Curing of an *Escherichia coli* Episome by Rifampicin

(acridine orange/F⁺/F⁻/Hfr/lac)

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Communicated by Rita Levi-Montalcini, November 16, 1971

ABSTRACT Low doses of rifampicin, an antibiotic that specifically inhibits RNA polymerase, cure F⁺ cells from the episome. An *E. coli* mutant, whose RNA polymerase is resistant to rifampicin, does not show the curing. The growth of Hfr strains, which replicate the chromosome by the F-replication system, is inhibited by low doses of the antibiotic.

An episome, according to Jacob and Wollman (1), is a genetic element that can be added to the genome of the cell and that, inside this cell, may exist in two distinct states, the autonomous state and the integrated state. F and λ , for example, are episomes.

All known facts support the notion that in the autonomous state, F behaves as a small supernumerary chromosome not essential to the cell, but stably maintained, replicating more or less synchronously with the chromosome and segregating with it (2). In the integrated state, F is replicated passively by the chromosomal replicating machinery. The evidence for this point is as follows: in the autonomous state, F replication is inhibited by concentrations of acridine orange that do not affect chromosome replication, whereas in the integrated state, F becomes insensitive to the same concentration of acridine orange (3).

We have shown that certain doses of an antibiotic of the rifamycin family selectively inhibit the synthesis of extra-chromosomal DNA that codes for ribosomal RNA in *Xenopus laevis* oocytes, without affecting chromosomal replication (4). This result aroused our interest in the study of the effect of rifamycins on bacterial episomes. In this report, we show that rifampicin*, a specific inhibitor of the initiation step in the reaction catalyzed by RNA polymerase (5), eliminates the F⁺lac episome from *E. coli*. Rifampicin does not affect the episome in *E. coli* mutants with an RNA polymerase resistant to the antibiotic (6).

MATERIALS AND METHODS

Strains. Strains used in these experiments are listed in Table 1. Strain E3002R is a spontaneous rifampicin-resistant mutant of E3002 (6). Rifampicin resistance is 60% cotransducible with Arg H (5). Moreover, RNA polymerase extracted from this strain is resistant *in vitro* (6).

Hfr strains in which the chromosome is replicated by the F-replication system (7), were obtained by plating strain LC 349 on TY plates and incubation at 40°C. The frequency of reversion was about 10⁻³. Three revertants, Hfr 1, 2, and 3,

that were sensitive to acridine orange at 40°C, and not at 30°C, were used in the experiment.

Media. TY broth and TY agar (1.2%) contain 10 g Bacto Tryptone (Difco), 5 g Yeast Extract (Difco), and 8 g NaCl per liter. Minimal medium is the Minimum Citrate medium of Vogel and Bonner (8), to which the indicated supplements were added. The lac indicator plates contain Lactose MacConkey Agar (Difco).

Rifampicin Treatment. An exponential culture of an F⁺lac strain is diluted to about 10² cells/ml in TY broth containing the indicated doses of rifampicin, and incubated at 37°C on a rotory shaker. At various times, aliquots are diluted in TY broth without rifampicin and plated on Lactose MacConkey Agar plates on which lac⁺ colonies are deep red, whereas lac⁻ colonies are white. Results are expressed in % of lac⁻ colonies formed. At a given rifampicin concentration, the extent of aeration, the physiological state of the bacteria in the inoculum, the size of inoculum, and the medium used all affect the % of lac⁻ colonies obtained.

Test for Rifampicin Sensitivity of Hfr Strains Sensitive to Acridine Orange. For this test, Hfr 1, 2, and 3 were grown to about 2 × 10⁸ cells/ml, then streaked on TY agar, TY agar adjusted to pH 7.6 with 2 N NaOH and supplemented with 20 µg/ml of acridine orange, and TY agar supplemented with 1 µg/ml of rifampicin. Growth was observed after overnight incubation at 30°C and 40°C (7).

RESULTS

When *E. coli* E3002 (F⁺lac pro⁺/∇ (lac pro) Sm^R B1⁻) is grown for 20 generations in TY broth in the presence of concentrations of rifampicin that permit bacterial growth, a fraction of the cell population, depending on the dose of antibiotic used, loses the capacity to ferment lactose (Fig. 1A). Other

TABLE 1. List of strains

Strain	Genetic markers	Origin
E3002	F ⁺ lac ⁺ pro ⁺ /(lac, pro)B1 ⁻ Str ^R Rif ^R	J. Beckwith
E3002R	F ⁺ lac ⁺ pro ⁺ /(lac, pro)B1 ⁻ Str ^R Rif ^R	See text
X5097	F ⁻ (lac, pro)B1 ⁻ Str ^R Rif ^R	J. Beckwith
E50141	F ⁺ lac ⁺ pro ⁺ /(lac, pro)B1 ⁻ Str ^R Rif ^R	J. Beckwith
LC349*	F ⁺ lac ⁺ /leu ⁻ thy ⁻ (proB, lac)T46*	L. Caro

* Rifampicin is the generic name for a semi-synthetic derivative of the rifamycins, and is also called rifampin and Rifadin.

* T46* is a temperature-sensitive mutation affecting initiation of chromosome replication (7).

TABLE 2. Effect of rifampicin on the growth of F^+ and F^- cells

Input		Final				
		− Rifampicin		+ Rifampicin		
	cells/ml	<i>lac</i> [−] %	cells/ml	<i>lac</i> [−] %	cells/ml	<i>lac</i> [−] %
F ⁺	2 × 10 ²	0	3 × 10 ⁸	0	2 × 10 ⁶	80
F [−]	2 × 10 ²	100	6.5 × 10 ⁸	100	8 × 10 ⁵	100

Strains E3002 ($F'lac$) and X5097 (F^-) were grown in TY broth at 37°C to about 2×10^8 cells/ml, then diluted; about 10^8 cells of each strain were inoculated into 5 ml of TY broth, either with or without 5 μ g/ml of rifampicin. All tubes were incubated at 37°C with gentle shaking. After 11 hr, cultures were diluted and plated for single colonies on Lactose MacConkey agar, and *lac*⁻ and *lac*⁺ colonies were counted. The size of inoculum was measured by plating it on Lactose MacConkey agar.

characteristics carried on the episome, such as the ability to synthesize proline and the ability to restrict phage T7 (9), are lost concomitantly. These losses are permanent, since after several generations of subculture without the drug there is no sign of reversion. This finding indicates that we are not dealing with phenocopies, but with cells lacking the episome. The episome can be introduced again in these cells by mating them with an $F'lac$ donor.

A strain with a rifampicin-resistant RNA polymerase, but otherwise with the same genetic background as the strain used in the previous experiment, was constructed (see *Methods*). A mutant strain of this kind possesses an altered β subunit (10). With this strain, even high concentrations (100 μ g/ml) of the antibiotic do not affect the episome (Fig. 1B). Thus, the interaction of rifampicin with the β subunit of RNA polymerase is presumably responsible for the curing effect we have observed.

Various doses (0.25–7.5 μ g/ml) of rifampicin are effective in the elimination of the episome; to reach the same frequency of

TABLE 3. Effect of rifampicin on cultures derived from single cells

Growth conditions	Number of tubes inoculated	Number of sterile tubes	Number of nonsterile tubes	Number of clones with more than 2% of cured cells
+ 5 μ g/ml rifampicin	42	34	8	8
Without rifampicin	48	17	31	1

An exponential culture of strain E3002 was grown in TY broth at 37°C to reach a titer of about 10^9 cells/ml, and then diluted in the same medium to about 1 cell/ml. Two series of tubes containing 1 ml of the diluted culture were incubated at 37°C. The first series contained 5 μ g/ml of rifampicin. After 7 hr, 0.1 ml from each tube was plated on Lactose MacConkey agar and *lac*⁻ and *lac*⁺ colonies were counted. 100 *lac*⁻ colonies, picked at random, were tested for maleness and all appeared to be F^- . In the rifampicin-treated clones, the actual % of cured cells was more than 50% for all clones; in the control clones, the actual % for 24 clones was less than 1% (no *lac*⁻ colonies were found), for 6 clones from 0.5 to 1.5%, and for only 1 clone about 50%.

FIG. 1A. Curing of $F'lac$ in a rifampicin-sensitive strain. 10^8 cells of exponential cultures of strains E3002 (Fig. 1A) and E3002R (Fig. 1B) were inoculated into 5 ml of TY broth containing the indicated doses of rifampicin, and were allowed to grow at 37°C with gentle shaking for about 20 generations; cultures were then diluted in TY broth without rifampicin and plated on Lactose MacConkey agar. After 18 hr of incubation, *lac*⁻ and *lac*⁺ colonies were counted.

1B. Effect of rifampicin on $F'lac$ in a rifampicin-resistant strain.

appearance of *lac*⁻ colonies, longer exposure to the drug is necessary at lower concentrations (Fig. 2).

The increase in the proportion of F^- colonies after rifampicin treatment could be attributed in principle either to curing or to selective enrichment of F^- cells already present in the F^+ population.

Table 2 shows that 5 μ g/ml rifampicin, used for a period of time sufficient to produce 80% of F^- colonies from an F^+ strain, does not preferentially affect the growth of F^+ versus F^- strains; the strains used were completely isogenic and differed only in the presence of the episome in the F^+ strain.

However, the most decisive experiments concern the fate of a small number of cells inoculated in the presence of the drug. Table 3 shows that when the cells are grown in the presence of rifampicin, out of eight clones, eight contain more than 50% of F^- colonies; in the control, on the contrary, out of 31 clones, 30 contain less than 2% of cured cells, and only one clone had a frequency of about 50% of F^- colonies.

These results support the conclusion that rifampicin induces the loss of the episome from growing cells containing F^+ .

FIG. 2. The effect of different doses of rifampicin. Cells were grown and treated with rifampicin as indicated in Fig. 1. The doses of rifampicin used were: ▲, 0.5 μ g/ml; ○, 2 μ g/ml; ●, 3 μ g/ml; △, 4 μ g/ml; ×, 5 μ g/ml; □, 7.5 μ g/ml.

TABLE 4. Effect of rifampicin on Hfr strains sensitive to acridine orange

Temperature of growth						
30°C			40°C			
	Con- trol	Acridine orange (20 µg/ml)	Rifam- picin (1 µg/ml)	Con- trol	Acridine orange (20 µg/ml)	Rifam- picin (1 µg/ml)
Hfr 1	+	+	+	+	—	—
Hfr 2	+	+	+	+	—	—
Hfr 3	+	+	+	+	—	—

Hfr 1, 2, and 3 were isolated as revertants, sensitive to acridine orange, of the strain LC 349 (7) that is temperature sensitive for initiation of DNA replication, and which harbors an F'*lac* episome.

The effect of rifampicin on the F'*lac* episome is reminiscent of the effect of acridine described by Hirota (3).

It could be argued that the effect of rifampicin on F⁺ cells is only a consequence of the state of disturbance induced by the drug that results in the elimination of the extrachromosomal DNA. We were, however, able to produce a strong argument against such an interpretation. We have constructed Hfr strains according to Nishimura *et al.* (7), who used a temperature-sensitive *E. coli* mutant with the T46 lesion, which presumably affects the initiator for DNA synthesis. If such a strain contains an episome, at 40°C the reversion frequency rises from about 5×10^{-5} to about 3×10^{-3} . Among the revertants are found Hfr strains in which the episome is integrated, but not repressed. In these Hfr strains, at high temperature, chromosome replication and cell division are under control of the F episome. Nishimura *et al.* (7) have shown that at 42°C, acridine orange inhibits cell division in these strains; we have shown, accordingly, that the strains that are sensitive to acridine are sensitive to low doses of rifampicin (Table 4). Thus, rifampicin at low doses, in these Hfr strains, blocks chromosome replication.

DISCUSSION

Our findings demonstrate that rifampicin cures *E. coli* from the episome F'*lac*. Because mutants with an altered RNA polymerase that are resistant to rifampicin are not cured, we conclude that the curing is caused by the interaction of the drug with the β subunit of RNA polymerase. Various possibilities can be considered to account for our results. RNA polymerase might, for example, denature some region of the episomal DNA to permit replication to occur; for example, a

disruption of the helical structure might be necessary for proper initiation. Alternatively, the β subunit of RNA polymerase could be shared by RNA polymerase itself, and another hypothetical enzyme responsible for the replication of the episome. It is also possible that a certain RNA molecule transcribed either from the chromosome or from the episome is required for the replication of the episome. Riva *et al.*† have observed that the expression of some episomal genes is preferentially inhibited by rifampicin.

On the other hand, the RNA chains transcribed from the episome might serve some function other than as messenger for protein synthesis; in particular, the RNA could be used as a template for RNA-dependent DNA synthesis, or could act as a primer terminus for covalent extension by a DNA polymerase. Further work is necessary to distinguish among the several alternatives. It is quite interesting that Kornberg *et al.* (11) have observed that rifampicin inhibits the conversion of parental single-stranded DNA from bacteriophage M13 to the double-stranded replicative form, as well as the further multiplication of the replicative form. A requirement for transcription for activation of the λ replicator has been reported by Dove *et al.* (12).

We thank J. Beckwith, E. Calef, and F. Graziosi for helpful discussions and L. Caro for providing a strain of bacteria used for these experiments.

1. Jacob, F. & Wollman, E. L. (1961) *Sexuality and the Genetics of Bacteria* (Academic Press, New York).
2. Campbell, A. M. (1969) *Episomes*, eds. Bell, E., Halvorson, H. O. & Roman, H. (Harper and Row).
3. Hirota, Y. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 57–64.
4. Tocchini-Valentini, G. P. & Crippa, M. (1971) *Second Lepetit Colloquium on Oncogenic Viruses*, ed. Silvestri, L. (North Holland Publishing Co., Amsterdam), pp. 237–243.
5. di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A. & Tocchini-Valentini, G. P. (1969) *Nature* **222**, 533–536.
6. Tocchini-Valentini, G. P., Marino, P. & Colvill, A. J. (1968) *Nature* **220**, 275–276.
7. Nishimura, Y., Caro, L., Berg, C. M. & Hirota, Y. (1971) *J. Mol. Biol.* **55**, 441–456.
8. Vogel, J. H. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97–105.
9. Morrison, T. G. & Malamy, M. H. (1971) *Nature New Biol.* **231**, 37–41.
10. Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A. & Seifert, W. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 47–58.
11. Brutlag, D., Schekman, R. & Kornberg, A. (1971) *Proc. Nat. Acad. Sci. USA*, **68**, 2826–2829.
12. Dove, W. F., Hargrove, E., Ohashi, M., Haugli, F. & Guha, A. (1969) *Japan. J. Genet.* **44**, suppl. 1, 11–22.

† Riva, S., Fietta, A. & Silvestri, L. G., submitted to *Nature*.