free F'*lac*, but instead had integrated the *lac*⁺ gene into the chromosome. These *lac*⁺ offspring were not analyzed further. It is possible that they were either *lac*⁺ mutants or abnormal recombinant types found with *recA* recipients.

Nature of the lac⁺ offspring obtained in crosses with rec⁺ males. To test whether the *lac*⁺ gene was integrated into the chromosome or still attached to the F episome, *lac*⁺ offspring obtained from crosses with the *rec*⁺ isogenic F⁻, F⁺, and Hfr recipients (AB253, AB284, and AB311, respectively) were analyzed. Five such recombinants from an F'*lac* × Hfr cross were used, two from an F'*lac* × F⁺ cross and one from an F*lac* × F⁻ cross.

After at least two purifications on MacConkey lactose-streptomycin-agar, these *lac*⁺ offspring were tested as donors for the *lac*⁺ gene as well as for chromosomal markers. Interrupted-mating experiments were carried out with recipient AB1353, which is *lac, thi, pro, his, arg*, and selection was for *lac*⁺, *pro*⁺, and *his*⁺ recombinants. Selection against the males was made by omission of threonine and leucine from the selection plates. F'*lac* donors inject the *pro* gene as an early marker, whereas AB311 injects the *his* gene as an early marker (28); therefore, selection for these markers should reveal the origin or origins of *lac*⁺ recombinants. The results of these experiments are shown in Fig. 1. The *lac*⁺ offspring derived from the F⁻ recipient, AB253, behaved like a typical F'*lac* donor (Fig. 1B), injecting the *lac*⁺ gene early and at high frequency, the *pro*⁺ gene as an early marker, and the *his*⁺ gene as a late marker. All five *lac*⁺ offspring derived from the Hfr recipient, AB311, on the other hand, transferred *his*⁺ as an early marker, but both *pro*⁺ and *lac*⁺ as late markers (Fig. 1A). They retained the AB311 origin and did not acquire the F'*lac* origin. In all crosses, some *pro*⁺ recombinants appeared at zero-time, in spite of interruption and immediate dilution of the mating mixtures; the reason for this is not known.

These results indicate that generally the *lac*⁺ gene is integrated into the chromosome in *lac*⁺ offspring of the Hfr recipients, and the transferred F' factor is either no longer present or is not expressed in the Hfr recipient.

Two *lac*⁺ offspring of the F⁺ recipient were also tested; they transferred *lac*⁺ early and at

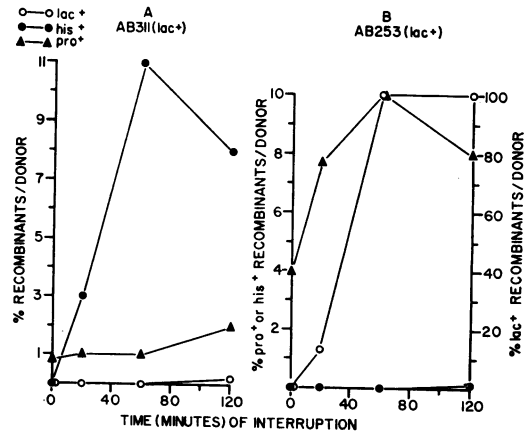

FIG. 1. *Interrupted-mating experiments with recipient AB1353. (A) The donor was a lac⁺ offspring derived from a cross between MA3020 and AB311. (B) The donor was a lac⁺ offspring derived from a cross between MA3020 and AB253.*

high frequency, *pro*$^+$ as an early marker, and *his*$^+$ as a late marker. Therefore, they are similar to the *lac*$^+$ offspring derived from F$^-$ recipients and they presumably carry free F'*lac*.

Replication of F'lac in Hfr recipients. To determine whether or not the *lac*$^+$ gene transferred by F'*lac* into Hfr recipients can replicate, the F'*lac* zygotes were diluted into fresh medium and assayed at various intervals for *lac*$^+$ offspring.

Mating between the F'*lac* donor, MA3020, and the *rec*$^+$ Hfr, AB311, was interrupted after 30 min by rapid agitation, and the mating mixture was diluted 30-fold into prewarmed broth containing streptomycin (100 μg/ml) to kill the F'*lac* donor. This culture, containing nonviable donor cells, *lac str* recipient cells, and *lac*$^+$/*lac str* zygotes, was incubated at 37 C with rapid shaking. At timed intervals, samples were removed and plated on MacConkey lactose-streptomycin-agar. Several hundred colonies were scored at each time point. The cells were kept growing exponentially by repeated dilution into fresh prewarmed broth whenever the OD exceeded 0.3 (about 3×10^8 cells per ml).

As shown in Fig. 2, the proportion of *lac*$^+$ *str* offspring obtained with Hfr recipients decreased with the number of generations and then leveled off. The decrease follows the theoretical curve for a gene which is neither replicating nor destroyed, i.e., the proportion of *lac*$^+$ *str* is halved with each generation. Since the *lac*$^+$ gene can integrate into the Hfr chromosome (*see above*), there also exists a growing population of *lac*$^+$ offspring among Hfr recipients which have integrated the *lac*$^+$ gene. This could account for the leveling off of the curve in Fig. 2. The figures in this part of the curve are less accurate because they represent a smaller number of *lac*$^+$ colonies scored. The proportion of the offspring *lac*$^+$ colonies which are variegated decreases with time, from 95% in the beginning of the experiment to 32% after about eight generations. Similar results were obtained with an F$^+$ recipient. The proportion of *lac*$^+$ offspring in the F$^-$ population, on the other hand, increases from 60 to 90% and then remains constant. This increase is probably due to secondary transfer of F'*lac* within the recipient population.

To show that the instability of the F'*lac* in Hfr recipients is specific to genes associated with the superinfecting F particle, experiments like those described above were carried out, but selection was made for the chromosomal genes *thr*$^+$ *leu*$^+$ as well as the episomal *lac*$^+$; streptomycin was used to counterselect the male parent in both cases (Fig. 3). Although the proportion of *lac*$^+$ offspring among the Hfr recipients dropped with each generation, the proportion of *thr*$^+$ *leu*$^+$

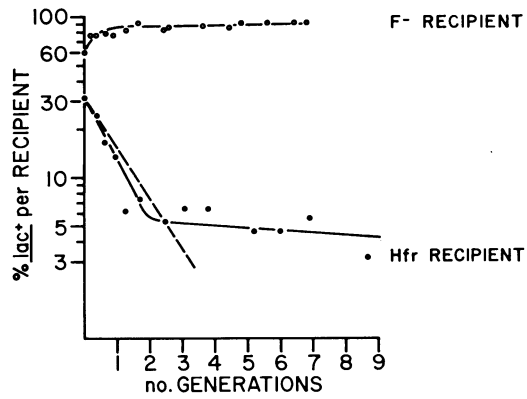

FIG. 2. *Frequency of lac*$^+$ *offspring per recipient plotted against the number of generations of growth of the recipient population. Generations were calculated from optical density measurements. The broken line represents the theoretical curve expected from dilution of a nonreplicating gene in a growing population.*

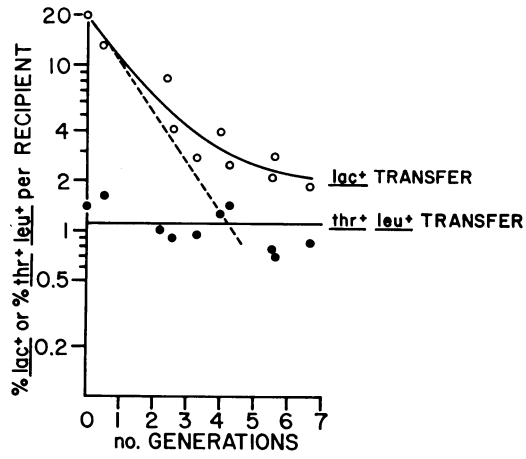

FIG. 3. *Frequency of lac*$^+$ *and of thr*$^+$ *leu*$^+$ *offspring with Hfr recipient AB311 plotted against the number of generations of growth of the recipient population. Generations were calculated from optical density measurements. The broken line represents the theoretical curve expected from dilution of a nonreplicating gene in a growing population.*

recombinants remained roughly constant at about 1% throughout the sampling period.

Expression of nonreplicating lac$^+$ *gene in recA and rec*$^+$ *Hfr recipients.* To determine whether the nonreplicating *lac*$^+$ gene is expressed in F'*lac* $\times$ Hfr zygotes, the rate of induced β-galactosidase synthesis was examined. The F'*lac* donor, MA-3020, and the *rec*$^+$ Hfr, AB311, were mated for 30 min. The mating mixture was diluted into

"conditioned broth" with streptomycin and TMG and incubated at 37 C with rapid shaking. Samples were removed at timed intervals and tested for β-galactosidase formation. The rate of induced enzyme synthesis was also examined in F'*lac* × F⁻ zygotes, with AB253 as the F⁻ recipient. The results are shown in Fig. 4, with the units of β-galactosidase per milliliter plotted against the log OD of the culture. This abcissa was used because the rate of enzyme synthesis in the F'*lac*-Hfr zygotes is a function of "biological time" rather than mass. Log OD is proportional to the number of generations in an exponentially growing culture.

F'*lac* × F⁻ zygotes synthesized β-galactosidase at an exponential rate after an initial lag, whereas the F'*lac* × Hfr zygotes synthesized enzyme at an approximately linear rate, also after an initial lag. The rate of enzyme synthesis in F'*lac* × Hfr zygotes increased after several hours and tended to become exponential. This is presumably due to the fact that some integration of the *lac*⁺ gene occurs and, therefore, in a fraction of the *lac*⁺ population, the *lac*⁺ gene is replicating. A purely linear rate would be expected if the *lac*⁺ gene were fully active, i.e., not repressed or destroyed, but also not replicated. The lag in enzyme synthesis follows a long (90 to 120 min) growth lag. This cellular inactivity is presumably due to the physiological effects of the "aeration phenocopy" conditions used for mating purposes. The same kinetics of β-galactosidase synthesis were obtained when the zygotes were diluted into broth containing 0.003% Dupanol, which prevents further F'*lac* transfer during induction of β-galactosidase. This shows that the increasing rate of enzyme synthesis is not due to formation of new zygotes.

Since no *lac*⁺ offspring had been obtained in matings between the F'*lac* donor and *recA* Hfr strains, it was of interest to test whether the *lac*⁺ gene is transferred and expressed in such a recipient. Therefore, similar experiments were carried out with the *recA* Hfr, MA1026. Rates of induced β-galactosidase synthesis were followed in F'*lac* zygotes of the *recA* Hfr, MA1026, and the *recA* F⁻, MA28. As controls, the *rec*⁺ Hfr, AB311, and the *rec*⁺ F⁻, AB253, were used. As shown in Fig. 5, the *recA* gene had no effect on the rate of β-galactosidase synthesis in the F⁻ recipient. As mentioned above, F'*lac* is transferred at high frequency and seems to replicate normally in *recA* females. The rate of synthesis in the *recA* Hfr recipient was linear and showed no tendency to increase, whereas, as mentioned above, in the *rec*⁺ Hfr recipient, the rate of synthesis increased after a period of fairly linear synthesis (Fig. 4 and 5). The initial rate of synthesis in the *rec*⁺ Hfr was 23.2 enzyme units per log OD unit and in the *recA* Hfr, 24.2 enzyme units per log OD unit.

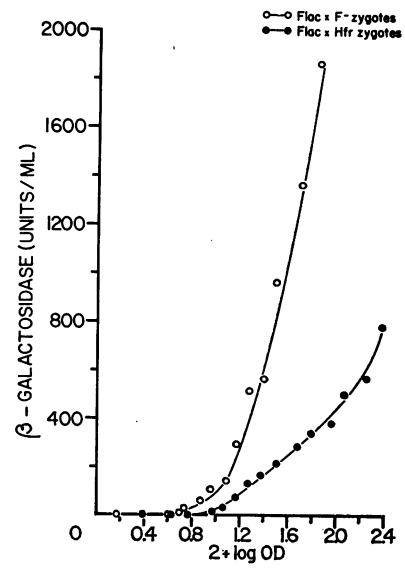

FIG. 4. *Induced synthesis of β-galactosidase in F'lac zygotes of AB253 and AB311 plotted against growth (log optical density).*

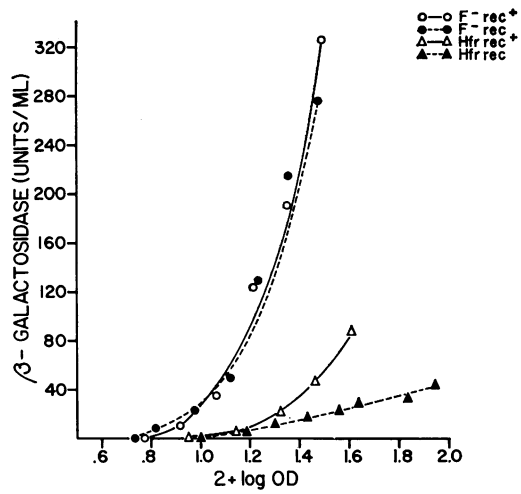

FIG. 5. *Induced synthesis of β-galactosidase in F'lac zygotes of rec⁺F⁻ AB253, recA F⁻ MA28, rec⁺ Hfr AB311, and recA Hfr MA1026, plotted against growth (log optical density).*

The uninduced synthesis of β-galactosidase in F'*lac* zygotes of these four recipients was measured, and, in all cases, low levels of enzyme synthesis similar to the "basal level" usually observed in K-12 cells could be detected.

Integrity of the lac⁺ gene in F'lac × rec⁺ Hfr

and F'lac × recA Hfr zygotes. Several experiments were done to determine whether there is any breakdown of the *lac*$^+$ gene after transfer by the F'*lac* episome to Hfr recipients. In the first experiment, the F'*lac* donor, MA3020 was mated with the *rec*$^+$ Hfr, AB311. After 30-min mating, the zygotes were diluted into broth with chloramphenicol (20 μg/ml) and streptomycin. *Lac*$^+$ offspring were assayed at various times on MacConkey lactose-streptomycin plates. No growth occurred under these conditions. There should be no decrease in the proportion of *lac*$^+$ offspring in the population under these conditions unless genes on the F'*lac* episome are destroyed. The proportion of *lac*$^+$ offspring remained constant (45%) for 4 hr in chloramphenicol. The cells remained 100% viable in the chloramphenicol.

A more direct method to determine whether the *lac*$^+$ gene is destroyed is to measure ability to synthesize β-galactosidase at various times after transfer of the gene. Therefore, in another cross between the F'*lac* donor, MA3020, and the *rec*$^+$ Hfr, AB311, the F'*lac* × Hfr zygotes were diluted (20-fold) into broth containing chloramphenicol (20 μg/ml) and streptomycin and incubated for various times before induction of β-galactosidase. If destruction of F'*lac* occurs under these conditions, the ability to make enzyme should decrease with time of incubation prior to induction. After 0, 93, and 185 min in chloramphenicol, 20-ml samples were removed, passed through a membrane filter, and resuspended in the same volume of conditioned broth with streptomycin and TMG. (Long growth lags of 1 to 2 hr occurred after incubation in chloramphenicol.) The rate of enzyme synthesis in F'*lac* × Hfr zygotes was not decreased by prior incubation in chloramphenicol. This indicates that the inhibition of F'*lac* replication in Hfr cells is not accompanied by extensive destruction of the *lac*$^+$ gene, at least under conditions of chloramphenicol inhibition.

Experiments similar to the one described above were done, but the F'*lac* zygotes were incubated in broth without added chloramphenicol, to test whether the transferred *lac*$^+$ gene is destroyed when the zygotes are incubated under conditions of growth. The F'*lac* donor, MA3020, was mated with the *rec*$^+$ Hfr, AB311, the *recA* Hfr, MA1026, and also, as controls, with the *rec*$^+$ F$^-$, AB25,3 and the *recA* F$^-$, MA28. After the usual 30-min mating, one sample of the mating mixture was induced immediately after mating, whereas another sample was allowed to grow and was induced 90 min later (Fig. 6). Any extensive destruction of the *lacZ*$^+$ gene in the zygotes during the 90-min incubation should result in a decreased rate of enzyme synthesis. In the case of all four recipients, however, the rate of β-galactosidase

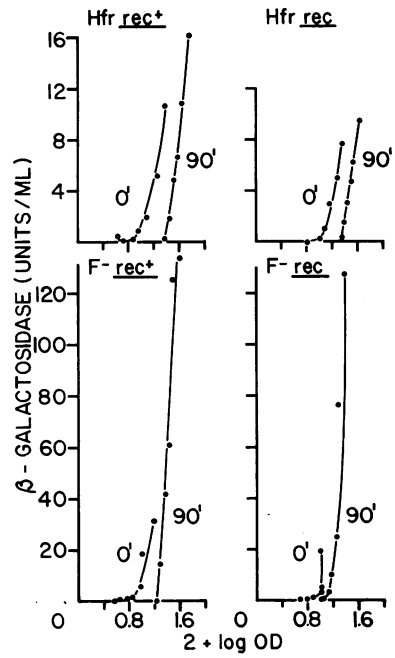

FIG. 6. *Induced synthesis of β-galactosidase in F'lac zygotes of rec*$^+$*F*$^-$ *AB253, recA F*$^-$ *MA28, rec*$^+$ *Hfr AB311, and recA Hfr MA1026, with and without 90-min preincubation before the addition of inducer.*

synthesis remained the same after the 90 min of incubation. The lag in enzyme synthesis observed in the sample induced immediately after mating did not appear in the sample induced 90 min later. This effect is presumably due to a further conditioning of the medium by the recipient cells during the 90-min growth period before induction.

DISCUSSION

The incompatibility between a resident, integrated F factor and a sex-duced, free F factor seems to be due to inhibition of replication of the superinfecting particle. There is no barrier to the entry of the episome under F$^-$ phenocopy conditions, nor is there any extensive destruction after entry. In contrast to replication, the function of the F$^-$ associated *lac*$^+$ gene is not inhibited in an Hfr cell. However, because of the inhibition of replication, there remains no trace of the *lac*$^+$ gene in offspring colonies, unless the *lac*$^+$ gene is "rescued" by recombination into the chromosome, as in *rec*$^+$ Hfr recipients. In *recA* Hfr recipients, however, in which no recombination occurs, transfer of the F'*lac* cannot be demonstrated except by the production of β-galactosidase in zygotes. In accord with the inhibition of replication of F'*lac* in Hfr zygotes, as shown by

the dilution kinetics of the segregation of the *lac*$^+$ gene, the rate of enzyme formation in the zygote suspensions remained constant, although the number of cells increased. With *rec*$^+$ Hfr recipients, the rate of enzyme formation, though initially constant, increased at later times. This deviation from linearity is presumably due to the occasional integration of the *lac*$^+$ gene into the chromosome and its subsequent replication.

Uninduced basal synthesis of β-galactosidase occurred in Hfr × F'*lac* zygotes, both with *rec*$^+$ and *recA* Hfr recipients. Since the *lac*$^+$ gene is not replicating at all in the case of the *recA* Hfr, and only rarely, upon integration into the chromosome, in the case of the *rec*$^+$ Hfr, we conclude that replication of the *lac*$^+$ gene is not required for uninduced synthesis of β-galactosidase. This is inconsistent with the idea that transcription of repressed genes occurs only at the time of deoxyribonucleic acid (DNA) replication (11). Cuzin and Jacob (*personal communication*) found that the addition of nalidixic acid, which inhibits DNA synthesis, to *lac*$^+$ zygotes of a *lacI* recipient, did not decrease the rate of β-galactosidase synthesis in the burst of enzyme synthesis which occurs prior to repression.

Incompatibility between superinfecting and resident bacterial genetic elements has become a familiar phenomenon. Two types of mechanisms have been elucidated. The first type, known as restriction, is associated with the breakdown of the superinfecting DNA and usually occurs between unlike genetic elements (9, 17). The classical example of restriction is the breakdown of phage lambda in cells of *E. coli* K-12 carrying prophage P1. We found no evidence of breakdown of the F'*lac* genetic material in the Hfr host. The second type of incompatibility, known as immunity, occurs between like genetic elements and is caused by inhibition of replication of the superinfecting particle. The most widely studied example is the immunity of cells lysogenic for lambda against superinfection by lambda (1, 13, 17, 33). It has been shown that an "immunity substance" present in the cytoplasm of the lysogenic cells is responsible for this effect, and the nature of the lambda repressor is under study at the present time (22).

Incompatibility has been reported between other plasmids, including the penicillinase plasmids of *Staphylococcus aureus* (20, 21), resistance transfer factors (30, 31), and col factors (27; B. Stocker, *unpublished data*). The incompatibility between resident integrated F and superinfecting F is in some ways like superinfection immunity of temperate phages. It involves inhibition of replication between genetic elements of the same type. Our data are consistent with this model. The substitution of F'*lac* for the F$^+$ could be analogous to prophage substitution (1).

Another possible mechanism, also consistent with our results, postulates that a single attachment site is present and that this site has to be occupied by the sex factor in order for it to replicate. In Hfr-F'*lac* zygotes, the integrated sex factor has priority and remains attached, whereas in F$^+$-F'*lac* zygotes there is no preference and either sex factor may be attached. There are certain observations which do not quite fit this hypothesis. A "double male" strain with two stably integrated sex factors has been isolated by crossing two different Hfr strains with each other (2). A derivative of this strain, MA1044, was shown above to still be immune to extrachromosomal F. Of course, if a mutation had occurred in this strain leading to the production of two F attachment sites, the strain would be immune to superinfecting F. Experiments are being carried out to determine whether or not such a mutation is present.

To gain some insight into the mechanism of the Hfr-F'*lac* incompatibility we are now trying to isolate mutants in which incompatibility is impaired. With the availability of *recA* Hfr strains, we have a powerful method for screening for such mutants, since the frequency of *lac*$^+$ recombinants in F'*lac* × *recA* Hfr matings is very low. Compatibility mutations, either in the Hfr element or the F'*lac* element, can be recognized by the production of F'*lac* offspring in such matings. It may be mentioned that the mechanism of immunity with phage lambda was clarified only after mutants affecting this immunity became available.

Finally, it should be pointed out that the incompatibility between F-prime elements and Hfr or between two different F-prime elements may not be absolute and that exceptional cases of apparent compatibility have been reported. These exceptional cases do not seem to involve gene mutations. Thus, Cuzin (4) and Cuzin and Jacob (5) showed that although, generally, superinfecting F factors cannot replicate in male recipients, certain Hfr strains can occasionally permit replication of F'*lac* and form "double male" strains containing integrated sex factors and free Flac elements. A similar case was reported by Maas (18). De Haan and Stouthamer (7) described the isolation of "double males" carrying F'*lac* and F'*gal*. These interesting strains have so far not been studied extensively enough to understand the cause of their exceptional behavior.

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LITERATURE CITED

1. BERTANI, G. 1958. Lysogeny. Advan. Virus Res. **5**:151–193.
2. CLARK, A. J. 1963. Genetic analysis of a 'Double Male' strain of *Escherichia coli* K12. Genetics **48**:115–120.
3. CLARK, A. J., AND A. D. MARGULIES. 1965. Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S. **53**:451–459.
4. CUZIN, F. 1962. Multiplication autonome de l'épisome sexual d'*Escherichia coli* K12 dans une souche Hfr. Compt. Rend. **254**: 4211–4213.
5. CUZIN, F., AND F. JACOB. 1967. Association stable de deux épisomes F différents dans un clone d'*Escherichia coli*. Ann. Inst. Pasteur **113**:145–155.
6. DAVIS, B. D., AND E. S. MINGIOLI. 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12} . J. Bacteriol. **60**:17–28.
7. DE HAAN, P. G., AND A. H. STOUTHAMER. 1963. F-prime transfer and multiplication of sex-duced cells. Genet. Res. **4**:30–41.
8. DEMEREC, M., E. A. ADELBERG, A. J. CLARK, AND P. E. HARTMAN. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61–76.
9. DUSSOIX, D., AND W. ARBER. 1962. Host specificity of DNA produced by *Escherichia coli* 11. Control over acceptance of DNA from infecting phage. J. Mol. Biol. **5**:37–49.
10. ECHOLS, H. 1963. Properties of F' strains of *Escherichia coli* superinfected with F-lactose and F-galactose episomes. J. Bacteriol. **85**:262–268.
11. HANAWALT, P., AND R. WAX. 1964. Transcription of a repressed gene: evidence that it requires DNA synthesis. Science **145**:1061.
12. JACOB, F., AND E. A. ADELBERG. 1959. Transfert de charactères génétiques par incorporation du facteur sexuel d'*Escherichia coli*. Compt. Rend. **249**:189–191.
13. JACOB, F., AND J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
14. JACOB, F., AND E. L. WOLLMAN. 1961. Sexuality and the genetics of bacteria. Academic Press, Inc., New York.
15. LEDERBERG, J., L. L. CAVALLI, AND E. M. LEDERBERG. 1952. Sex compatibility in *E. coli*. Genetics **37**:720–730.
16. LOW, B., AND T. H. WOOD. 1965. A quick and efficient method for interruption of bacterial conjugation. Genet. Res. **6**:300–303.
17. LWOFF, A. 1953. Lysogeny. Bacteriol. Rev. **17**: 269–337.
18. MAAS, R. 1963. Exclusion of an Flac episome by an Hfr gene. Proc. Natl. Acad. Sci. U.S. **50**: 1051–1055.
19. MAAS, R., AND W. K. MAAS. 1962. Introduction of a gene from *Escherichia coli* B into Hfr and F^- strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S. **48**:1887–1893.
20. NOVICK, R. 1967. Penicillinase plasmids of *Staphylococcus aureus*. Federation Proc. **26**: 29–38.
21. NOVICK, R., AND M. H. RICHMOND. 1965. Nature and interactions of the genetic elements governing penicillinase synthesis in *Staphylococcus aureus*. J. Bacteriol. **90**:467–480.
22. PTASHNE, M. 1967. Isolation of the λ phage repressor. Proc. Natl. Acad. Sci. U.S. **57**:306–313.
23. REVEL, H. R. 1965. Synthesis of β-galactosidase after F-duction of lac^+ genes into *Escherichia coli*. J. Mol. Biol. **11**:23–34.
24. REVEL, H. R., S. E. LURIA, AND B. ROTMAN. 1961. Biosynthesis of β-D-galactosidase controlled by phage-carried genes. 1. Induced β-D-galactosidase biosynthesis after transduction of gene z^+ by phage. Proc. Natl. Acad. Sci. U.S. **47**:1956–1967.
25. RILEY, M., A. B. PARDEE, F. JACOB, AND J. MONOD. 1960. On the expression of a structural gene. J. Mol. Biol. **2**:216–225.
26. SCAIFE, J., AND J. D. GROSS. 1962. Inhibition of multiplication of an F-lac factor in Hfr cells of *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **7**:403–407.
27. STROBEL, M., AND M. NOMURA. 1966. Restriction of the growth of bacteriophage BF23 by a colicine I (col I-P9) factor. Virology **28**:763–765.
28. TAYLOR, A. L., AND E. A. ADELBERG. 1960. Linkage analysis with very high frequency males of *Escherichia coli*. Genetics **45**:1233–1243.
29. WATANABE, T., AND T. FUKASAWA. 1962. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. IV. Interactions between resistance transfer factor and F-factor in *Escherichia coli* K-12. J. Bacteriol. **83**:727–735.
30. WATANABE, T., AND K. W. LYANG. 1962. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. V. Spontaneous segregation and recombination of resistance factors in *Salmonella typhimurium*. J. Bacteriol. **84**:422–430.
31. WATANABE, T., H. NISHIDA, C. OGATA, T. ARAI, AND S. SATO. 1964. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. VII. Two types of naturally occurring R factors. J. Bacteriol. **88**:716–726.
32. WATANABE, T., T. TAKANO, T. ARAI, H. NISHIDA, AND S. SATO. 1966. Episome-mediated transfer of drug resistance in *Enterobacteriaceae* X. Restriction and modification of phages by fi^- R factors. J. Bacteriol. **92**:477–486.
33. WOLF, B., AND M. MESELSON. 1963. Repression of the replication of superinfecting bacteriophage DNA in immune cells. J. Mol. Biol. **7**:636–644.