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Properties of Ribosomes from Erythromycin Resistant Mutants of *Escherichia coli*

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Summary. We have studied the in vitro properties of ribosomes from several mutants resistant to erythromycin. Mutations in three different genes may confer resistance to erythromycin. Two of them are structural genes for proteins L4 and L22 of the large subunit. The third mutation (in *eryC* gene) seems to affect mainly the small subunit. The mechanism of action of the antibiotic may involve both subunits.

Introduction

The antibiotic erythromycin inhibits protein synthesis in different species of bacteria (Oleinick, 1975). The target of this drug is the ribosome, probably the large subunit (Oleinick, 1975).

In *Escherichia coli*, mutations in at least three genes may confer resistance to this antibiotic. These genes are *rplD*, *rplV* and *eryC* (Wittmann et al., 1973; Pardo and Rosset, 1977; Bachman, 1976).

In a previous paper, we reported the behaviour of different classes of ery^R mutants isolated as spontaneous mutants (Pardo and Rosset, 1974). Now we describe the in vitro analysis of ribosomes from four of these ribosomal mutants. We analyzed different aspects of the structure and functional properties of these ribosomes:

- binding of ^{14}C-erythromycin
- interaction of the two subunits
- initiation step formation
- peptide bond formation.

The results presented here suggest that the mechanism of action of erythromycin involves both subunits since resistance can be conferred to ribosomes by alterations in either the 50S (*rplD* and *rplV*) or the 30S (*eryC*) subunit.

Material and Methods

1. Bacterial Strains

Strains used are listed in Table 1. They were previously described (Pardo and Rosset, 1974) and are spontaneous mutants. Mutations conferring erythromycin resistance have pleiotropic effects: in addition to resistance to the drug the strains are temperature-sensitive. They were located either around minute 72 on the *E. coli* chromosome (Pardo and Rosset, 1974) in strains 345-05, 345-20, 345-09 or around minute 85 in strain 345-08 (Pardo and Rosset, 1977). Erythromycin resistant mutants are defined as being resistant to 500 μg/ml of the antibiotic.

2. In vitro Assay of the Associativity of Subunits

was carried out following the technique of Debey et al. (1975). Equilibrium association curves were followed by the turbidimetric technique described by Debey et al. (1975). An Aminco DW2 spectrophotometer was used in dual mode; the measurement beam was fixed at $\lambda I = 340$ nm and the reference beam at 500 nm. Ribosomal solutions were incubated at 37° C for 15 min before use. The assay begins with the introduction of ribosomal solution in cacodylate buffer 50 mM pH 7, NH_4Cl 60 mM where the ribosomal subunits are dissociated. The magnesium concentration is then increased by steps from 2 mM to 30 mM. The relative turbidimetry change at 20 mM magnesium concentration of the parental strain 345 was taken as reference of fully associated subunits. The ribosomal concentration was 7 A_{260}/ml. The ribosomes used in this assay

Table 1. Strains used

Strain	Genotype	Phenotype[a]	Origin
AT2472	*aroE24* B_1		L. Gorini
345	as AT2472 but *rpsE345*		our lab
345-05	as 345 but *rplD05*	ts	,,
345-08	as 345 but *eryC*	cs	,,
345-09	as 345 but *rplV09*	cs	,,
345-20	as 345 but *rplD20*	cs ts	,,

[a] cs and ts mean cold-sensitive or thermo-sensitive. We define cold-sensitivity or thermosensitivity as the inability of strain to grow at 20° C or 42° C respectively

were prepared according the technique described by Kurland (1966).

3. Binding of ^{14}C-erythromycin in vitro

was done as described by Pestka (1974). KCl concentration is 50 mM in all assays.

4. In vitro Assay of Ribosome Activity

a) Preparation of Ribosomes and S150 Supernatant. 6 g of cells were resuspended in 10 ml of TMNSH buffer (Tris pH 7.5 10 mM, $MgCl_2$ 10 mM, NH_4Cl 60 mM, β-mercaptoethanol 6 mM) and homogeneized in a French pressure cell at 12,000 psi. Unbroken cells and debris were eliminated at 10,000 g for 10 min. The supernatant was centrifuged at 30,000 g for 30 min and the upper two thirds of this second supernatant were centrifuged at 150,000 g for 3 h. The upper two thirds of this supernatant (S150) were dialyzed against TMNSH buffer and stored at −70° C. The protein concentration of this supernatant which contains enzymes necessary for protein synthesis was determined with the Folin technique with Bovin serum albumin as standard. The ribosomal pellet was washed with TMNSH, resuspended in the same buffer and centrifuged once again under the same conditions. The resulting pellet was resuspended in 5 ml of buffer TIII (10 mM Tris pH 7.5, 1 M NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol) and gently stirred overnight at 4° C. The suspension was centrifuged at 150.000 g for 4 h, the pellet was resuspended in TMNSH buffer and dialyzed for 10 h against this buffer. Ribosomes were divided in aliquots and stored at −70° C.

b) In vitro Assay of Polypeptide Synthesis. We used the technique described by Traub et al. (1971). The same S150 extract (from parental strain 345) was used in all experiments.

5. In vitro Assay of Initiation Step and in vitro Assay of Peptide Bound Formation

We used the technique of Leder and Bursztyn (1966) with (^{3}H)fMet-tRNA instead of (^{14}C)fMet-tRNA.

6. Two-Dimensional Polyacrylamide Gel Electrophoresis

We used the method of Kaltschmidt and Wittmann (1970). The ribosomes used were prepared by the technique described by Kurland (1966). Ribosomal proteins were extracted with acetic acid (Hardy et al., 1969).

7. Material Used

(^{3}H)fMet-tRNA and crude initiation factors (I.F.) were given by Monique Graffe and Jacques Dondon; ^{14}C-erythromycin was a gift of Dr. S. Pestka.

8. Expression of the Results

When activities of ribosomes were tested, five experiments were performed for each assay, each with the same result. In each experiment we used 5 to 7 A_{260} of ribosomes. The results are expressed as activity for 1 UA_{260} of ribosomes. In all experiments blanks, without ribosome, were substracted.

Results

Analysis of Ribosomal Proteins

We have analyzed ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis. Protein L4 is altered in mutants 345-05 and 345-20 (Fig. 1). In Figure 2 gels of proteins from parental and mutant strains (345 and 345-09) are shown. As can be seen, L22 is altered in the mutant. The characterization of the components altered allows us to identify 345-05 and 345-20 as *rplD* mutants and 345-09 as an *rplV* mutant in accordance with previous results (Pardo and Rosset, 1974). Furthermore, in strain 345-09 the genetic background of strain AT2472 does not mask

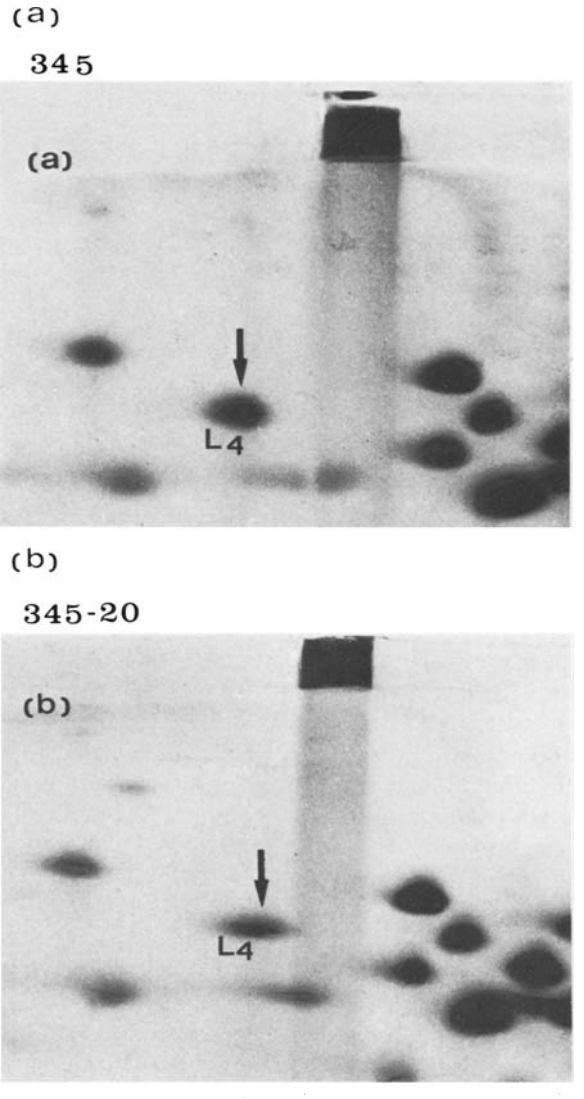

Fig. 1a and b. Two-dimensional gel electrophoresis of ribosomal proteins TP70S from **a** parental strain 345 and **b** mutant 345-20. Only the portions of gel containing L4 are shown

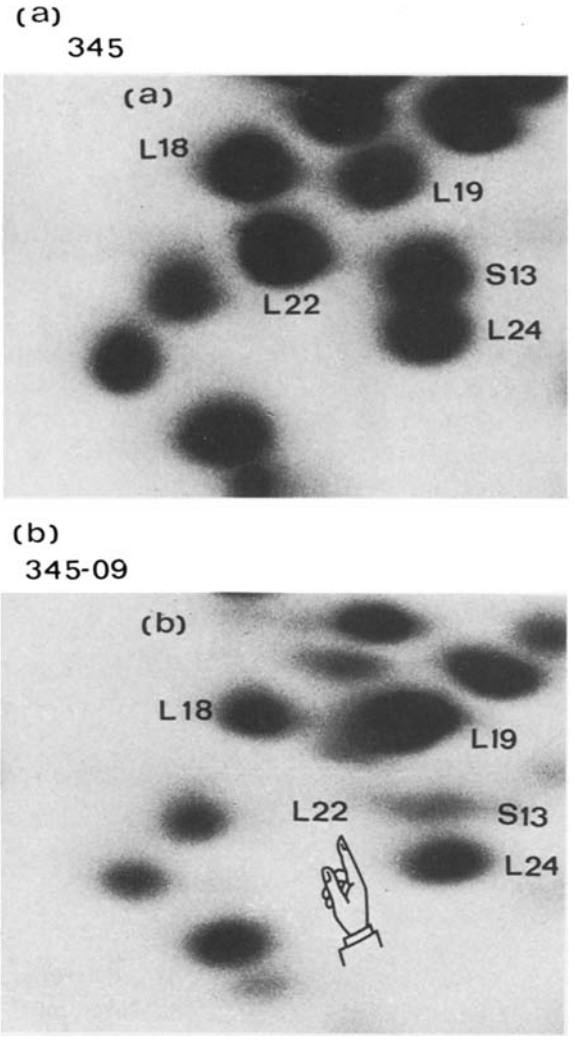

Fig. 2a and b. Two dimensional gel electrophoresis of ribosomal proteins TP70S from **a** parental strain 345 and **b** mutant 345-09. Only the portions of gel containing L22 are shown

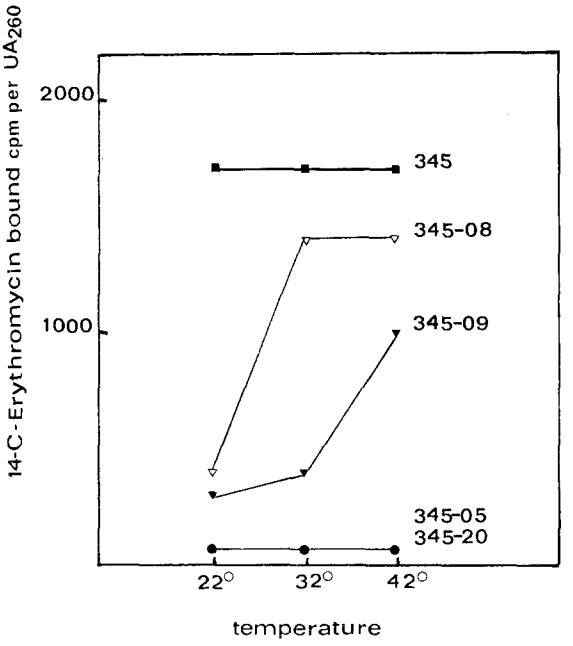

Fig. 3. Binding of ^{14}C-erythromycin in vitro

Table 2. Association of ribosomal subunits in vitro

Strains used	Association		Requirement of Mg^{++}
	at 22° C	at 42° C	
345	100%	100%	20 mM
345-05	100%	85%	20 mM
345-20	100%	100%	20 mM
345-08	0%	100%	30 mM
345-09	0%	100%	30 mM

the erythromycin resistance bound to an altered L22 protein (Wittmann et al., 1973). In the conditions used patterns concerning ribosomal proteins from 345-08 and 345 were similar. We cannot therefore unambiguously identify the product of gene *ery*C.

Binding of ^{14}C-erythromycin

Figure 3 shows that we can distinguish two groups of erythromycin resistant mutants. Ribosomes isolated from 345-05 or 345-20 do not bind erythromycin. Ribosomes isolated from 345-09 and 345-08 cannot bind the antibiotic at 22° C but can do it at 32° C and 42° C. Although the binding level of the parental strain 345 is never reached we can state that ribosomes from 345-08 and 345-09 bind erythromycin at the permissive temperature. The behaviour of these ribosomes at 22° C and 42° C is significantly different. This means that the conformation of the ribosomes from 345-08 and 345-09 is altered at 22° C in vitro. We can assume that this temperature is also critical in vitro because strains 345-08 and 345-09 are cold-sensitive.

Interaction of the Two Ribosomal Subunits

We tested the associativity of the two ribosomal subunits with the technique described by Debey (1975). Two parameters were varied: the temperature and the concentration of magnesium. The results summarized in Table 2 show again that the groups of ribosomes can be distinguished. Ribosomes from 345-

Table 3. In vitro assay of ribosome activity

Incorporation of ^{14}C-Phe cpm per 1 A_{260} of ribosome

Temperature	Strains used			
	345	345-09	345-05	345-08
22° C	500	225	300	100
32° C	800	750	800	1300
42° C	1300	1000	1100	1300

Table 4. In vitro assay of initiation step

cpm of F-met tRNA bound per 1 A_{260} of ribosome

	Strains used				
	345	345-09	345-05	345-20	345-08
cpm	2000	1900	2100	2100	220

Table 5. In vitro assay of peptide-bond formation

cpm of F-met-puromycin per 1 A_{260} of ribosome

Temperature	Strains used				
	345	345-09	345-05	345-20	345-08
22° C	700	440	150	1000	250
32° C	1150	1200	900	700	700
42° C	1160	1500	700	470	700

05 and 345-20 have the same behaviour as the ribosomes from the parental strain 345; i.e. at 20 mM of magnesium they form 70S complexes at 22° C and 42° C. In contrast, ribosomes from strains 345-08 and 345-09 cannot form 70S at 22° C and require 30 mM of magnesium for association at 42° C.

In vitro Activity

We chose to study two fundamental activities of the translational process in vitro: initiation and translocation. In addition, we have tested the overall activity of the ribosome as can be seen on Table 3. These results indicate that at 22° C the ribosomes from mutants are less active than parental ribosomes. The difference is marginal except for ribosomes from strain 345-08 which are five-fold less active at 22° C than parental ribosomes.

In vitro Assay of Initiation Step

As can be seen in Table 4, ribosomes from erythromycin resistant mutants with altered L4 or L22 proteins and from the parental strain bind similar amounts (^{3}H)fMet-rRNA. However, ribosomes from mutant 345-08 have a very low activity in this test, only 10% of the parental activity. Since this initiation step is considered to occur on the small subunit we can conclude that the activity of the 30S subunit is affected in this mutant.

In vitro Assay of Peptide Bond Formation

The results of this assay are reported in Table 5. The first remark concerns the ribosomes from 345-09 which have higher activity than the parental ribosomes. The activity tested increases with the temperature for the ribosomes from strains 345, 345-09 and 345-08. The low activity at 22° C for 345-08 is related to the defective initiation step so we cannot state that the elongation step is particularly affected in this mutant.

The second observation concerns the ribosomes from 345-05 and 345-20. The elongation step seems to be altered in these strains whose L4 protein is altered. The activity is defective at 22° C and 42° C for 345-05 ribosomes and decreases with temperature for 345-20 ribosomes.

Conclusion

Different mutants isolated as erythromycin resistant display altered ribosomal properties (Table 6). We confirm that a defect in ribosomal protein L4 which leads to resistance can be correlated with a decrease in the activity of the peptidyl transferase (Tables 5, 6, Wittmann et al., 1973). At the same time the optimum in NH_4^+ concentration required for ribosome activity is increased (Pardo and Rosset, 1974; Otaka et al., 1970). So, these properties can be used to characterize mutants of this type.

A mutant altered in protein L22, strain 345-09, shows a cold-sensitive phenotype in vivo which is correlated with severely affected activities of ribo-

Table 6. Summary of the different parameters used to defined the *ery*^{R} mutants studied

Strain	Gene affected	Map location	Component altered	Binding of erythromycin	Association	Initiation step	Translocation step
345-05	*rplD*	72′	L4	0	+	+	0
345-20	*rplD*	72′	L4	0	+	+	0
345-09	*rplV*	72′	L22	+	0	+	+
345-08	*eryC*	85′	?	+	0	0	

somes at the non-permissive temperature in vitro (Fig. 3, Table 3). At the permissive temperature ribosomes show no special characteristics except an increased requirement for magnesium to obtain complete association of subunits (Table 2).

The third type of mutant presented here is different from the previous two. 345-08 is cold-sensitive in vivo and ribosome activity in vitro is impaired at 22° C, but one can notice that the binding of fMet-tRNA, a property of the small subunit, is drastically reduced (Table 4). Furthermore we have not be able to detect abnormality at the level of the 50S subunit. Although an alteration of the large subunit cannot be totally excluded at this point it seems therefore that the small ribosomal subunit, in the mutant 345-08, may be implicated in the mechanism leading to erythromycin resistance.

Mutants of the first type, affected in L4, have lost the property to bind erythromycin. This inability can be considered as the origin of resistance. For mutants of the two other types however resistance cannot be explained on these grounds.

Our previous in vitro results (Pardo and Rosset, 1974) have clearly shown that resistance to the drug in 345-09 is an intrinsic property of the ribosome. Therefore, the resistance does not necessarily involve an external component (Wittmann et al., 1973).

The binding test used in the present studies detects only large differences in the affinity of the ribosome for the drug. Ribosomes from 345-09 and 345-08 have markedly cold-sensitive activities (Table 3). At 22° C, ribosomes from 345-09 and 345-08 do not bind the drug: this is in agreement with the idea that their structures are grossly affected. At the permissive temperature, binding of erythromycin is restored but at a lower level than in the parent. It could, in our opinion, suggest a slightly reduced affinity of these ribosomes for the drug. Such a situation has been carefully studied by Oleinick and Corcoran (1969).

An alternative (but not exclusive) possibility would be that the binding of the erythromycin to wild-type ribosomes from *Escherichia coli* blocks a specific reaction which is the actual target of the drug. A structural alteration of the ribosome (in resistant strains like 345-09 or 345-08) would act by preventing this inhibition without preventing binding of the antibiotic.

Both hypotheses can explain the existence of various types of ery^R mutants. Recently it has been shown that the inability to bind erythromycin to 50S subunits from an ery^R strain can be reversed by 30S subunits with S5 and S12 proteins altered (Saltzmann and Apirion, 1976). This result shows an intimate relationship between 30S and 50S ribosomal elements in the function and the structure of the ribosome.

Therefore, occurrence of a mutant like 345-08, where the major defect seems to be in the 30S subunit, could point to the participation of both subunits in the mechanism of action of erythromycin.

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