ISOLATION OF COLD SENSITIVE-RIFAMPICIN RESISTANT RNA POLYMERASE MUTANTS OF *ESCHERICHIA COLI*

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SUMMARY: A selection procedure was performed to isolate mutants of *E. coli* which were temperature sensitive due to a structural modification of the enzyme DNA-dependent RNA polymerase. This was accomplished by using a tritium suicide procedure and isolating surviving cells resistant to the antibiotic rifampicin. The use of a tritium suicide procedure made it possible to virtually eliminate cells which were not temperature sensitive, while the use of high concentrations of rifampicin ensured the isolation of mutants with an altered RNA polymerase. Although an attempt was made to isolate spontaneous mutants which were inhibited in growth at either 30 or 40°C, only in mutants failing to grow at 30°C was resistance to high concentrations of rifampicin found. The mutants appear to have one mutation which alters RNA polymerase activity.

The isolation of several temperature sensitive RNA polymerase mutants has been described (1,2,3). Thus far, all the mutants described are heat sensitive, i.e., they synthesize RNA and grow at low temperature ($\sim$ 30°C) but fail to grow and exhibit defective RNA synthesis at higher temperatures. In addition to these mutants, others have been described which are rifampicin sensitive at 28°C and rifampicin resistant at 36°C (4) or have a heat sensitive mutation which decreases the rifampicin resistance of a strain containing a rifampicin allele (5). This paper describes the isolation of mutants that are growth inhibited at low temperatures and rifampicin resistant due to a mutation which affects *in vitro* RNA polymerase activity.

MATERIALS AND METHODS

A derivative of *E. coli* strain W3110 which is an arginine and tryptophan auxotroph (*arg H*, *trp A23*; ref. 6) was used for the selection of mutants. Cultures were grown in M9 minimal medium (7) supplemented with 0.25% casamino acids and 20 µg tryptophan/ml (M9S), tryptone medium (1.0% Bacto-tryptone, 0.5% NaCl/liter of water) or L-broth (8). Rifampicin (Calbiochem, Los

Angeles, California) was made fresh prior to each use; its concentration was determined by optical density at 475 nm using a molar extinction coefficient of 15.4 (9). Radioactive isotopes were obtained from Schwartz-Mann Bioresearch (Freehold, New Jersey).

Measurements of RNA Synthesis

The rate of RNA synthesis *in vivo* at 15°C was measured by determining the incorporation of uridine-5-^{3}H into RNA (6) at 5, 10, 15 and 20 minutes after exposing cells to the isotope. *In vitro* RNA polymerase assays were performed using the method of Burgess (10) except that (5-^{3}H) uridine-5'-triphosphate was used (at a specific activity of 67 mC/mmole) and the concentration of bovine serum albumin was reduced to a final concentration of 0.2 mg/ml.

Procedure for Mutant Isolation

The procedure used for tritium suicide is a modification of that described by Person (11). Sixteen individual cultures, each derived from a single colony were used to select mutants at two temperatures; eight cultures at 30°C, eight at 42°C. Overnight cultures in M9S medium at 37°C were diluted into the same medium and grown for three generations at 37°C to a concentration of 10^{8} cells/ml before harvesting by quick chilling and centrifugation. Cells were resuspended in 0.01 the original volume of M9S supplemented with arginine (60 µg/ml), tryptophan (40 µg/ml) and uridine (30 µg/ml). At time zero 1.0 ml of each resuspended pellet was added to 8.3 ml prewarmed medium of the same composition. After ten minutes of incubation at the non-permissive temperature (either 30 or 42°C) 0.7 ml uridine-5-^{3}H was added to give a final specific activity of 0.5 C/mmole and incubation continued for 1.5 generations of growth (Fig. 1a). Immediately following this incorporation period, cultures were quickly chilled and the cells of each captured on a 0.45 µ Millipore filter. The cells were washed with ten times the culture volume of water at 0°C, resuspended in 10 ml M9 medium and stored at 4°C (Fig. 1b). After approximately three weeks of storage at 4°C,

when 0.002% of the original number of cells were viable, cultures were incubated for one hour at 37°C in M9S medium containing 100 µg rifampicin/ml. Each culture was then plated at 37°C on tryptone plates containing 100 µg/ml rifampicin. Colonies which grew on these plates were tested for growth on tryptone plates at 30, 37 and 42°C.

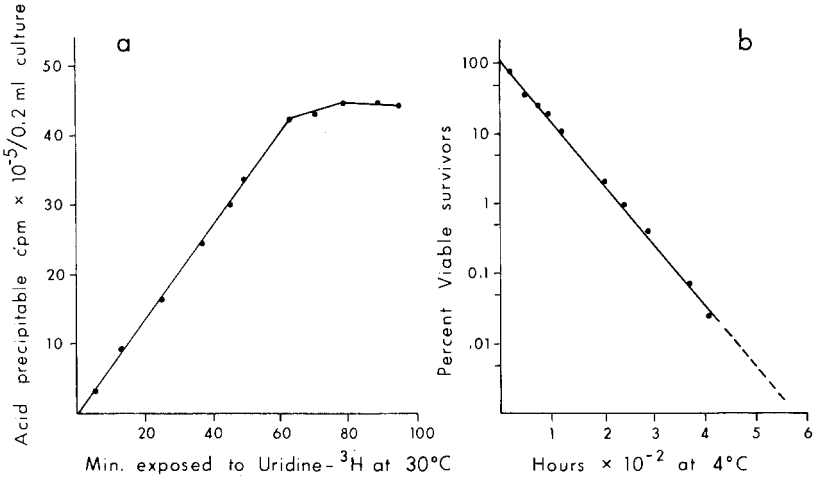

FIGURE 1. a) Incorporation of tritium by parental cells prior to tritium suicide. Cells were prepared for tritium incorporation at the non-permissive temperature (in this case 30°C) as described in the text. Cultures were harvested after 90 minutes of uridine-5-^{3}H incorporation. A similar curve was obtained at 42°C except that the total time scale was 60 minutes. b) Tritium suicide of parental cells from the 30°C procedure. Cultures harvested from the incorporation procedure represented by Fig. 1a were stored at 4°C; viability for one culture was determined with time. Cell concentration at time zero was 1.2 x 10^{9} cells/ml. Cells not exposed to tritium exhibited no change in viability for 200 hours then died at a rate about one-third of that shown. This decrease might be prevented by including glycerol in the storage medium (5).

RESULTS

Three-hundred seventy rifampicin resistant colonies were recovered from the original 8 x 10^{10} cells in the 42°C procedure; 148 were recovered from the same number of cells in the 30°C procedure. When these were tested for colony formation at 30, 37 and 42°C, three from the 30°C procedure failed to make colonies in 16 hours at 30°C, all the others grew at all three temperatures. These three colonies, designated cold sensitive (CS), were able to form colonies at 37 and 42°C in the presence or absence of 100 µg rifampicin/

ml and thus are cold sensitive and rifampicin resistant. Two of them (CS-1 and CS-2) were isolated from one culture, the third (CS-3) from a different culture. The two from the same culture are probably of independent origin as one of them (CS-1) has RNA polymerase activity which is thermolabile compared to parental activity (Reid, unpublished result). The three spontaneous mutants were isolated with a frequency of approximately 10^{-10}.

Evidence that the mutants have an altered RNA polymerase which is responsible for their cold sensitivity. Two lines of evidence support this conclusion: genetic evidence suggests that one mutation is responsible for the observed *in vivo* phenotype of rifampicin resistance, poor growth and poor RNA synthesis at low temperature; *in vitro* assays of RNA polymerase activity in crude extracts shows that mutant activity is rifampicin resistant and different than the rifampicin sensitive activity of crude extracts from the wild type parent.

The hypothesis that a single mutation is responsible for both characteristics is supported by the failure to separate the rifampicin resistant and cold sensitive phenotypes of the mutants by transduction (Table 1). In addition, the hypothesis is supported by the observation that spontaneous revertants exhibiting simultaneous reversion of both phenotypes can be iso-

TABLE 1

Transductant phenotype	Mutant recipient strains CS-1	CS-2	CS-3
Rif^s, Cld^r (Parental Donor)	196	197	300
Rif^s, Cld^s	0	0	0
Rif^r, Cld^r	0	0	0
Rif^r, Cld^s (Mutant recipient)	28	18	19
Total Arg+ transductant colonies	224	215	319
Cotransduction of rif^r, cld^s with arg+	88%	92%	94%

Table 1. Inseparability of rifampicin resistant and cold sensitive phenotypes by transduction. Transduction was performed according to the methods of Luria, Adams and Ting (12). Phage PI (arg+, rif^s, cld^r) from the parental strain W3110 (made Arg+ by transduction) was mixed with mutant cells (arg^-, rif^r, cld^s) and plated on minimal medium at 37°C to select for Arg+ recombinants. Donor, recipients and all transductants were also Trp^-. Numbers in the table are the numbers of Arg+ transductant colonies found with the phenotype indicated. Rif = rifampicin, Cld = cold, s = sensitive, r = resistant, CS = cold sensitive mutant.

lated at a frequency approximately 1000 times greater than expected for a double mutant. The spontaneous temperature insensitive revertants were found at a frequency no less than 10^{-10} and were indistinguishable from the wild type parent *in vivo* (with regard to growth rate and rifampicin sensitivity) and *in vitro* (Table 2).

TABLE 2

	(a) Assays for rifampicin resistance			(b) Assays of mixed extracts at 37°C		
	37°C					
Strain	+Rif	-Rif	+Rif / -Rif	alone	mixed	expected
Parent	50	800	0.06	712	---	---
CS-1	173	192	0.90	266	494	490
CS-1 revertant	50	789	0.06			
CS-2	267	272	0.98	258	496	486
CS-2 revertant	71	867	0.08			
CS-3	223	242	0.92	262	496	492
CS-3 revertant	11	846	0.01			

Table 2. *In vitro assays for RNA polymerase activity.* Extracts were prepared by sonication and centrifugation at 18,000 xg for 15 minutes. Assays without centrifugation or on Brij lysates gave essentially the same results as those shown here. Numbers are picomoles UTP incorporated/mg protein in a 10 minute incubation. Rif = rifampicin, CS = cold sensitive mutants. a) *Rifampicin resistance of parent, mutant and revertant extracts.* Extracts were preincubated with rifampicin at a final concentration of 1 µg/ml (+Rif) or without rifampicin (-Rif) for five minutes prior to adding the assay mixture. b) *Assays of mixed extracts.* Equal volumes of parent and mutant extract were mixed prior to assay to give the same protein concentration (*mixed*) as that used to determine the activity of each extract *alone*.

As indicated in Table 1, the mutation in each mutant cotransduces with *arg H* approximately 90% of the time.

RNA polymerase activities of mutant crude extracts are rifampicin resistant compared to parental extracts (Table 2a). In addition, as the data indicate, the specific activity of mutant extracts has repeatedly been seen to be 20-40% of parental activity. The reduced activity of the mutant extracts is not due to the presence of an inhibitor as shown by mixing each mutant extract with wild type parental extract (Table 2b). The combined activities are additive, thus revealing no inhibitor in mutant extracts and

suggesting that, under these conditions, the defective activity in mutant extracts cannot be improved by additions from parental extracts. At assay temperatures lower than 37°C (30, 20 and 15°C) mutant extracts have lower in vitro activity compared to parental extracts than at 37°C. However, the kinetics of UTP-H^3 incorporation is complicated and different for each mutant. These effects of low temperature on in vitro activity are under investigation.

RNA synthesis and growth of the cold sensitive mutants. The growth rate of the cold sensitive mutants is 65-75% of parental growth rate at 37°C in M9S medium, and becomes progressively less compared to parental growth rate as the temperature is lowered. The growth of mutant and parental cells at 15°C, after transfer from 37°C, is shown in Fig. 2. Ten minutes after such a shift the rate of RNA synthesis in the mutants compared to the parental strain had decreased approximately six fold (from 65-75% of parental rate at

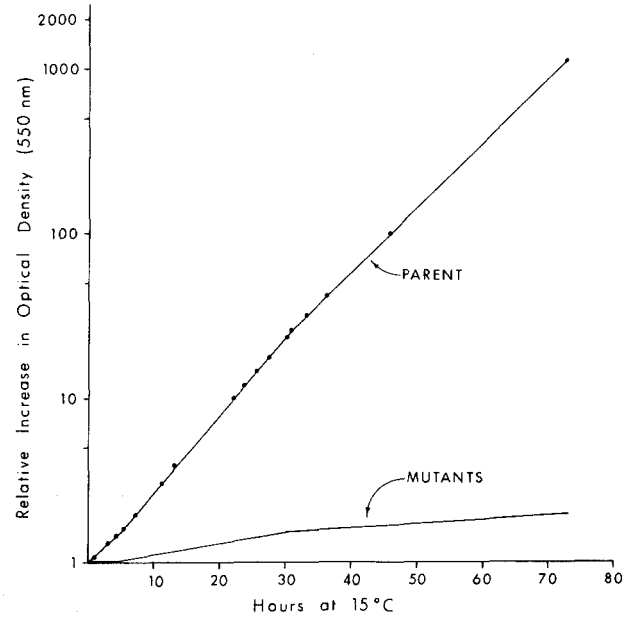

FIGURE 2. Growth of mutants and parent at 15°C in M9S medium. Mutant and parental cells growing exponentially in M9S medium at 37°C were transferred to 15°C at time zero. Samples for the three mutants were taken at the times shown for the parental strain; the line representing their increase in optical density is the average for all three. The final growth rate achieved was 7.5 hours/doubling for parental cells; approximately 110 hours/doubling for the mutants.

37°C to approximately 12% of parental rate at 15°C). At later times further decreases in the rate occurred so that mutant rates were approximately 5% of the parental rate by 2 hours and 2% at 12 hours or later when the mutants were growing at a rate roughly 7% that of their parent. The data for mutant growth shown in Fig. 2 is also compatible with a linear increase in optical density over the time interval shown, suggesting that either overall RNA synthesis or synthesis of a class of RNA might be growth limiting. This phenomenon is being studied.

As has been pointed out (13), multimeric E. coli RNA polymerase is approximately five times larger than phage T7 RNA polymerase which may indicate a regulatory function for part of the E. coli enzyme. Since it is known that subunit interactions in some enzymes are decreased by lower temperatures (14) and sigma dependent RNA polymerase-DNA complex formation fails below 17.5°C in vitro (15), the isolation and characterization of cold sensitive RNA polymerase mutants may be helpful in elucidating the presumed regulatory functions of this enzyme.

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REFERENCES

1. Jacobson, A. and Gillespie, D., Cold Spring Harbor Symp. Quant. Biol., 35, 85 (1970).
2. Khesin, R.B., Shemyakin, M.F., Gorlenko, Zh. M., Mindlin, S.Z. and Ilyina, T.S., J. Mol. Biol., 42, 401 (1969).
3. Yura, T., Igarashi, K. and Masukata, K., Lepetit colloquia on biology and medicine; RNA polymerase and transcription (Silvestri, L., editor), 1, 71 (1970).
4. Alikhanian, S.I., Debabov, V.J., Tomasion, C.N., Kopcickina, S.T., Nickifovova, I.D., Severina, T.A., Piruzian, E.S. and Pogosov, V.Z., Lepetit colloquia on biology and medicine; RNA polymerase and transcription (Silvestri, L., editor), 1, 28 (1970).

5. Babinet, C., Lepetit colloquia on biology and medicine; RNA polymerase and transcription (Silvestri, L., editor), 1, 37 (1970).
6. Reid, P. and Speyer, J., J. Bact. 104, 376 (1970).
7. Adams, M.H., Bacteriophages, John Wiley & Sons, Inc., New York (1959).
8. Lennox, E.S., Virology, 1, 190 (1955).
9. Maggi, N., Pasqualucci, G.R., Ballotta, R. and Sensi, P., Chemotherapia, 11, 285 (1966).
10. Burgess, R.R., J. Biol. Chem., 244, 6160 (1969).
11. Person, S., Biophys. Jour., 3, 183 (1963).
12. Luria, S.F., Adams, J.N. and Ting, P.C., Virology, 12, 348 (1960).
13. Chamberlin, M. and McGrath, J., Cold Spring Harbor Symp. Quant. Biol., 35, 259 (1970).
14. Valentine, R.C., Wrigley, N.G., Scrutton, M.C., Irias, J.J. and Utter, M.F., Biochem., 5, 3111 (1966).
15. Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V.S., Palm, P., Heil, A., Seifert, W., Cold Spring Harbor Symp. Quant. Biol., 35, 47 (1970).