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Drastic Alteration of Ribosomal RNA and Ribosomal Proteins in Showdomycin-Resistant *Escherichia coli*

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ABSTRACT In a mutant of *Escherichia coli* resistant to showdomycin, both the 50S and 30S ribosomal subunits contain RNA species in which the purine concentration greatly exceeds that of pyrimidines. The same is true for total rapidly-labeled RNA. The modified ribosomal RNA hybridizes poorly with homologous DNA, which is apparently unchanged in base composition. Acrylamide gel electrophoresis of mutant ribosomal proteins shows a highly altered protein pattern for both ribosomal subunits, although the activity of these ribosomes is not decreased.

In bacteria, the ribosomes play an important role in the translation mechanism for protein synthesis (1) and constitute the primary site of action of several antibiotics (2–6). Each ribosomal subunit, 50S and 30S, has a specific functional role essentially linked to a group of proteins (7, 8), whose synthesis is under genetic control (9–12). Structural genes for ribosomal proteins may be the loci conferring sensitivity (12, 13) to and dependence (14) on antibiotics. Thus, it has been shown that certain antibiotics provoke the alteration exclusively of a single protein in 50S ribosomal subunits, while others affect a single protein of the 30S subunits. In a mutant of *E. coli* resistant to erythromycin or lincomycin, a single protein associated with 50S ribosomal subunits seems to be altered (4, 15), while in the case of resistance to streptomycin (11, 14) and spectinomycin (5) a single protein of the 30S subunits is functionally modified. The biological activity of ribosomes seems to depend on the presence of the correct ribosomal proteins while the ribosomal RNA is needed for the assembly of these proteins (16).

We have previously shown that showdomycin, a naturally occurring "nucleoside" (17, 18), rapidly provokes in *E. coli* (19, 20) a drastic change in the base composition of 23S ribosomal RNA and a large fraction of the rapidly-labeled RNA (20). In a mutant of *E. coli* resistant to low concentrations of showdomycin, these types of RNA are modified and the activity of DNA-dependent RNA polymerase is strongly reduced, while that of polynucleotide phosphorylase is greatly altered (20). On the basis of these observations, we expected that some proteins of both the 50S and 30S ribosomal subunits would be altered in a mutant of *E. coli* resistant to high concentrations of showdomycin.

EXPERIMENTAL

Isolation of a showdomycin-resistant mutant of *E. coli*

Mutant Shor M 500 of *E. coli* HFr (Hayes), resistant to 500 μg of showdomycin/ml in synthetic medium containing glucose, was isolated from a mutant resistant to 10 μg of antibiotic per ml (21). After many transfers in synthetic medium in the absence of antibiotic, mutant Shor M 500 grows in the presence of 500 μg of showdomycin/ml as well as wild type *E. coli* in its absence. It is as sensitive to phage λ, phage T_4, and to phage f_2 as wild type (M. Beljanski, in preparation). Mutant cells and parental ones grown in synthetic medium in the total absence of showdomycin were used in this work.

Ribosomal RNAs in mutant Shor M 500 have a new base composition

The ratio of (G+A)/(C+U) in the RNA of *E. coli* is approximatively equal to 1.1–1.2 (22, 23). We have shown that showdomycin induces in *E. coli* the production of several altered RNA species that are no longer complementary to DNA (19–21), and leads to the massive production of resistant mutants (manuscript in preparation). We have isolated two classes of mutants. In the first class, mutants resistant to low concentrations of showdomycin (1–10 μg/ml) have their 23S RNA, 5S RNA, and a large fraction (80%) of rapidly-labeled RNA altered, while 16S RNA and 4S RNA are apparently not modified (21). Mutants of the second class, resistant to high concentrations of showdomycin (20–500 μg/ml), contain highly altered ribosomal RNAs. In fact, Dowex column analysis of nucleotides after alkaline hydrolysis showed a drastic change in the ratio of purine to pyrimidine nucleotides in 23S, 16S, and 5S RNA (Table 1). In the altered RNA, G is equal to A, and C is equal to U, indicating the absence of complementarity between nucleotides. A + G nucleotides present in excess in altered rRNA are an integral part of this RNA, as previously shown for 23S RNA isolated from *E. coli*

TABLE 1. *Base composition of ribosomal RNAs in wild type and mutant E. coli*

	Mol per 100 mol of nucleotide					
	Wild type			Mutant Shor M 500		
Nucleotide	23S	16S	5S	23S	16S	5S
A	25.2	24.2	18.7	31.0	32.0	31.6
G	28.0	27.9	34.2	34.3	35.0	32.4
C	24.0	24.2	28.8	17.7	16.7	18.3
U	23.0	23.6	17.0	16.1	16.3	16.7
(G+A)/(C+U)	1.11	1.10	1.06	1.96	2.01	1.83

Ribosomal RNA (23S, 16S, and 5S) was isolated by the phenol method and separated as described (20). After alkaline hydrolysis (KOH, 0.5 N, 18 hr at 37°C), the nucleotides were analyzed using a Dowex 1 × 2 column, 200–400 mesh (20). For base-ratio analysis, 1 mg of each type of RNA was used.

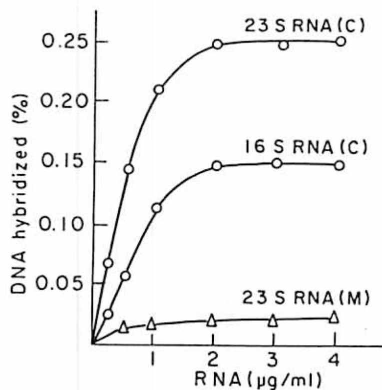

FIG. 1. Hybrid DNA–ribosomal RNA. [32P]ribosomal RNA (23S and 16S) was isolated from normal and mutant bacteria grown overnight in synthetic medium in the presence of neutralized [32P]H_3PO_4 at 30°C. RNA was isolated from washed ribosomes by the phenol method and separated as described (20). Denatured mutant DNA, 5 µg (isolated as described in Table 2), was immobilized on filters (Schleicher and Schull, Bact T-Filex, type B_6) and incubated with 32P-labeled rRNA (600–800 cpm/µg) at 66°C in 4 × SSC solution (0.60 M NaCl–0.06 M sodium citrate) for 6 hr as described (20) for DNA–mRNA hybrid formation. Wild type (*C*), mutant (*M*).

resistant to low concentrations of showdomycin (20, 21). Lack of complementarity between altered rRNA and mutant DNA (or DNA of wild type) is shown by hybridization experiments (Fig. 1) as was previously demonstrated for altered rapidly-labeled RNA (20). Modified 23S RNA (or 23S + 16S), labeled with 32P, hybridizes very poorly with DNA, as compared to the RNA from the parental strain (Fig. 1). Analysis of nucleotides of DNA from mutant cells did not reveal any significant change in the base composition, although the mRNA is profoundly modified (Table 2). However, this observation does not eliminate the possibility that mutant DNA was modified in its sequences of nucleotides, or in some other important physical parameters.

Protein pattern of 70S ribosomes is altered in mutant

We speculated that drastically altered RNA, either rRNA or rapidly-labeled RNA, might act as a messenger RNA with new coding potential. In that case, we expected that some proteins in the ribosomes of mutant Shor M 500 could be significantly altered. 70S ribosomes were isolated as described in the legend to Fig. 2, and their proteins analyzed by acrylamide gel electrophoresis (Fig. 2). The protein pattern of 70S ribosomal proteins in mutant cells shows important alterations when compared with that of wild type. On the densitometer tracings (Fig. 3), the most significant qualitative and quantitative differences are marked by arrows. In several reproducible trials, two bands present in the wild type (arbitrarily designated *Y* and *Z*) have practically disappeared in mutant cells, while a new band, *X*, appeared. This band has never been observed at the corresponding position in the pattern from wild-type ribosomes. It moves slower on acrylamide gel than the bands *Y* and *Z*, suggesting a change in the net charge of protein *X*, i.e., a possible modification in the amino acid composition of that particular protein. The observed patterns (qualitative and quantitative differences) of proteins in 70S ribosomes are unchanged after the ribosomes of both types of bacteria were washed with 1 M ammonium chloride. If showdomycin affected only one specific ribosomal structural gene, then the primary mutational modification in the mutant should reside in one particular protein of either the

TABLE 2. *Base composition of mRNA and DNA in wild type and mutant E. coli*

Nucleotide	Mol per 100 mol of nucleotide			
	Wild type		Mutant	
	DNA	mRNA	DNA	mRNA
A	24.5	25.0	25.0	31.3
G	24.5	27.5	24.0	34.5
C	25.0	24.5	25.6	16.8
T (U)	26.2	28.0	25.5	17.4
{G+A}/{C+T(U)}	0.97	1.00	0.96	1.93

DNA was isolated and purified as described (22). Purified DNA (1 mg) was hydrolyzed by $HClO_4$ at 100°C according to Wyatt (27). After separation of hydrolyzed products on Whatman 1 paper (isopropanol–HCl–H_2O 170:41:250), the concentration of each nucleotide was determined at 260 nm; rapidly-labeled RNA (mRNA) was labeled with 32P for 60 sec and isolated with total RNA as described (20). [32P]mRNA (1 × 10^4 cpm) contained in 1 mg of total RNA (unlabeled) was analyzed for base composition on a Dowex column. Purity of [32P]nucleotides was controlled by paper electrophoresis. Only the ratio of labeled nucleotides is reported.

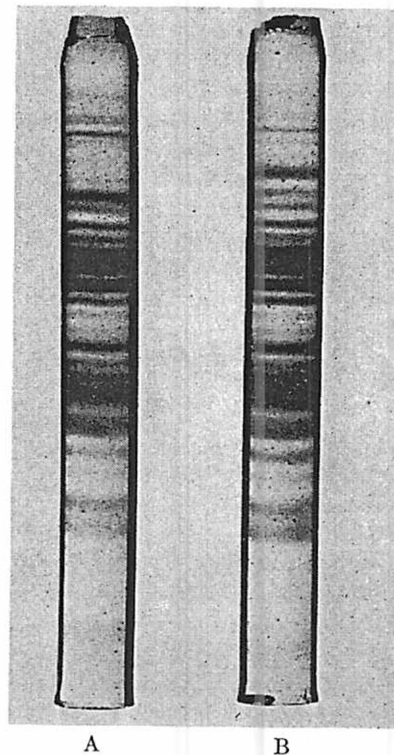

FIG. 2. Electrophoretic patterns of 70S ribosomal proteins. 70S ribosomes were isolated from freshly-grown bacteria (exponential phase). After dialysis for 16 hr against Tris·HCl buffer (0.01 M, pH 7.6) containing 10^{-2} M magnesium acetate, the ribosomes were pelleted at 105,000 × *g* for 2 hr; after an additional washing, they were pelleted again. Ribosomal proteins were extracted by acetic acid (65% final concentration) (24). Ribosomal proteins (80 µg) were separated on a column of polyacrylamide gel at pH (28). Migration was 200 min at 3 mA per tube at 4°C in 7.5% (w/v) acrylamide gel. Separated proteins were colored by amino black dye for 20 min, and the excess of dye was removed with acetic acid (7%) for 24 hr. Wild type, *A*; mutant, *B*.

50S or 30S ribosomal subunits. However, an attack of the antibiotic on several genes could not be excluded, although it seems less probable. On the other hand if, as it appears, showdomycin has acted primarily on some "dormancy" gene or genes involved in RNA synthesis, then new classes of RNA are synthesized, as we have shown (19, 20), and some alteration of proteins in both types of ribosomes can be expected.

Altered protein pattern in the 50S ribosomal subunits of the mutant

50S ribosomal subunits were separated from 30S subunits by ultracentrifugation on a sucrose gradient, as described in Fig. 4. Isolated proteins (24) were separated by acrylamide gel electrophoresis (Fig. 4). The densitometer tracings show the most important qualitative and quantitative differences between 50S ribosomal proteins of wild type and mutant. First, the band Y present in the wild-type 50S ribosomal proteins does not appear at the corresponding position in the pattern of mutant ribosomes. In contrast, the band X, which regularly appeared in the mutant 50S ribosomes, has never been observed at the corresponding position in the pattern of wild type. Second, quantitative differences between several bands (see arrows) were repeatedly observed between wild type and mutant. These differences may be of importance if the corresponding proteins participate in the organization of the "catalytic" center of 50S ribosomal subunits; this center is organized by the interaction of several proteins and is required for peptide bond formation in protein synthesis (25). Reconstitution experiments should assist in defining the "catalytic" center of 50S ribosomal subunits in the mutant Shor M 500.

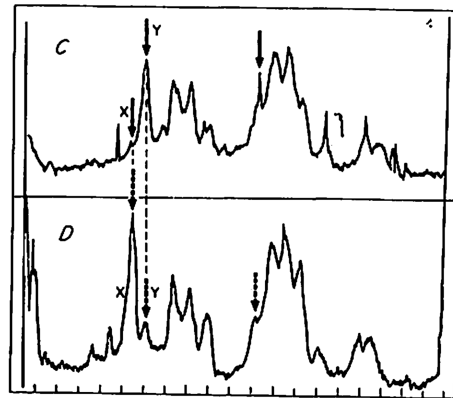

FIG. 4. 50S ribosomal protein patterns. 50S ribosomal subunits were separated by ultracentrifugation on sucrose gradients (5–20%) of 70S ribosomes dialyzed against Tris buffer (0.01 M, pH 7.6) containing 10^{-4} M magnesium acetate. Separation for 16 hr at 16,000 × g in a Spinco SW25 rotor. Collected fractions containing 50S ribosomal subunits, and those containing 30S ribosomal subunits, were separately treated with ammonium sulfate at 40% saturation; the pellet was collected by centrifugation and dialyzed against Tris buffer for 16 hr. Proteins were separated from RNA by acetic acid (24), dialyzed against distilled water, separated (90 μg) by acrylamide gel electrophoresis, and treated with amido black dye as described in Fig. 2. Densitometer tracings in the Cary Spectrophotometer show the protein patterns of wild type, C, and of mutant, D.

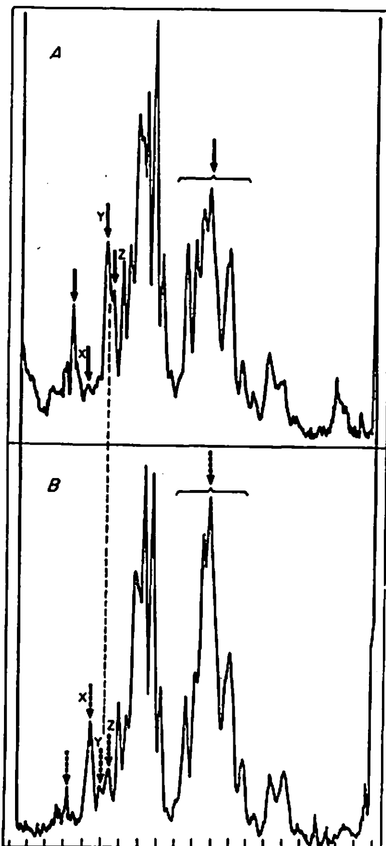

FIG. 3. Densitometer tracings of 70S ribosomal proteins, from the gels pictured in Fig. 2, at 570 nm in the Cary spectrophotometer (20). Arrows mark qualitative and quantitative differences between 70S ribosomal proteins of wild type, A, and mutant, B.

Altered protein pattern in the 30S ribosomal subunits of the mutant

30S ribosomal subunits were separated from 50S subunits as described in Fig. 4. The presence of drastically modified 16S RNA in 30S ribosomal subunits suggested that their proteins could also be modified. Densitometer tracings (Fig. 5) show

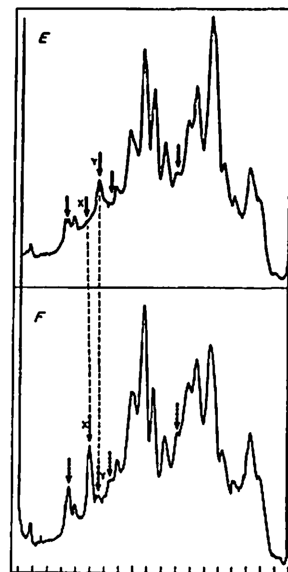

FIG. 5. 30S ribosomal protein patterns. 30S ribosomal subunits were separated from 50S subunits as described in Fig. 3. Proteins (60 μg) were separated by acrylamide gel electrophoresis and treated with amido black dye as described in Fig. 2. Densitometer tracings: wild type, E, and mutant, F.

TABLE 3. *Effect of mRNA on the activity of ribosomes from wild type and mutant*

	cpm × 10^{-3} per 100 μg of ribosomal protein							
	Wild type				Mutant			
[14C]Amino acids	Nil	Poly(U)	Poly(C)	TYMV RNA	Nil	Poly(U)	Poly(C)	TYMV RNA
Phe	6.0	61.0	—	11.8	5.5	148.0	—	11.2
Leu	8.0	9.5	—	—	8.0	10.6	—	—
Ile	1.7	2.6	—	—	1.6	3.4	—	—
Pro	1.7	—	2.9	3.2	3.2	—	4.2	5.8

For *in vitro* amino acid incorporation, the activity of ribosomes isolated from exponentially growing bacteria was assayed in a volume of 0.3 ml of 50 mM Tris·HCl (pH 7.65); 15 mM $MgCl_2$; 2 mM ATP; 1 mM each of GTP, CTP, UTP; 10 mM phosphoenol pyruvate (Na-K); 5 μg of pyruvate kinase; 1 mM mercaptoethanol; 0.2 mg of total tRNA; 20 μg of poly(U); [14C]L-phenylalanine (or other [14C]-amino acid) 10 nmol (2 × 10^6 cpm); 0.1 mg of 70S ribosomes (protein); 0.3 mg of dialyzed 105,000 × *g* supernatant (either from wild type or from mutant cells). Incubation was at 34°C for 10 min. The reaction was stopped with 1 ml of 10% trichloroacetic acid and the mixture was heated for 15 min at 100°C. The precipitate was filtered on a Millipore filter, washed with cold trichloroacetic acid, dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

clearly a profound change in the position and intensity of several proteins in mutant bacteria.

The band *Y* present in the wild type has almost completely disappeared from 30S subunits of mutant cells. Conversely, a new protein (band *X*) constantly appeared in ribosomes of the mutant strain; it has never been observed in the 30S proteins of wild type. Although there is no question about the individual existence of bands *Y* and *X*, one cannot exclude the possibility that in mutant cells the protein *Y* was greatly altered (amino acid composition) such that it moves in acrylamide gels at the position of protein *X*.

In vitro activity of 70S ribosomes from wild-type and mutant cells

The normal growth (20) of showdomycin-resistant mutants indicated that their ribosomes could be as active as ribosomes from the wild-type strain. In fact, the incorporation of different [14C]amino acids (Table 3) shows that isolated ribosomes from mutant bacteria usually exhibit an increased capacity for polyphenylalanine synthesis in the presence of poly(U) as compared with control ribosomes. No significant difference was observed for [14C]leucine, [14C]isoleucine, and [14C]proline incorporation. In the presence of a natural "messenger RNA" (turnip yellow mosaic virus (TYMV) RNA) no difference was observed between control and mutant ribosomes for [14C]-valine and [14C]phenylalanine incorporation, although TYMV RNA does increase significantly the incorporation of these two amino acids into protein.

EVALUATION OF THE RESULTS

Ribosomes in the mutant Shor M 500 of *E. coli* contain greatly altered rRNAs (23S, 16S, and 5S) and proteins of the 50S and 30S ribosomal subunits. rRNAs in which purines are in excess over pyrimidines hybridize poorly with DNA of *E. coli*. These facts suggest that in mutant cells, DNA-dependent RNA polymerase does not transcribe some segments of DNA that are normally transcribed in the wild-type strain. Three plausible explanations can be proposed. First, in the mutant, very small segments of DNA that are usually not copied could be rapidly transcribed, and we may postulate that transcription products (rRNA) with particular chemical and physical properties (20) do not hybridize *in vitro* with DNA. This does not appear likely since we have shown previously that even altered rapidly-labeled RNA, synthesized at a normal rate in mutant of *E. coli* resistant to low concentrations of showdomycin, hybridizes very poorly with homologous DNA (20). A second explanation could be that an enzyme like polynucleotide phosphorylase that is altered in mutant cells is capable, under given physiological conditions *in vivo*, to replace the DNA-dependent RNA polymerase, thus creating new functional species of RNA that permit the cells to survive. It should be pointed out that the polynucleotide phosphorylase from mutant cells synthesizes *in vitro* polynucleotides in which purines are in excess over pyrimidines ($G + A/C + U \neq 2.0$). In addition, the activity *in vitro* of DNA-dependent RNA polymerase of mutant bacteria is very much reduced compared with that isolated from wild type (20). A third possibility is the existence in mutant bacteria of an enzyme which leads to the appearance of some kind of functional "RNA" genome or episome apparently independent of DNA. In any case, new and altered RNA must act as messenger RNA since we find that the mutant multiplies normally, that several enzymes studied in mutant bacteria have new properties (ref. 20 and manuscript in preparation), and that ribosomal proteins are also altered. In fact, acrylamide gel electrophoresis of ribosomal proteins shows that in both the 50S and the 30S ribosomal subunits the protein patterns clearly differ between wild type and mutant. The same is true for proteins of 70S ribosomes. Disappearance of proteins *Y* and *Z* in mutant 70S ribosomes, and the appearance of a new protein *X*, are the consequence of the presence of modified "messenger RNA" which could possess new coding properties. Qualitative and quantitative changes in ribosomal proteins of mutant bacteria do not lead to less-active ribosomes, either *in vivo* (mutant cells grow as well as the wild strain [20]) or *in vitro*.

The mutation that leads to resistance to showdomycin in *E. coli* did not lead to less active "messenger RNA" nor less active ribosomes. One would expect that a mutation of almost all of the RNAs of *E. coli* and of ribosomal proteins, as is the case with mutant Shor M 500, would be lethal.

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