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Relationships Between Curing of the F Episome by Rifampin and by Acridine Orange in *Escherichia coli*

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Subinhibitory doses of rifampin cured F+ *Escherichia coli* cells from the episome. The target of the drug was transcription because *E. coli* mutants with a ribonucleic acid polymerase resistant to rifampin were not cured. The experimental conditions required for optimal curing with rifampin very closely resembled those required for curing with acridine orange. Mutants were found which are more resistant to curing by both acridine orange and rifampin. Probably the two drugs affect a common metabolic step, or alternatively they may inhibit the synthesis of a factor which is necessary for the replication of the episome.

In a previous paper (13), we reported that male (F+, F', and Hfr) cells grown for a few generations in the presence of low doses of rifampin (2 to 5 μg/ml) reversibly lose their capacity to express several male characters (MS2 absorption, T7 restriction, and conjugation). In our previous experiments, we occasionally observed a certain amount of curing (13). We report here the conditions which are required for regularly obtaining curing of the F episome. We have also compared the curing by rifampin with the curing obtained by use of acridine orange.

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1.

Strain JC6583 is an F'*lac* (JCFLO) derivative of JC6256 which carries the *lac* deletion Δ x 74 (1). JC6583-*rif-r* is a spontaneous rifampin-resistant mutant derived from JC6583 by plating 10^8 cells on plates containing 100 μg of rifampin/ml. JC5499 is an F'*lac*, rec+ derivative of AB2463 and is noncurable with acridine orange (2). L447 and L446 are F'*lac* (JCFLO) derivatives of strains W4626 and W4626 *eps-6*, respectively (16). Strains JC6583 (R_1) and JC6583 (R_2) were obtained by introducing the R factors R136 and R163, respectively, into strain JC6583 F-. The donor strains harboring R factors R136 and R163 were kindly provided by E. Meynell.

Media. Oxoid nutrient broth no. 2, Oxoid nutrient agar (1.7% agar), Luria (L) broth adjusted to pH 7.8 with NaOH, and Penassay broth (Difco) were used. The *lac* indicator plates contained MacConkey lactose agar (Oxoid).

Treatment with rifampin. An exponentially growing culture of an F'*lac* strain was diluted to about 10^3 cells/ml into prewarmed Oxoid nutrient broth containing the indicated doses of rifampin. The cultures were then incubated without aeration or shaking for various lengths of time at 37 C. Samples were then plated on MacConkey lactose agar and incubated for 18 h at 37 C for the identification of *lac*+ and *lac*- colonies.

The pulse treatment with rifampin was performed as follows: a culture of about 10^8 cells/ml (in the exponential phase of growth) was incubated for 15 min at 37 C with 50 to 100 μg of rifampin/ml; after this time, the culture was diluted 10- to 20-fold into prewarmed medium to obtain the appropriate concentration of the drug, and the treatment was con-

TABLE 1. *List of strains*

Strain	Genetic markers	Origin
JC6583	*lac*-, *trp*-, *sup*-*D*/F'*lac*	N. Willetts
JC6583-Rif	*lac*-, *trp*- *sup*-*D*, *rif-r*/F'*lac*	See text
JC5499	*arg*-, *his-4*-, *leu*-, *str-r*, *thi*-, *thr*-/F'*lac*	N. Willetts
L447	*purE*-, *trp*-, lac_{85}-, *xyl*-, *mtl*-, mal_1-, gal_2-(λ), *str-r*, ara_2-/F'*lac*	H. Uchida, See text
L446	*purE*-, *trp*-, lac_{85}-, *xyl*-, *mtl*-, mal_1-, gal_2-(λ), *str-r*, ara_2-, *eps-6*/F'*lac*	H. Uchida, See text
JC6583 (R_1)	*lac*-, *trp*-, *sup*-*D*/R136 (Tc, Su, fi+drd)	See text
JC6583 (R_2)	*lac*-, *trp*-, *sup*-*D*/R163 (Km, Col l, fi-drd)	See text

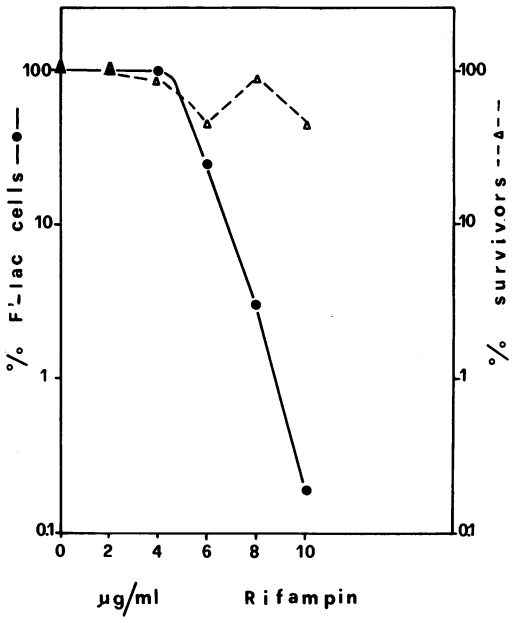

FIG. 1. *Curing of F'lac episome as a function of rifampin concentration. An inoculum of 10^8 cells/ml from a log-phase culture was added to 10 ml of prewarmed nutrient broth (Oxoid no. 2) containing the indicated doses of rifampin. The cells were allowed to grow without aeration at 37 C for 18 to 24 h. Cultures were then plated on MacConkey lactose agar (Oxoid), and after 20 h of incubation lac⁺ and lac⁻ colonies were counted. Strain: JC6583.*

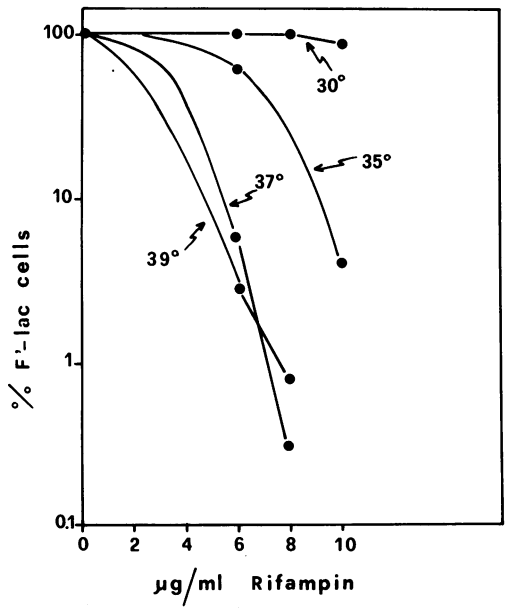

FIG. 2. *Dependence of curing on temperature. Experimental conditions as in Fig. 1. Strain: JC6583.*

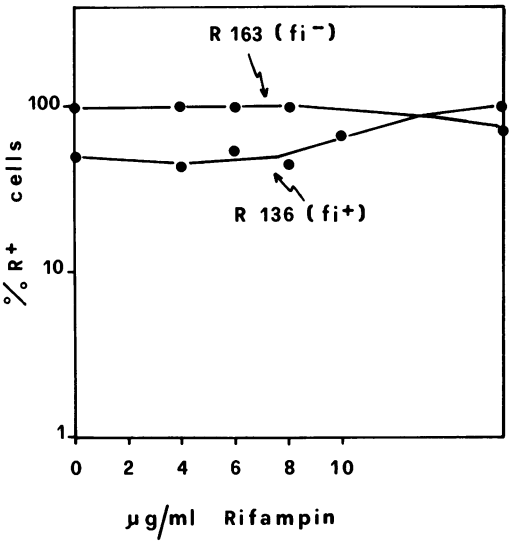

FIG. 3. *Curing of R factors by rifampin. Strains JC6583 (R_1) and JC6583 (R_2) were obtained as described in Materials and Methods. The curing procedure was as described in Fig. 1. Curing was detected by plating samples on plates with and without the selective drugs: kanamycin (100 µg/ml) for R163 and tetracycline (50 µg/ml) for R136.*

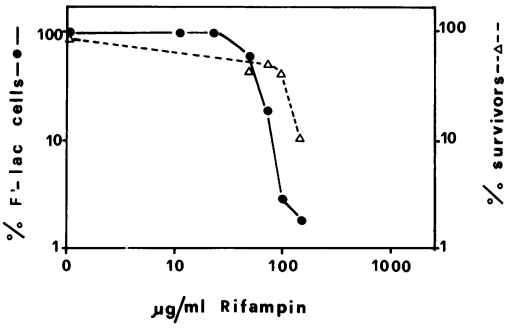

FIG. 4. *Curing of the F'lac episome in a rifampin-resistant mutant. Minimal inhibitory concentration of rifampin = 250 µg/ml. Experimental conditions as in Fig. 1. Strain: JC6583 Rif-r.*

tinued for the indicated times as previously described.

Acridine orange curing. Cells from an overnight culture were diluted to about 20 cells/ml into L broth (pH 7.8) containing the indicated concentration of acridine orange. Cultures were incubated in light-proof flasks at 37 C with shaking for 24 h. Cured *lac⁻* clones were detected by plating dilutions on MacConkey lactose plates and incubating the plates for 20 h at 37 C.

RESULTS

Conditions for curing. The effect of rifampin on the stability of the F'*lac* episome resem-

bles the effect of acridine orange described by Hirota (6). When *E. coli* JC6583 (Table 1) was grown for 24 h in nutrient broth in the presence of subinhibitory concentrations of rifampin, a certain fraction of the cells lost the capacity to ferment lactose and to express some F-specific characters. These characters were lost irreversibly; and the episome could be reintroduced into the cured cells by mating them with an F'*lac* donor.

Figure 1 shows typical kinetics of curing of the F'*lac* episome as a function of the concentration of rifampin. It can be seen that, at the higher concentration (10 μg/ml), although the bacterial duplication time was only doubled, more than 99% of the cells had lost the episome. On the other hand, rifampin did not preferentially inhibit the growth of F^+ versus F^- strains (3; unpublished data). The efficiency of the curing process was influenced by the size of the inoculum, the composition of the medium, and the temperature.

At a given concentration of rifampin, the percentage of curing decreased as the size of the inoculum increased. For the JC6583 strain, the optimal size was $<10^4$ cells/ml; at higher concentrations of cells, curing was less efficient. For other strains, inocula lower than 10^3 cells/ml had to be used for significant curing to occur. We have no clear explanation for this effect. It was not due to a soluble factor present in dense cultures because the addition of samples of the supernatant fluid of a stationary-phase culture did not prevent the curing.

Good curing was obtained in complete media (nutrient broth [Oxoid], L broth, and PY broth [Difco]). Curing was dramatically reduced in cells growing in synthetic medium (data not shown).

The efficiency of elimination of F'*lac* by rifampin was reduced at low temperatures and was virtually zero at temperatures below 30 C (see Fig. 2), whereas temperature had no effect on the viability of the cells treated with the antibiotic. These conditions are very similar to those described by Hirota (6) for curing with acridine orange. Similar results have also been reported by Bazzicalupo and Tocchini-Valentini (3).

Curing by rifampin did not seem to be dependent on the size of the episome; the efficiency of curing of F^+, F'*lac*, and F'*lac*, *pro* episomes was, in fact, roughly the same (data not shown). R factors, however, (of both the fi^+ and fi^- type), were not cured by treatment with rifampin under the conditions described above. Figure 3 shows the results of an attempt at the curing of two R factors, R163 and R136, introduced into strain JC6583. No appreciable curing is observable. Erratic curing of R factors has, however, been observed by other authors (9, 10) and by us. It is possible that conditions can be found under which reproducible curing by rifampin could be obtained for R factors.

Very likely, the primary target of rifampin for the curing effect is transcription. Rifampin-resistant mutants with a rifampin-resistant polymerase were much more resistant to curing than the sensitive parental strain. Figure 4 shows the kinetics of curing of a *rif-r* strain isolated from JC6583. It can be seen that curing takes place at concentrations close to the inhibitory concentrations for growth. Other mutants with a much higher level of resistance are not cured by concentrations as high as 1,000 μg/ml (data not shown).

Kinetics of curing. Figure 5 shows the kinetics of curing of JC6583 as a function of time of contact with 8 μg of rifampin/ml. An

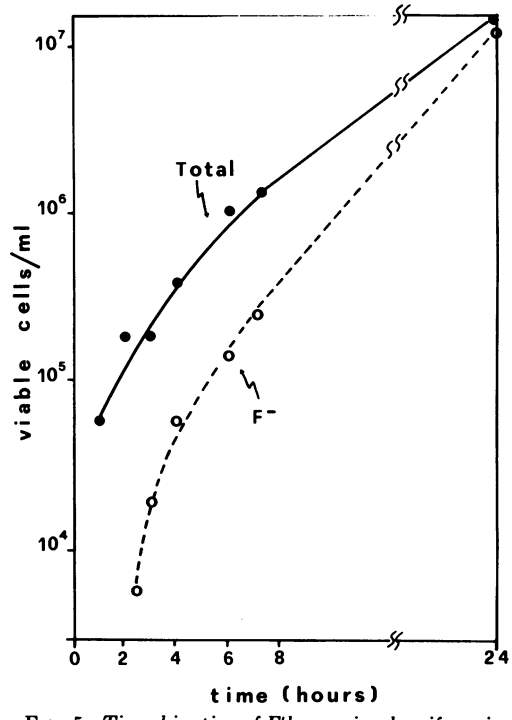

FIG. 5. *Time kinetics of F'lac curing by rifampin. 5 × 10^4 cells/ml from a log-phase culture was inoculated into prewarmed nutrient broth (Oxoid no. 2) containing 8 μg of rifampin/ml at 37 C without aeration. At the times indicated, samples were plated on MacConkey lactose agar (Oxoid). The solid line represents the total number of cells ($lac^+ + lac^-$); the dotted line represents the number of cured (lac^-) cells. Strain: JC6583.*

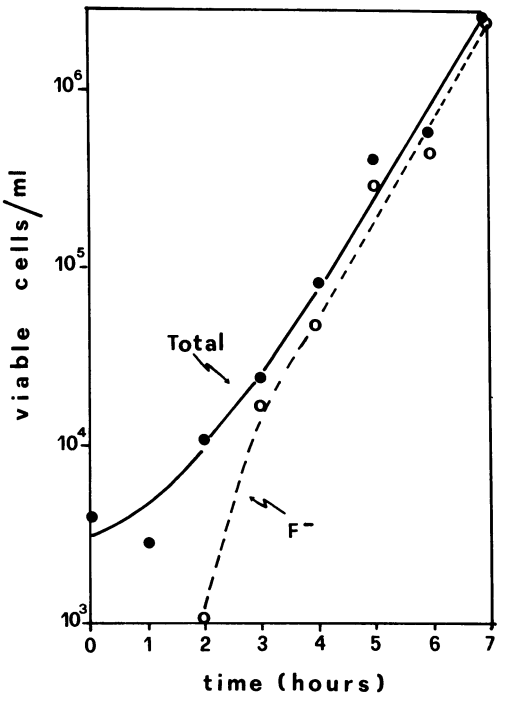

FIG. 6. *Time kinetics of F'lac curing by rifampin after pretreatment of cells with high doses of the drug. An inoculum of 4 × 10⁴ cells/ml from a log-phase culture was inoculated into prewarmed nutrient broth (Oxoid no. 2) containing 50 μg of rifampin/ml and incubated for 15 min at 37 C. The culture was then diluted to 6 μg of drug/ml and incubated at 37 C without aeration. At the times indicated, samples were plated on MacConkey lactose agar (Oxoid). Strain: JC6583.*

initial inoculum of about 5 × 10^4 cells/ml was used. Under these conditions, cells grew with a doubling time of about 2 h. At time zero, less than 0.1% of the cells were F^-; after one to two duplications in the presence of the drug, about 10% of the cells had lost the episome, and this function increased gradually with time. These results indicate that, under these conditions, the drug partially inhibits the replication of the episome in such a way that, at any generation, only a fraction of the cells lose the episome.

We have found conditions under which essentially immediate and complete loss of the episome can be observed. Cells are preincubated for 15 min in the presence of 50 μg of rifampin/ml and then diluted into prewarmed medium so as to obtain 6 μg of rifampin/ml. The results of this experiment are shown in Fig. 6. This pulse treatment caused extensive cell death (about 90% of the cells were killed). The surviving cells, however, lost the episome at a much greater rate than in the case of the experiment described in Fig. 5. Within two to three generations from the time of dilution to 6 μg of rifampin/ml, more than 80% of the cells had been cured. When the percentage of F'-containing cells was plotted against the number of generations, the result shown in Fig. 7 was obtained. The experimental points indicate that the loss of the episome follows first-order kinetics (after a certain lag) at a rate indicating that rifampin produces an immediate and complete block of episome replication. This result is similar to that observed by other authors for curing with acridine orange (7). These results are in agreement with the hypothesis that for fast curing rifampin must be allowed to saturate its receptors within the cells which are needed for episome replication. When this happens, the replication of sex factors is arrested and they are not segregated into daughter cells.

Incurability of acridine orange-resistant mutants. Mutants have been described which

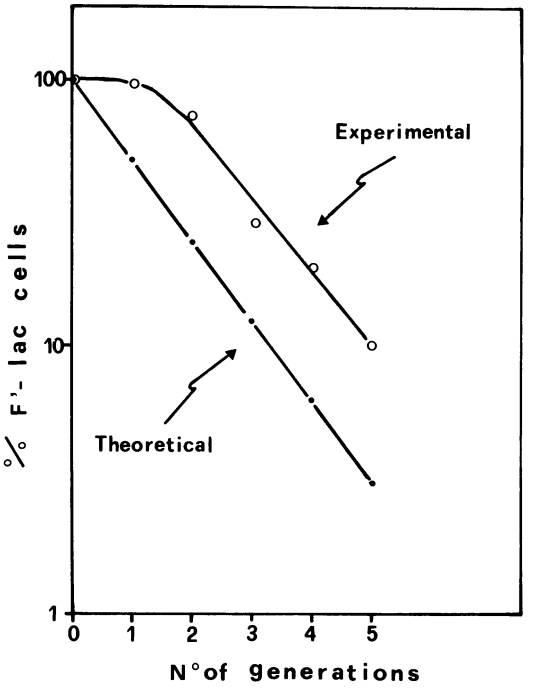

FIG. 7. *Kinetics of curing of JC6583 by rifampin. The experimental points are derived from the data of an experiment similar to that described in Fig. 6. The theoretical line predicts the rate of loss of F' from a population in which each bacterium contains a single chromosome and a single episome, assuming that rifampin produces an immediate and complete block of episome replication.*

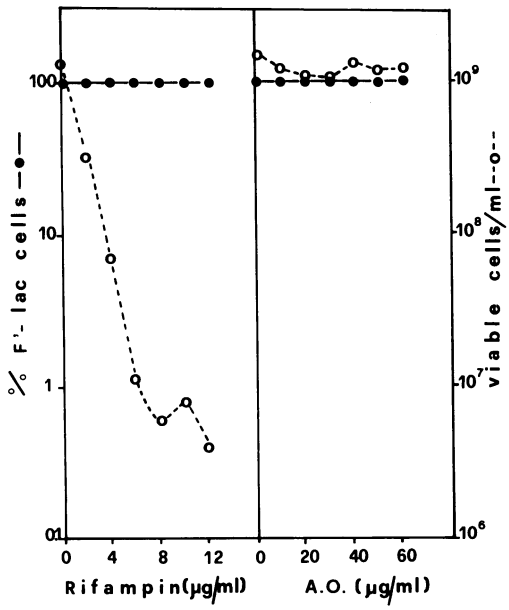

FIG. 8. *Curing of F'lac episome by rifampin in a strain "not curable" with acridine orange (A.O.). (left) F'lac curing by rifampin: inoculum size, 10^3 cells/ml. Other experimental conditions as in Fig. 1. (right) F'lac curing by acridine orange: 20 to 50 cells/ml from an overnight culture were inoculated into 4 ml of L broth containing the indicated doses of acridine orange and were incubated with shaking in darkness at 37 C for 24 h. Cultures were then plated on MacConkey lactose agar (Oxoid). Strain: JC5499.*

cannot be cured with acridine orange (2). For one class of such mutants, this property was originally attributed to the *rec* mutation, but later it was found that it is due to a mutation that is genetically separable from the *rec* locus. Its location on the chromosome, however, has not yet been established. Two such mutants were tested for ability to be cured with rifampin. Figure 8 shows the results for one mutant (JC5499). It can be seen that JC5499 was cured neither by acridine orange nor by rifampin. The results for the other mutant were similar and are not shown. More recently, another class of chromosomal mutation has been described which confers partial resistance to acridine orange curing (16). It has been called *eps* (episome stabilizer mutation), since in these mutants the thermosensitive sex factor, F-*lac* T62 (8), is able to multiply at high temperature. The mutation is cotransduced with the *str-A* gene by phage P1, and the suppressing activity of the mutation is counteracted by superimposed *spc-r* mutations (17). It was found that *eps* mutants also decrease the sensitivity of the episome toward curing with acridine orange (16). Figure 9 shows that one of these mutants (*eps-6*) is also much more resistant to the curing by rifampin.

DISCUSSION

The results presented here suggest that rifampin cures the F'*lac* episome by blocking its replication and therefore its segregation into daughter cells. Because mutants with *rif-r* ribonucleic acid (RNA) polymerase are not cured, we conclude that the curing is due to the inhibition of transcription produced by the drug and not to some other (unknown) effect of rifampin. In a previous paper, we showed that under less drastic treatment conditions than the ones described here rifampin reversibly inhibits the expression of some episomal genes (13). We believe that the curing is the ultimate consequence of such an inhibition, and that both phenomena have a common mechanism.

These results can be explained by two hypotheses for the mechanism of action of rifampin. (i) Rifampin preferentially inhibits the transcription of the episomal deoxyribonucleic acid (DNA) as compared with that of the chromosome. Curing could be a consequence of the reduction of the level of synthesis of an episome-specific protein, required for its rep-

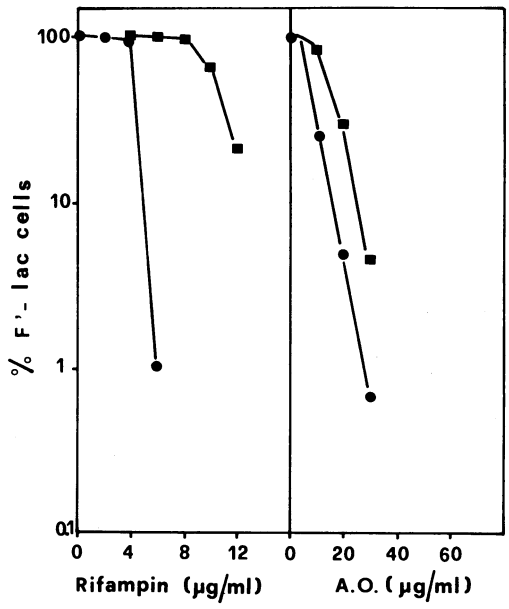

FIG. 9. *Curing of F'lac episome in a strain carrying eps mutation. (left) F'lac curing by rifampin: inoculum size, 10^2 cells/ml. Other experimental conditions as in Fig. 1. (right) F'lac curing by acridine orange: experimental conditions as in Fig. 8. Strains: L447 and L446.*

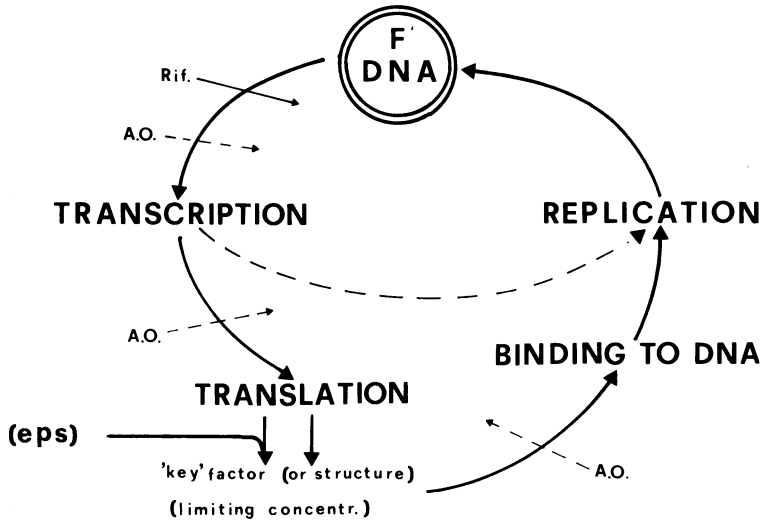

FIG. 10. *Hypothetical model for the mechanism of action of rifampin (Rif.) and acridine orange (A.O.) in curing the F'lac episome; (eps) = episome stabilizer mutation (16). The arrows (→, rifampin; ⇢, acridine orange) indicate the levels at which the two drugs may act.*

lication. This hypothesis receives some support from the observation that rifampin preferentially inhibits the expression of some F genes (13). (ii) A certain RNA molecule is required for the replication of sex factor, and the transcription of this RNA is particularly sensitive to rifampin. This RNA molecule could be synthesized either on the chromosome or on the episome. The requirement of RNA priming for DNA replication has already been described for other systems such as M13 RF synthesis and M13 RF replication (4, 15), M13 single-strand synthesis (14), ϕX174 RF synthesis (R. Schekman et al., Proc. Nat. Acad. Sci. U.S.A., in press), *col* E_1 duplication (5), and for initiation of *E. coli* chromosome replication (11).

Other possible hypotheses can be put forward to explain our results. The act of transcription per se could be required to initiate replication of the episome (e.g., local unwinding of the helical structure), or the replicative machinery of the episome has the RNA polymerase or its β subunit as an essential component. Curing by rifampin closely resembles curing with acridine orange, both in its cellular physiological requirements (exponential growth) and in the kinetics of loss of the episome (6, 7). Furthermore, we have observed that mutants which are poorly cured with acridine orange are also more resistant to curing with rifampin. It seems unlikely that these mutants are permeability mutants, since acridine orange and rifampin are structurally unrelated. Furthermore, these mutants are normally sensitive to rifampin (see Fig. 8). It appears, therefore, that the two drugs either act at the same level or affect at different levels the same physiological function that is essential for episome replication. The mechanism of action of acridine orange on the episome has not yet been clarified. It seems, however, that the drug directly inhibits the replication of the episome, while it does not dissociate the episome from the cellular element that is believed to constitute the bacterial unit of segregation (7).

On this basis, the simplest explanation of these observations is to assume that, like rifampin, acridine orange inhibits transcription in vivo. If this is the case, how can we explain the behavior of the *eps* mutants? Evidently the *eps* mutation is able to repair partially the damage produced by acridine orange or rifampin on transcription. If we assume that the episome is lost because the inhibition of transcription depletes the cell of an essential protein (or structure), then the *eps* mutation which might affect the ribosome (16) modifies the translational control of the essential protein (12). However, although we have good evidence that rifampin specifically impairs transcription in the cell, the same is not true for acridine orange. This latter, in fact, might interfere at other levels with the synthesis, or with the function, of the "key" product (or structure) itself. Also, in this case the behavior of the *eps* mutants can be explained by assuming that mutants might produce the "key"

product in large excess and therefore overcome the effect both of acridine orange and of rifampin.

The model in Fig. 10 schematically summarizes the above-mentioned considerations and shows the possible levels at which rifampin and acridine orange might act in curing the F'*lac* episome.

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