RNA POLYMERASE MUTANTS OF ESCHERICHIA COLI, I. MUTANTS RESISTANT TO STREPTOVARICIN

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Streptovaricin inhibits RNA and protein synthesis in microorganisms, particularly in gram-positive bacteria.[1] This antibiotic also inhibits *in vitro* RNA synthesis catalyzed by the DNA-dependent RNA polymerase of *Escherichia coli*.[2] Unlike other antibiotics, such as actinomycin D, it seems to act by binding to RNA polymerase rather than to template DNA. This property immediately suggests the possibility that, simply by selecting for streptovaricin-resistant mutants, one could isolate mutants that produce RNA polymerase with an altered sensitivity to this drug. Genetic and biochemical analyses of such mutants could provide valuable information not only on the structure and function of this enzyme but also on its role in the growth of bacteria and bacteriophages.

The present communication describes the isolation and some properties of streptovaricin-resistant mutants obtained from several strains of *E. coli*.[3]

Materials and Methods.—Bacterial strains: The bacterial strains employed are all derivatives of *E. coli* K-12 and are listed in Table 1.

Media: The broth medium used contained 20 gm of polypeptone (Wako Drug Co.) and 5 gm of NaCl per liter; the pH was adjusted to 7.2. Medium E[4] with appropriate supplements of amino acids (20 μg/ml), thiamine (2 μg/ml), or Difco Casamino acids (2 mg/ml) was used as a standard minimal medium.

Selection of streptovaricin-resistant mutants: A number of independently grown cultures in broth (1 ml) were either treated with nitrosoguanidine (NG) or grown in the presence of 2-aminopurine (AP, 50 μg/ml) at 37°. Cultures were centrifuged, cells resuspended in 1 ml of 0.01 *M* tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) containing 0.001 *M* ethylenediaminetetraacetate (EDTA) and incubated at 37° for 5 min. A loopful from each tube was then inoculated into 0.5 ml of broth containing 400 μg/ml of streptovaricin (Upjohn Co. U-7750, complex). After 1 or 2 days' incubation at 37°, most of the tubes became turbid, an indication of growth of streptovaricin-resistant clones. An aliquot of each cell suspension was spread on broth-agar medium, and single colonies were picked and tested for streptovaricin resistance. One clone from each tube was established as a mutant stock to ensure the independent origin of the mutants.

Preparation of RNA polymerase: A modification[5] of the procedure of Chamberlin and Berg[6] has been used, except that cells were disrupted by sonication for 3 min in a Raytheon sonic oscillator (10 kc) and the partially purified enzyme was dialyzed overnight against buffer B containing 0.05 *M* KCl. The enzyme thus obtained had a sedimentation coeffi-

TABLE 1. *Bacterial strains used.*

Strain	Relevant genetic characters	Source
KY118	HfrH: *metA thi*	
KY5102	HfrH: *stv-72 metA thi*	KY118
JC1569	F⁻: *leu his argG metB rec*	J. A. Clark
KY5405	F⁻: *stv-202 leu his argG metB rec*	JC1569
KY5418	F⁻: *stv-215 leu his argG metB rec*	JC1569
AB1206	F'14: *pro his* deletion(*ilv-metB-argH*)/F14	E. A. Adelberg
KY5605	F'14: *pro his* deletion(*ilv-metB-argH*)/F14 *stv-279*	AB1206
KY5615	F'14: *pro his* deletion(*ilv-metB-argH*)/F14 *stv-293*	AB1206
GHA_1T_1	F⁻: *thr leu his argH metB ppc thi*	N. Glansdorff

cient of about 22S following centrifugation in a glycerol density gradient. In some experiments, crude extracts (supernatant after the 8000 × *g* centrifugation) were also employed.

Assay of RNA polymerase: The reaction mixture (0.25 ml) contained the following in μmoles: Tris buffer (pH 7.8), 10; β-mercaptoethanol, 3; $MgCl_2$, 1; $MnCl_2$, 0.25; the standard nucleoside triphosphates (ATP, CTP, GTP, UTP), 0.025 each; H^3-UTP, 0.1–0.5 μc; phosphoenolpyruvate; 1; pyruvate kinase, 10 μg; DNA, 20 μg; enzyme, 10–50 units. Incubation was carried out at 30° and the reaction was stopped by the addition of perchloric acid (PCA) in the cold. The RNA in the precipitate was collected by centrifugation using bovine serum albumin as carrier. After two washings with cold PCA, the precipitate was dissolved in 0.3 ml of 5% aqueous ammonia and the radioactivity determined in a Beckman scintillation counter. RNA synthesis continued linearly for at least 30 min under these conditions. When crude extracts were used, DNA was omitted from the reaction mixture and the reaction was carried out for 10 min at 30°.

Messenger RNA determination: Pulse-labeled RNA preparations used for sucrose density-gradient analysis (Fig. 2) were treated with 0.1 *M* HCl, precipitated with ethanol, and hybridized with denatured DNA of *E. coli* by the method of Gillespie and Spiegelman.[7]

Results.—Isolation of resistant mutants: A number of streptovaricin-resistant mutants have been isolated from several strains of *E. coli* K-12. Cells were pretreated with EDTA to facilitate the permeation of streptovaricin into the cells and at the same time to eliminate the class of mutants that might have become impermeable to the drug. Thus, all the mutants obtained, unlike the parental strains, could grow when first treated with EDTA and inoculated into broth containing 400 μg/ml of streptovaricin. The frequency of such mutations could not be estimated accurately due to the type of selection used, but was of the order of 10^{-8} spontaneously and 10^{-6} or higher following NG- or AP-mutagenesis. About two thirds of these streptovaricin-resistant (Stv-R) strains were also found to be resistant to another antibiotic, rifampicin, which is known to inhibit RNA synthesis in *E. coli*, presumably by similar mechanisms.[8]

RNA synthesis in vivo: The comparative effects of streptovaricin upon the RNA synthesis of the drug-resistant mutants (Stv-R) and of the parental, sensitive strain (Stv-S) were determined. Figure 1 presents the results of experiments with one of the Stv-R mutants derived from strain KY118. Both continuous labeling and pulse-labeling of RNA were carried out. As shown in Figure 1(*A-D*), streptovaricin markedly inhibits the synthesis of stable RNA's with the Stv-S strain used (KY118), but only slightly inhibits RNA synthesis with the Stv-R mutant (KY5102). The rate of RNA synthesis in several other Stv-R mutants also was only slightly inhibited by streptovaricin.

To test the possibility that streptovaricin might differentially affect the synthesis of ribosomal, transfer, and messenger RNA, pulse-labeled RNA was prepared from cells at various times after the drug had been added. The labeled RNA was examined by sucrose density-gradient centrifugation and by DNA-RNA hybridization. The results (Fig. 2) revealed little, if any, differential effect on the synthesis of RNA by the Stv-S strain (KY118). The rate of synthesis of bulk messenger RNA as judged by hybridization with denatured *E. coli* DNA was reduced to a somewhat greater extent than was the synthesis of ribosomal or transfer RNA. RNA patterns almost identical to those in Figure 2*A* were also obtained with the Stv-R mutant (KY5102) when it was pulse-labeled in the presence or absence of streptovaricin. It thus appears that, at least under

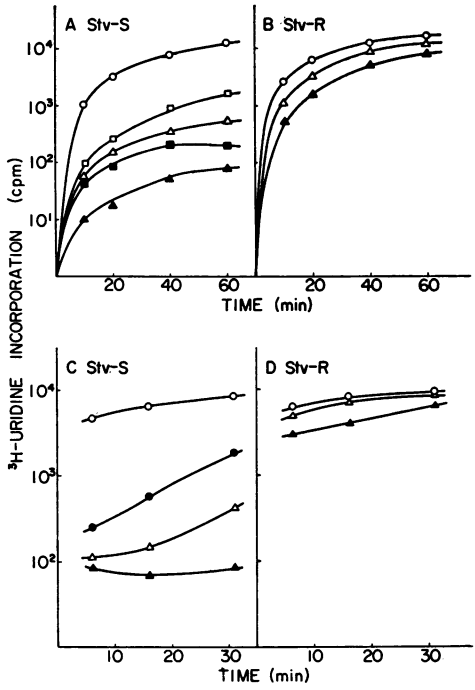

FIG. 1.—Effect of streptovaricin on RNA synthesis with strains KY118 (Stv-S) and KY5102 (Stv-R). (*A*, *B*) Bacteria were grown to an exponential phase in medium E supplemented with 2 mg/ml Casamino acids and 2 μg/ml thiamine. Cells were collected by centrifugation, treated with 0.001 *M* EDTA at 37° for 5 min, and immediately mixed with prewarmed medium containing various concentrations of SV and uridine-5-H^3 (0.2 μc/10 μg/ml). After a shaking at 37°, aliquots were taken at intervals and were added to 3 ml of cold 3.5% PCA.

(*C*, *D*) RNA was pulse-labeled with H^3-uridine (0.2 μc/1 μg/ml) for 1 min at various times after EDTA treatment; otherwise the conditions were the same as for (*A*) and (*B*).

All PCA-treated cells were kept in ice for 30 min or longer, and the radioactivity in the PCA-insoluble fraction was determined. Without streptovaricin, —○—; with the following amounts of streptovaricin: 25 μg/ml, —□— ; 50 μg/ml, —●—; 100 μg/ml, —△—; 200 μg/ml, —■—; and 400 μg/ml, —▲—.

the conditions we employed, the synthesis of all major species of RNA is inhibited to comparable degrees by this antibiotic.

RNA synthesis in vitro: To determine whether the Stv-R mutations affect the properties of RNA polymerase, the effect of streptovaricin on *in vitro* RNA synthesis was studied. As is shown in Figure 3, RNA synthesis is severely inhibited when the wild-type RNA polymerase is used but not when the mutant enzyme is employed. Mixing the two enzyme extracts results in an intermediate resistance to the drug, an indication that there is no interaction between the enzyme preparations. Similar results were obtained with enzyme that was dialyzed in the presence of 0.5 *M* KCl. Following this treatment the enzyme has a sedimentation coefficient of about 15*S* in a glycerol density gradient. Experiments with crude extracts also gave similar results. About 20 other Stv-R mutants similarly tested were all found to produce enzymes partially or completely resistant to streptovaricin. None of them, however, showed specific enzyme activities appreciably different from the parental strain. These results suggest that the Stv-R mutations indeed affect the RNA polymerase molecule directly rather than other factors involved in RNA synthesis.

Mapping of the stv gene: The gene determining the streptovaricin resistance of RNA polymerase in Stv-R strain KY5102 was mapped by conjugation and by P1 phage transduction. The results of conjugation experiments suggested that a single gene (designated *stv*) located between *ilv* and *metA* was involved in the Stv-R mutation. It was subsequently found that *stv* is cotransducible with *argH* at high frequency by phage P1. As indicated by Table 2, the distribution of unselected markers in the latter experiment suggests the following gene order,

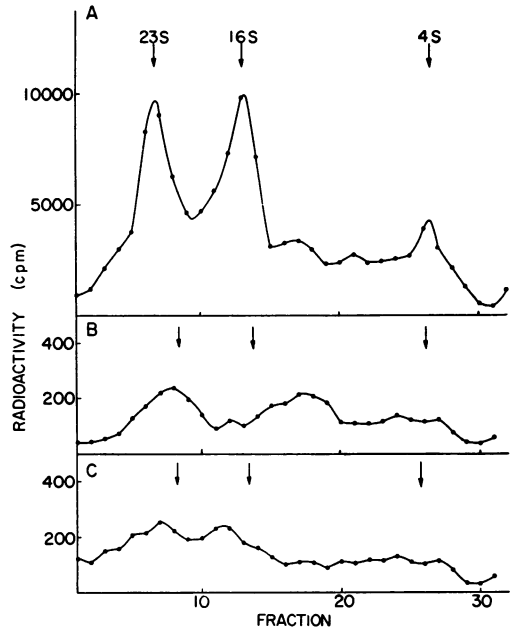

FIG. 2.—Sucrose density-gradient centrifugation of pulse-labeled H^3-RNA of strain KY118 (Stv-S). RNA was pulse-labeled with H^3-uridine (10 μc/1 μg/ml) from 5 to 7 min in the absence (*A*) or presence (*B*) of 400 μg/ml of streptovaricin, or from 20 to 22 min in the presence of streptovaricin (*C*). The labeling was stopped by addition of 500 μg/ml of unlabeled uridine and 0.01 *M* sodium azide in the cold. Cells were washed twice in buffer with sodium azide, lysed with sodium lauryl sulfate and RNA prepared by phenol extraction in the presence of 0.5 *M* KCl at 37°. RNA was twice precipitated with ethanol, dissolved in 0.01 *M* Tris buffer (pH 7.8) containing 0.1 *M* NaCl, filtered through a Millipore filter (HA, 0.45-μ pore size), and then applied on a sucrose density gradient (5–20%). Centrifugation was in a SW-39 rotor at 100,000 × *g* for 6 hr. Fractions were collected and the radioactivity was determined after PCA precipitation. Arrows indicate the positions of 23*S*, 16*S*, and 4*S* RNA as determined by C^{14}-RNA included in each tube for density reference (not shown).

metB-ppc-argH-stv. Several other Stv-R mutants tested also gave similar results, the cotransduction frequency of *stv* with *argH* varying between 20 and 35 per cent. Thus it may be concluded that a gene or a group of genes located near *argH* primarily determines the streptovaricin resistance of the *E. coli* RNA polymerase.

Dominance test: To test whether the *stv*$^+$ gene is located on the F14 episome,[11] which is known to carry the chromosomal segment *ilv-metB-argH* attached to the

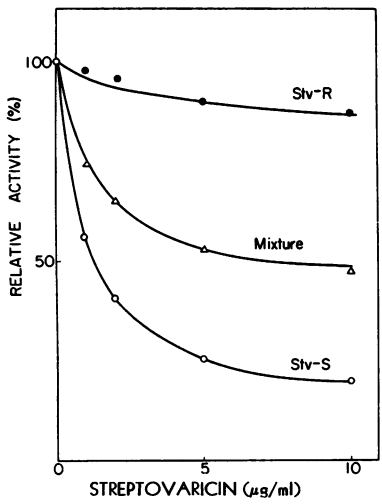

FIG. 3.—Effect of streptovaricin on enzymatic RNA synthesis catalyzed by RNA polymerase from strain KY118 (Stv-S) and KY5102 (Stv-R). Dialyzed partially purified enzyme preparations (50 units) were used. Streptovaricin was added before enzyme and other reaction components were mixed.

TABLE 2. *Transduction of the streptovaricin-resistant gene (stv).*

Selection	Unselected Marker			Number of transductants
	stv	*ppc*	*metB*	
argH$^+$	1*	1	1	6
	1	1	0	55
	0	1	1	4
	1	0	0	19
	0	1	0	90
	0	0	0	54
			Total	228

The procedure is as described previously,[9] except that a virulent P1 phage[10] was used. Recombinants (*argH*$^+$) were picked, purified by streaking on the same selective media, and the unselected markers scored by replica plating on appropriate media. Streptovaricin resistance was scored by inoculating about 10^6 cells of each recombinant into broth containing 400 μg/ml of the drug and letting this stand at 37° for about 24 hr. Donor (KY5102): *argH*$^+$ *ppc*$^+$ *metB*$^+$ *stv;* Recipient (GHA_1T_1): *argH-1 ppc metB stv*$^+$.

* 1 represents markers from the donor and 0 represents markers from the recipient.

F factor, a number of Stv-R mutants isolated from an F$^-$ strain (JC1569) and an F'14 strain (AB1206) were investigated. All these mutants had been shown to produce streptovaricin-resistant RNA polymerase. Thus, merodiploids were constructed by two series of crosses: (*A*) between JC1569 and each of the Stv-R derivatives of AB1206, and (*B*) between AB1206 and each of the Stv-R derivatives of JC1569. Recombinants (*met*$^+$ *pro*$^+$) were selected from each cross, purified, and tested for their streptovaricin resistance in the same selective medium.

Among the merodiploids obtained from (*A*) crosses, two were able to grow in the presence of streptovaricin while ten others were not. That all merodiploid strains indeed carry F14 was verified by their ability to transfer the episome to another F$^-$ strain (GHA_1T_1) at high frequency. It should be emphasized that the original F$^-$ strain used (JC1569) is defective in recombination;[12] therefore, complications due to recombination between the episome and the chromosome are virtually excluded. It thus seems likely that two of the Stv-R mutants carry dominant mutations at the chromosomal region covered by F14. With respect to the other ten mutants, two possibilities were considered: (1) that they occurred within the region covered by F14 and were *recessive* to the wild-type *stv*$^+$ allele, or (2) that they occurred outside the region covered by F14. The second possibility is excluded by the fact that the merodiploid strains that received the episome from such Stv-R strains do carry the *stv* allele as shown by its cotransduction with *argH*$^+$ (recipient strain GHA_1T_1) by phage P1. The occurrence of these recessive Stv-R mutations also indicates that the *stv*$^+$ gene is deleted from the chromosome of strain AB1206.

Merodiploid strains from the (*B*) crosses were similarly examined, and 13 out of 21 were shown to be Stv-R while the rest were Stv-S. The latter mutants must then represent recessive mutations in the chromosomal region covered by F14 episome, whereas the former may represent dominant mutations like those described above. All these results indicate that a gene (or genes) determining streptovaricin resistance is located on the F14 episome and that an *stv* mutant allele can be either dominant or recessive.

RNA polymerase from the merodiploids: The merodiploid strains were also examined enzymatically; typical results are presented in Table 3. It can be

TABLE 3. *Streptovaricin-resistance of merodiploids in vivo and in vitro.*

Strain	*stv* Allele on Chr.*	*stv* Allele on Epis.	*Stv* phenotype	Per Cent Inhibition *in vitro* by Streptovaricin 2 μg/ml	5 μg/ml	10 μg/ml
JC1569	+		S	72.4	81.7	85.0
AB1206	Del.	+	S	76.4	83.3	86.3
KY5700	+	+	S	73.4	82.3	83.6
KY5615	Del.	289	R	5.0	10.5	17.4
KY5715	+	289	R	46.5	53.9	54.9
KY5605	Del.	279	R	12.2	25.7	32.3
KY5705	+	279	S	75.2	82.7	89.4
KY5405	202		R	1.4	1.6	2.4
KY5805	202	+	R	4.8	6.3	7.2
KY5418	215		R	0.4	1.3	0.5
KY5818	215	+	S	48.7	56.0	60.8

Merodiploid strains were constructed as described in the text. They were grown in minimal medium supplemented with leucine, histidine, and arginine to prevent growth of possible F^- segregants. Aliquots were taken from all cultures used for enzyme extraction, spread on broth agar for single colony isolation, and ten colonies each were examined to assure their merodiploidy by cross-streaking against cells of GHA_1T_1 on the appropriate medium. Crude extracts (40–60 units) were employed in these experiments.

* Chr., chromosome; Epis., episome (F14); Del, deletion.

seen that some merodiploid strains (KY5715 and KY5818) produced RNA polymerase whose sensitivity to streptovaricin is intermediate between that of the parental strains used, whereas others (KY5705 and KY5805) produced enzyme with a sensitivity similar to that of the chromosomal parent. The level of sensitivity correlates well with the dominance relationships noted above. However, *in vivo*, strain KY5715 is resistant while KY5818 is sensitive to streptovaricin, despite the fact that they are almost equally resistant *in vitro*. Quantitative studies of streptovaricin resistance *in vivo* may clarify these apparently contradictory findings. It should also be noted that none of the merodiploids so far tested exhibits significantly higher specific enzyme activity, which might be expected if the F14 episome carries all structural genes for this enzyme.

Discussion.—The results presented suggest that streptovaricin-resistance mutations modify the structure of RNA polymerase, resulting in the formation of an enzyme resistant to this drug. This implies that these mutations affect the structural gene(s) for the enzyme. Further support for this interpretation has been provided by our recent detection of temperature-sensitivity mutations affecting both the streptovaricin resistance *and* the heat stability of RNA polymerase.[13] These mutations also map close to the *stv* mutations reported here.

Since streptovaricin is believed to inhibit specifically the initiation of RNA synthesis,[14] the *stv* mutations may affect the site on RNA polymerase that is involved in one of the initial steps of RNA synthesis, such as recognition of the initiation sites (promotor) on DNA, selection of the DNA strand to be transcribed, and binding and/or selection of the first ribonucleoside triphosphate. Experiments designed to discriminate between the various possibilities are in progress.

The fact that streptovaricin inhibits the synthesis of messenger, transfer, and ribosomal RNA in the Stv-S strain but not in the Stv-R strain is consistent with

the notion that the RNA polymerase is the major, if not the only, enzyme involved in genetic transcription *in vivo*. This possibility is directly supported by the observation, to be reported elsewhere, that the synthesis of all major RNA species is arrested more or less simultaneously when cultures of a temperature-sensitive RNA polymerase mutant are transferred from low to high temperature.

The dominance relationships found between *stv* and *stv*$^+$ in the merodiploid strains seem to be generally correlated with the level of streptovaricin resistance of the RNA polymerase extracted from these strains. However, the molecular mechanisms involved in the interaction between the two *stv* genes present in a single cell remain obscure at the present time. Complementation studies *in vivo* and *in vitro* between different *stv* alleles may provide a useful approach to problems of formation as well as of structure and function of this important enzyme.

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