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Biochemical and Genetic Studies on Two Different Types of Erythromycin Resistant Mutants of *Escherichia coli* with Altered Ribosomal Proteins

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Summary. Ribosomes from nine *E. coli* mutants with high level resistance to the antibiotic erythromycin were isolated and their proteins were compared with those of the parental strains by two-dimensional polyacrylamide gel electrophoresis, by carboxymethylcellulose column chromatography and by immunological techniques. Two 50S proteins were found to be altered in the mutants: either L 4 or L 22.

Ribosomes with an altered L4 protein bound erythromycin rather poorly and the formation of N-acetylphenylalanyl puromycin was drastically decreased. On the other hand ribosomes with an altered L22 protein bound erythromycin as efficiently as wild type ribosomes and their puromycin reaction was at least as good as that of wild type ribosomes.

Transduction experiments showed that the mutations affecting both proteins, L4 and L22, are located very close to the *str* and *spc* genes, nearer to the *spc* than to *str* gene.

Introduction

E. coli is quite resistant to the macrolide antibiotic erythromycin (Hahn, 1967). Sensitive (*lir*) as well as high level resistant (*ery*) mutants were isolated from *E. coli* K12 strains and both types of changes could be due to ribosomal alterations (Apirion, 1967, Krembel and Apirion, 1968). Studies with *E. coli* mutants resistant to high levels of erythromycin suggested that a 50S ribosomal protein (50-8 which corresponds to L4 in the nomenclature of Kaltschmidt and Wittmann, 1970a), is altered in such mutants and that the gene for this protein is closely linked to the *str* (streptomycin) gene (Otaka *et al.*, 1970; Takata *et al.*, 1970).

The studies reported here suggest that two 50S proteins, either L4 or L22, can be altered in mutants resistant to high levels of erythromycin and that the mutations causing these alterations are in two very closely linked genes both of which are located in the *str* region of the *E. coli* chromosome.

* Paper No. 61 on "Ribosomal Proteins". Preceding paper is by Hasenbank *et al.*, Molec. gen. Genet., **127**, 1—18 (1973).

Material and Methods

Strains used in this study are listed in Table 1. Mutants N281–N287 were derived from *E. coli* K12 strain AB301 and mutants N288–N289 from strain N314. They are spontaneous *ery* mutants resistant to high level (at least 200 μg/ml) of erythromycin. For the phenotypic characterization of mutants N281, N282, and N288 see Apirion (1967). The other mutants are very similar to the three mentioned above. As comparison, *ery* mutants of the QE series (QE004, QE107, and QE201; see Otaka *et al.*, 1970) were used in some experiments.

Two-Dimensional Polyacrylamide Gel Electrophoresis was done according to Kaltschmidt and Wittmann (1970b). Ribosomes isolated by differential centrifugation and washed once with 0.5 ammonium chloride were employed in the experiments.

Carboxymethyl-Cellulose Column Chromatography of labelled ribosomal proteins was essentially according to Osawa *et al.* (1969).

Peptide Analyses of the tryptic digests of labelled ribosomal proteins were performed on a column of Dowex 50 according to Otaka *et al.* (1971).

Immunology. The preparation of antisera against individual ribosomal proteins of *E. coli* and the Ouchterlony double diffusion experiments were described by Stöffler and Wittmann

Table 1. Strains used

Strain	Genotype	Phenotype with respect to ribosomal antibiotics			Ref. or origin
		Ery	Spc	Str	
AB301	*met*$_7$	R	S	S	Apirion (1966)
D10	*met*$_7$ *rns*10	R	S	S	Gesteland (1966)
A19	*met*$_7$ *aux rns*19	R	S	S	Gesteland (1966)
Q13	*ts*452 *met*$_7$ *aux rns*19 *pnp*13 *tyr*451	S	S	S	Weatherford *et al.* (1972)
N21	*met*$_7$ *str*21	R	S	R	sp. from AB301
N54	*met*$_7$ *str*21 *spc*54	R	R	R	sp. from N21
N314	*lac*1 *xyl thi gal*2 *arg*3 *his*4 *ilv*158 *pro*2 *str*314	R	S	R	sp. from AB774 (Apirion, 1967)
N485	*aro*E *spc*485	R	R	S	sp. from AT2472
N486	*aro*E *spc*485 *met*1 *rns*10	R	R	S	Transduction: N485 donor; D10 recipient
AT2472	*Hfr* H *aro*E *B1*	S	S	S	Brooks Low, Yale Univ.
WH107	*met lp*	S or R	R	R	Dekio and Takata (1969)
SH108	as WH107	S or R	R	R	Dekio and Takata (1969)
JCS PCO2	*F*$^-$ *leu*2 *his*1 *arg*6 *met*1 *lac*2,4 *mal*1 λ^r *xyl mtl*	S or R	S	S	N.G. induced from JC411
S4354	*F*$^-$ *met*	S or R	S	S	
C600	*F*$^-$ *su*II *thr leu B1 lac*	S or R	S	S	
BE11OT	*Hfr*C try	S or R	S	S	N.G. induced from BE110
AB2826	*F*$^-$ *aro*B	S or R	S	S	

sp: spontaneous mutant; N.G.: nitrosoguanidine; For designation with respect to erythromycin see Table 2.

(1971) and the quantitative immuno-precipitation methods by Stöffler, Tischendorf, Hasenbank and Wittmann (1973).

Binding of Erythromycin and Puromycin Reaction. The ribosomes were prepared from the cells harvested at early log phase of growth and washed three times with NH_4Cl according to Pestka (1968). C^{14}-Erythromycin was prepared by short-term incubation method according to Kaneda *et al.* (1962) The binding of C^{14}-erythromycin to ribosomes was measured as described by Teraoka (1970). Ac-C^{14}-Phe-tRNA was prepared by the method of Lapidot *et al.* (1967). The formation of Ac-C^{14}-Phe-puromycin was assayed as previously described (Teraoka and Tanaka, 1971).

Genetic Analysis was carried out according to Lennette and Apirion (1971). In order to select for Str-R or Spc-R transductants, infected recipient cells were plated, incubated at 37° for 4–6 hours on enriched medium and then covered with 3 ml of soft agar containing 2000 μg streptomycin or spectinomycin per ml. Selection of Ery-HR transductants is rather difficult. However, a number of successful experiments were carried out when erythromycin (400 μg/ml) was added to the soft agar of plates that were preincubated for 4 h at 37°. Transduction experiments done at Hiroshima were essentially according to Takata *et al.* (1970). The following concentrations of antibiotics were used. Streptomycin: 100 μg/ml; spectinomycin: 200 μg/ml; erythromycin: 500 μg/ml unless otherwise stated.

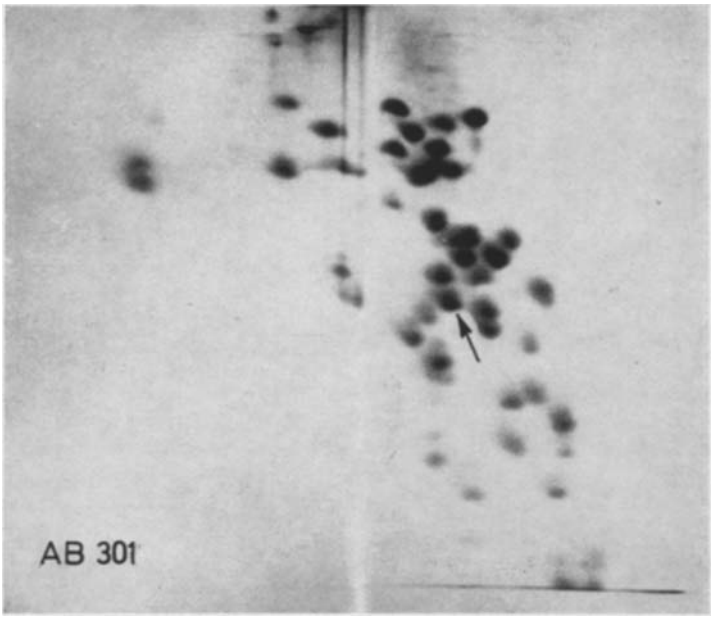
Results

Altered Ribosomal Proteins. Ribosomal proteins from mutants N281 to N289 were analyzed by two-dimensional polyacrylamide gel electrophoresis. In two of these mutants, N281 and N289, a clear change in the 50S protein L22 was detected. The changes in both mutants are probably identical as they lead to identical positions of the altered L22 proteins on the two-dimensional map of ribosomal proteins. In Fig. 1 the ribosomal proteins of N281 are compared by two-dimensional gel electrophoresis with those of the parental strain AB 301. From this comparison it can be concluded that the altered L22 protein has a more negative net charge than protein L22 from the parental strain.

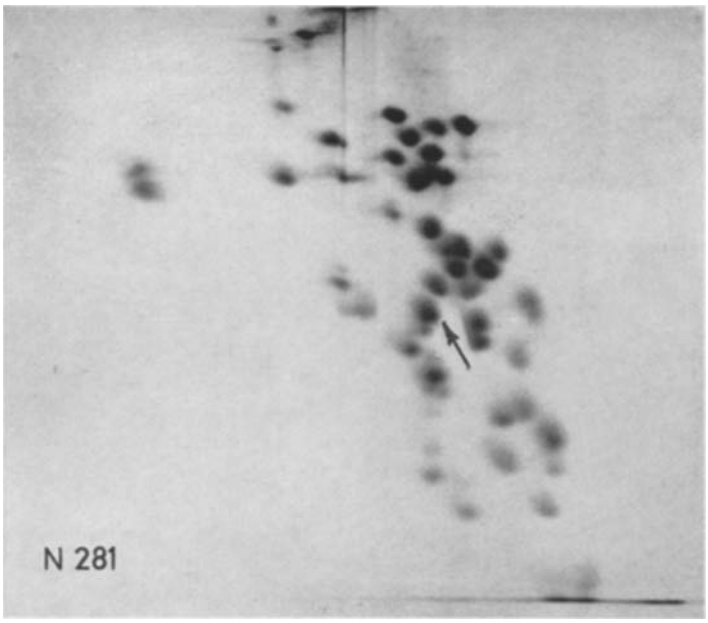
The ribosomal proteins from N281, N282, and N288 were also analyzed on carboxymethyl-cellulose columns. No altered proteins were found in mutant N288. However, as can be seen in Fig. 2, in the other two mutants (N281 and N282) altered 50S proteins were clearly discernible: Protein 50-13 (= L22) in mutant N281 and protein 50-8 (= L4) in mutant N282. The alteration in the L4 protein of N282 was very similar to alterations found in other *ery* mutants isolated from strain Q13 (Otaka *et al.*, 1970). The correlation between proteins L4 and 50-8 as well as L22 and 50-13 respectively, was carried out by two-dimensional gel electrophoresis with radioactive proteins eluted from the CM cellulose column.

As both methods, namely two-dimensional polyacrylamide gel electrophoresis and CM-cellulose column chromatography, depend on charge differences between the proteins to be compared ,immunological techniques were used by which exchanges of neutral amino acids can be detected. Double diffusion experiments and quantitative immuno-precipitation studies with antisera against protein L4 and L22 respectively were done in order to rule out as far as possible the possibility that in a given mutant, e.g. N281, not only one protein (L22) but also the other (L4) was altered in a way which could not be detected by electrophoresis and chromatography.

In double diffusion experiments antisera against protein L4 from *E. coli* K strain A19 were comparatively developed against total ribosomal proteins from A19, N281, N282 and three erythromycin resistant mutants (QE004, QE105,



a



b

Fig. 1a and b. Comparison by two-dimensional gel electrophoresis of the ribosomal patterns from *E. coli* mutant N281 and its parental strain AB301. Arrows point to protein L22

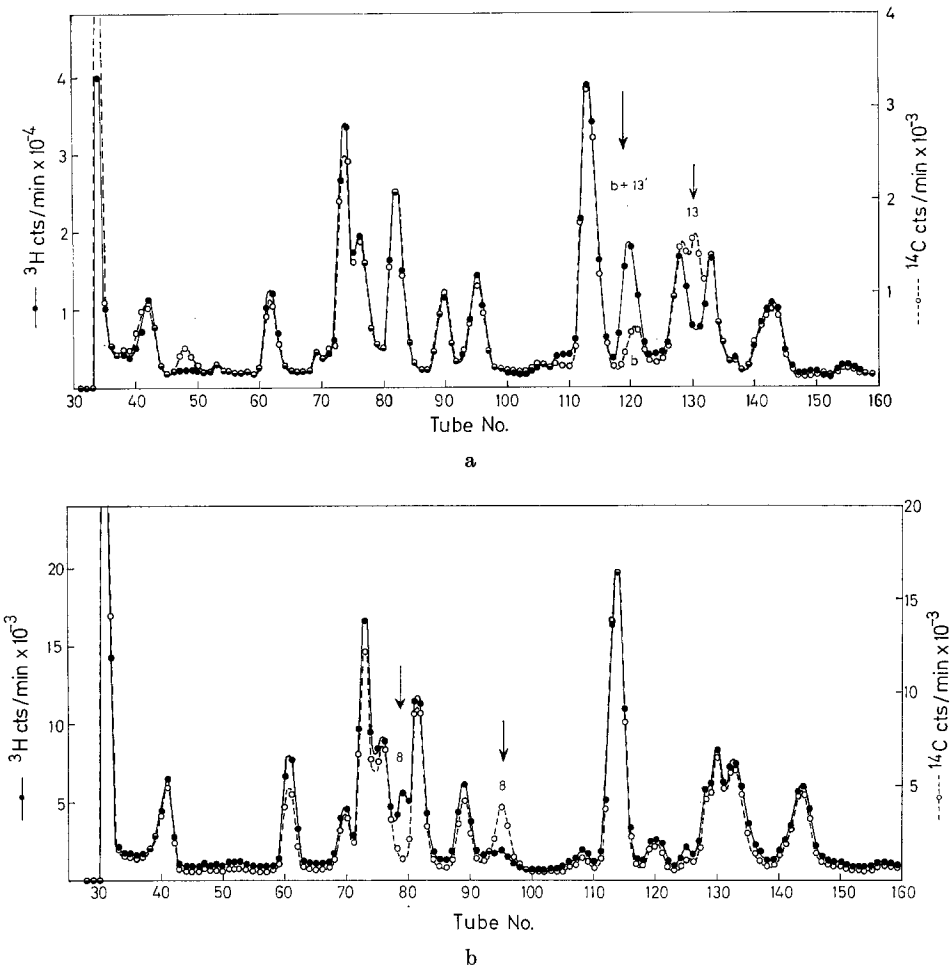


Fig. 2a and b. Chromatography on a CM-cellulose column of 50S ribosomal protein from *E. coli* Q13 and N281 (a) or N282 (b). o—o—o C^{14}-lysine-labelled Q13 protein; •—•—• H^3-lysine-labelled N281 (a) or N282 (b) protein

QE107) which have been previously shown to be altered in protein L4 (Otaka *et al.*, 1970). Spur formation was observed between A19 on one hand and N282 as well as QE004, QE105 and QE107 on the other hand. This result demonstrates that protein L4 of A19 is different from that of the four mutants. Furthermore complete cross-reaction was obtained between N282, QE004, QE105 and QE107 indicating that the amino acid exchanges in all mutants are in the same antigenic determinant.

For protein L4 complete cross-reaction, i.e. immunological identity, was detected between A19 and N281. It is therefore unlikely that mutant N281, with an electrophoretically altered L22, has an additional alteration in protein L4.

Antisera against protein L22 of A19 showed no decrease in cross-reactivity when tested against the electrophoretically altered L22 of N281. This indicates

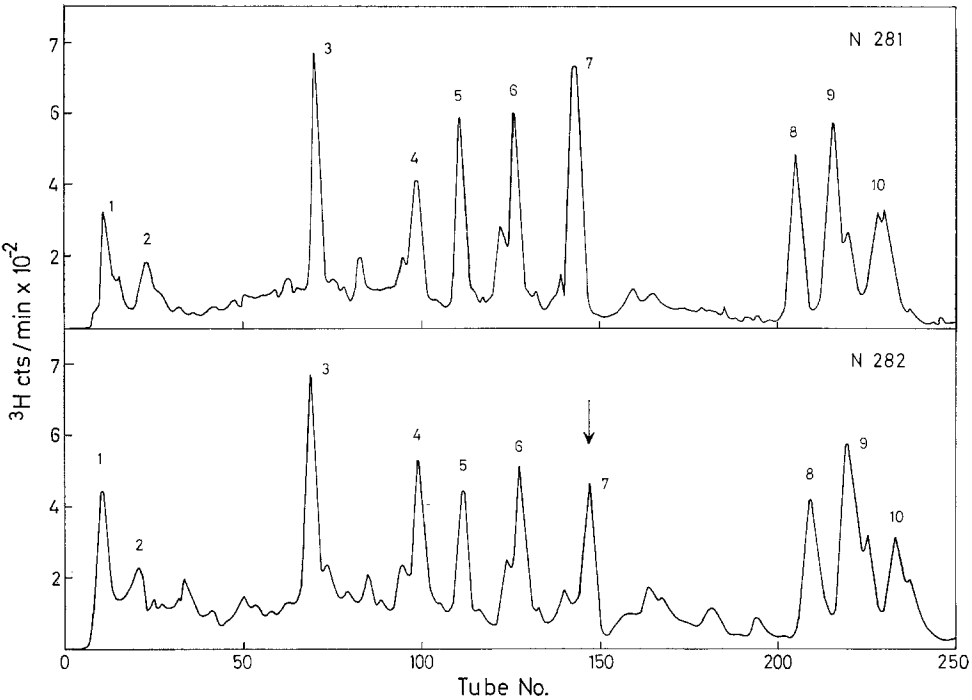


Fig. 3a and b. Chromatography on Dowex 50 columns of tryptic digests of H^3-arginine labelled 50-8 (= L4) protein of N281 (a) or N282 (b). In these experiments, proteins were not extensively purified. For the chromatogram of a similar peptide analysis on L4 protein of Q13 see Otaka *et al.* (1971). Note a decrease of No 7 peak to about half in size in N282

that the amino acid exchange is not located within the antigenic determinants recognized, and therefore immunochemical investigations as described above for anti-L4 are not possible with anti-L22.

Peptide Analyses. H^3-arginine-labelled L4 proteins from Q13, N281 and N282 were isolated. After digestion with trypsin the peptides were chromatographed on a Dowex 50 column (Fig. 3). No difference was detected between the L4 peptides of N281 and Q13. On the other hand one of the L4 peptides (No. 7) of N282 differs from that of Q13 (for the chromatogram of Q13–L4, see Otaka *et al.*, 1971). Similar analyses were performed on H^3-lysine labelled L4 proteins. No difference was observed between Q13, N281 and N282.

The observed alteration in the arginine peptides (No. 7) is very similar to the alteration found previously with other erythromycin resistant mutants (Otaka *et al.*, 1971), especially QE201, in which also peptide No. 7 was altered .This result can be taken as further evidence that the amino acid exchanges in proteins L4 from erythromycin resistant mutants are located within the same tryptic peptide.

Functional Differences among Ribosomes. As can be seen in Fig. 4, erythromycin binds as poorly to ribosomes from N282 as to ribosomes from other *ery* mutants which have altered L4 proteins (Otaka *et al.*, 1970), but it binds quite normally to ribosomes from N281.

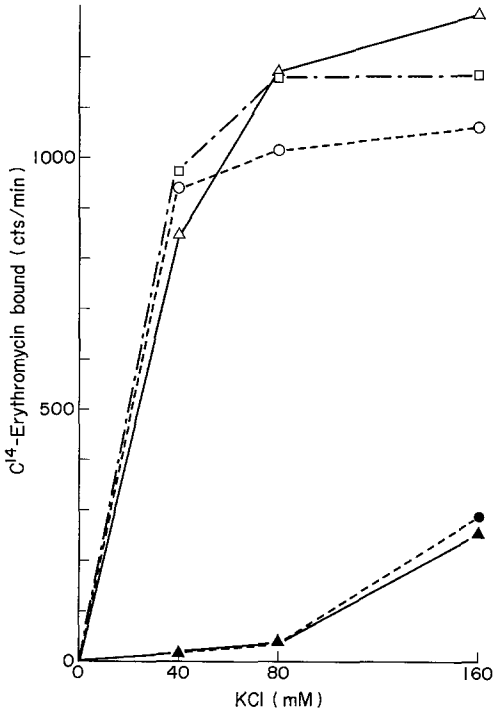


Fig. 4. Binding of C^{14}-erythromycin to ribosomes from E. coli Q13, QE201, N281, N282 and N288. The incubation mixture (125 µl) contained three A_{260} units of ribosomes, 3200 cts/min (207 µµmoles) of C^{14}-erythromycin and indicated conncentration of KCl as well as 16 mM magnesium acetate. For further experimental details see Teraoka (1970). o——o Q13 ribosomes; ▵——▵ N281 ribosomes; ▫——▫ N288 ribosomes; •——• QE201 ribosomes; ▲——▲ N282 ribosomes

When the formation of N-acetylphenylalanyl puromycin was measured (Fig. 5), ribosomes from N282 had a low ability to participate in this reaction similar to other *ery* mutants with altered L4 proteins, while ribosomes from strain N281 had an increased ability as compared with ribosomes from strain Q13.

Genetic Analysis. In transduction experiments strains N281 and N282 were used as donors or recipients. The *spc*, *str* and *aroE* alleles were selected or their segregation was followed when they were not selected. These studies suggested that the order of these genes is *str ery spc* (with both alleles *ery* 281 and *ery* 282), and that *ery* and *spc* are extremely closely linked.

Experiments with the mutants N281 (Table 2) and N282 (Table 3) are shown. The order of the genes deduced from these studies is *str ery spc*. On the other hand, when JCSPCO2 (*str*^{r} *spc*^{r}), and WH107 or WH108 (*str*^{r} *spc*^{r}) were used as the donor, *ery* of N282 was mapped in the order of *str spc ery* (data not shown). The possible reason for this discrepancy will be discussed later.

Since extensive analysis with *ery* mutants that have an altered L4 protein (Takata *et al.*, 1970) suggested that the same mutation causes the resistance to erythromycin and the altered L4 protein, we shall concentrate here on N281

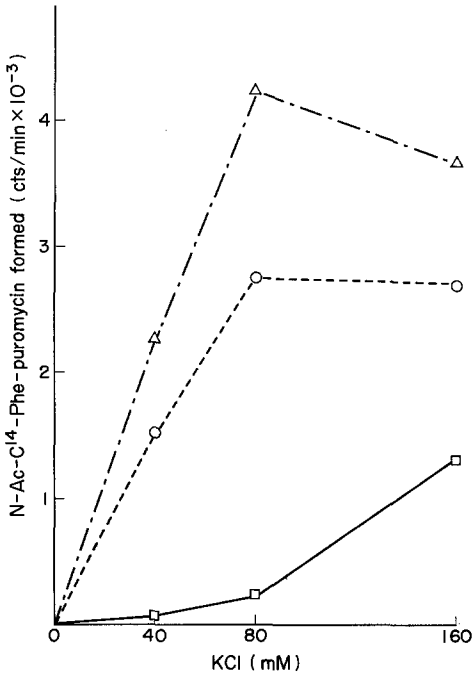


Fig. 5. N-Acetyl-C^{14}-Phe-puromycin formation by the ribosomes from E. coli Q13, N281 and N282. The reaction mixture (125 µl) contained 3.5 A_{260} units of ribosomes, 12000 cts/min (specific activity of C^{14}-Phe ≡ 455 mC/mmol) and indicated concentration of KCl as well as 16 mM magnesium acetate. For further experimental details see Teraoka and Tanaka (1971). o---o Q13 ribosomes; Δ—Δ N281 ribosomes; □——□ N282 ribosomes

which has an altered L22 protein. Twenty *ery* transductants were analyzed from crosses where N281 was the donor and selection was made for erythromycin high resistance. The recipients used were N21 (Str-R), N54 (Str-R, Spc-R) and N486 (Spc-R). Their ribosomal proteins were analyzed by two-dimensional gel electrophoresis. All twenty transductants had the modified L22 protein. From the first cross 11 transductants (7 Str-S and 4 Str-R), from the second cross 5 (3 Str-R Spc-S and 2 Str-S Spc-S) and from the third cross 4 (all Spc-S) were analyzed.

Masking the Genotype of an ery Mutant. It was already described that the erythromycin resistance of N281 having altered L22 protein was well expressed in strains N21, N54 or N486. We also examined several other *E. coli* strains in this respect. In these transduction experiments, a str^r derivative from N281 (N281S) was the donor and the respective strains (all ery^s str^s) the recipient. Str-R transductants were selected and their resistance to erythromycin was examined on a plate containing 200 and 500 µg/ml of erythromycin. It was found that 70% of Str-R transductants were resistant to either 200 or 500 µg/ml of erythromycin when W4354 was used as recipient, while about 60% of such transductants were resistant only to 200 µg/ml of erythromycin but not to 500 µg/ml when C600 or BE110T were the recipients. Furthermore no Str-R Ery-HR transductants were found in either concentration of erythromycin when AB 2826, Q13 or AT2472

Table 2

N54 Donor — *spc* + *str*

N281 Recipient — + ery_{281} +

Selection for	No. of colonies tested	Segregation of unselected markers	Percentage
Str-R	100	*Spc-R Ery-R Str-R*	47
		Spc-S Ery-HR *Str-R*	46
		Spc-S *Ery-R Str-R*	6
		Spc-R Ery-HR *Str-R*	1
Spc-R	100	*Spc-R Ery-R* Str-S	66
		Spc-R Ery-R Str-R	32
		Spc-R Ery-HR Str-S	2
		Spc-R Ery-HR *Str-R*	0
Order and Distance		*spc* — ery_{281} — *str*; ←0.87–0.98→ ←0.33–0.53→; ← 0.32–0.48 →	

The phenotypes characteristic to the donor strain found in the transductants are in italics. From the segregation of markers and the frequency of cotransduction, the order and the distance, in cotransduction frequencies, between markers are inferred.
Ery-R designates medium level of resistance to erythromycin.
Ery-HR designates high level of resistance to erythromycin.
Most *E. coli* strains used in laboratories are Ery-R. From them strains more sensitive to erythromycin can be isolated. These strains are designated Ery-S (for more details see Apirion, 1967).

were recipients (For designation of phenotypes with respect to erythromycin see Table 2).

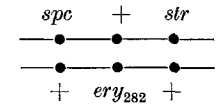
From a transduction experiment where N218 was the donor and strain AT2472 (*aroE* ery^s) the recipient, selection for Aro^+ transductants was carried out. The ribosomal proteins from five transductants all of which were sensitive to erythromycin were analyzed by CM-cellulose chromatography. Three of them carried the altered L22 protein. No such phenomenon was observed in a similar cross between AT2472 and N282 having altered L4. In this case Aro^+ Ery-HR transductants appeared and all of them had an altered L4 protein.

Discussion

The experiments presented here suggest that alteration in one of two different 50S proteins, L4 or L22, can endow an *E. coli* cell with resistance to high levels of erythromycin. The alterations are caused by mutations, probably in the structural genes that code for these proteins. These two genes are located very near the *str* and *spc* genes in the *E. coli* chromosome, nearer to the *spc* gene than to the *str* gene. The experiments reported here raise a number of questions which deserve some comments.

Table 3

N 54 Donor
N282 Recipient



Selection for	No. of colonies tested	Segregation of unselected markers	Percentage
Str-R	72	*Spc-R Ery-R Str-R*	58.3
		Spc-S Ery-HR *Str-R*	23.6
		Spc-S *Ery-R Str-R*	15.3
		Spc-R Ery-HR *Str-R*	2.8
Spc-R	150	*Spc-R Ery-R* Str-S	62.0
		Spc-R Ery-R Str-R	32.7
		Spc-R Ery-HR Str-S	5.3
		Spc-R Ery-HR *Str-R*	0.0
Order and Distance		*spc* *ery*282 *str*	
		←0.79–0.95→ ←0.35–0.74→	
		←0.33–0.61→	

In vitro Studies with Altered and Normal Ribosomes. Thus far among mutants resistant to high levels of erythromycin only two kinds of mutants with altered ribosomal proteins were observed, those with modified L4 or L22.

Ribosomes with a modified L4 protein bind erythromycin less well than ribosomes from the parental sensitive strains. A similar situation exists with streptomycin resistant mutants (Kaji and Tanaka, 1968; Ozaki *et al.*, 1969; Schreiner and Nierhaus, 1973). On the other hand the ribosomes from mutant N281 which has an altered L22 protein bind erythromycin as efficiently as ribosomes from sensitive strains (Fig. 4). Mutants of a similar type have recently described for *Bacillus subtilis* by Tanaka *et al.* (1973). These mutants are resistant to erythromycin and have an altered ribosomal 50S protein but their ribosomes bind erythromycin *in vitro* as efficiently as ribosomes from the parental type.

There is also a drastic difference between the two mutants with respect to formation of N-acetyl-phenylalanyl puromycin (Fig. 5). Ribosomes from mutant N282 with an altered L4 protein have a poor capacity to react with puromycin, whereas those from mutant N281 with an altered L22 protein react at least as efficiently as ribosomes from the erythromycin sensitive strain Q13.

Possible Mechanism of Resistance. In mutants such as *ery* 282, the ribosomes of which do not bind erythromycin, resistance to the antibiotic is probably achieved by an alteration in the ribosome that prevents adequate binding of the inhibitory antibiotic molecule.

However, when the antibiotic can bind *in vitro* to the ribosome, as in the case with mutant *ery* 281 (see Fig. 4), other alternatives should be considered. While the alteration in the ribosome may not prevent the binding of the antibiotic, it

may interfere with the effect of the antibiotic on a ribosomal active site; thus the mutation can cause an ineffective action of the antibiotic. Since ribosomal elements can interact, as has been demonstrated for several ribosomal proteins, such a possibility is not too far fetched.

Another possibility is that a mutation from erythromycin sensitivity to resistance alters the structure of the ribosomes in such a way that an unknown substance binds at or near the erythromycin binding site of the ribosome. As a consequence, erythromycin cannot bind to the mutant ribosomes *in vivo* and the cells are resistant to this drug. During the isolation and purification of the mutant ribosomes the substance is removed from the erythromycin-binding site and, therefore, the mutant ribosomes retain their full binding capability.

The finding that intact cells are resistant to erythromycin whereas there is no difference between isolated wild type and mutant ribosomes with respect to erythromycin binding, would suggest that the mutation has led to an altered membrane which prevents the transport of erythromycin into the cell. However this is unlikely because the mutation to erythromycin resistance maps within the region of the chromosome where the genes for ribosomal proteins are located and furthermore because a ribosomal protein is altered in these spontaneous one step mutants. These findings can be explained by the hypothesis that ribosomes interact with the cell membrane via a ribosomal component, possibly protein L22. This interaction does not interfere with the transport of erythromycin through the membrane in wild type cells, whereas it prevents the transport into mutant cells because of the altered L22 protein. Therefore the mutant cells are resistant because erythromycin cannot be transported through the membrane but the binding of erythromycin is not impaired by the altered L22 protein. This would explain why ribosomes from mutants with altered L22 protein bind erythromycin as efficiently as those from wild type cells (Fig. 4).

Masking the Genotype of an ery Mutant. The results in certain crosses suggested that it is possible to mask the *ery* genotype. In one of the crosses (see "Results") recombinants with an altered L22 protein were found to be sensitive to erythromycin. This was demonstrated in a transduction experiment in which a Str-R derivative from N281 was the donor and strain AT2472 the recipient. This situation is a very striking case of a masked genotype and a further example for interaction between ribosomal elements to produce the various phenotypes (Apirion and Schlessinger, 1969).

The mentioned experiments suggests that the genetic background of strain AT2472 with regard to ribosomal genes is different from that of strain AB301. (Mutants N281, N282, and N54 were all derived from strain AB301, see Table 1) Indeed strain AT2472 contains an altered 30S protein, namely S17 (Dekio, 1971; Wittmann and Osawa, unpublished). Whether this is the only difference between the strains, and whether S17 is the protein that contributes to the masking of the erythromycin genotype cannot be answered at present.

Since strain AT2472 carries the *aro*E marker which is cotransduced with *str* and *spc* alleles, it is widely used in genetic analyses of ribosomal mutations. Strain N486 (see Table 1) on the other hand also carries the *aro*E mutation but it does not have an altered S17 ribosomal protein which can be detected by two-dimensional gel electrophoresis (Wittmann, unpublished observations).

Thus, the genetic analysis indicates that indeed in a suitable genetic environment the altered L22 protein is responsible for the phenotypic resistance to erythromycin, since in crosses with three different strains as recipients (see "Results") all the Ery-HR transductants tested had the altered L22 protein. A similar example of a masked genotype have been recently found by Osawa *et al.* (1973) in mutants of *Bacillus subtilis* resistant to chloramphenicol.

Are ery Mutations in Structural Genes for Ribosomal Proteins? There is growing evidence that the allele *ery* 282 is in the structural gene for protein L4 (Otaka *et al.* 1970, Takata *et al.*, 1970; Otaka *et al.*, 1971). In the studies reported here a difference in a specific peptide of protein L4 from N282 has been detected (Fig. 3) and in studies with other *ery* mutants changes in the same peptide were found (Otaka *et al.*, 1971). Similar clusters of single amino acid exchanges have also been detected in mutants resistant to spectinomycin (Funatsu *et al.*, 1971; Funatsu *et al.*, 1972a; DeWilde and Wittmann-Liebold, 1973) and streptomycin (Funatsu and Wittmann, 1972; Funatsu *et al.*, 1972b).

Thus far we have found two mutants (N281 and N289) with altered L22 proteins among nine mutants tested. From the two-dimensional gel electrophoretic studies it can be concluded that the alterations in L22 of both mutants are very similar if not identical. (These two mutants were isolated about six years apart). It remains to be seen by sequence analyses of protein L22 of these mutants whether the postulated identity of the mutant L22 proteins will be confirmed. These analyses, now in progress, are also expected to clarify whether the more negative net charge of the altered L22 protein is due to a replacement of a neutral by an acidic or to that of a basic by a neutral amino acid. A secondary modification of protein L22, e.g. by phosphorylation or acetylation, is possible but rather unlikely since thus far such modifications have not been found in any other ribosomal mutant protein. Since the *ery* 281 mutation maps very closely to the *spc* and *str* genes and since evidence accumulated that many genes that code for ribosomal proteins map in the *str* region of the *E. coli* chromosome (Osawa *et al.*, 1972; Takata, 1972) it is very likely that the *ery* 281 and 289 mutations are in the structural gene that codes for protein L22.

Isolations of ery Mutations in Different Laboratories. In these studies we found that *ery* mutants isolated by Apirion (1967) could result from an alteration in at least one of two 50S ribosomal proteins while numerous isolation by Otaka *et al.* (1970) and Takata *et al.* (1970) encountered only *ery* mutants with altered L4 proteins.

One possibility to explain this difference is the following: Mutants N281–289 were spontaneous ery^r mutants while most of the mutants reported by Otaka *et al.* (1970) were obtained by N-methyl-N′-nitrosoguanidine treatment. Therefore there may be some difference in mutation frequency between spontaneous mutations and the mutagen induced mutations in these two protein genes. Another possibility could be the genetic background of parental strains used for the isolation of the *ery* mutants. The mutants studied by Otaka *et al.* (1970) were derived from strain Q13 while mutants N281–289 originated from strains AB301 and N314. Although strain Q13 is a derivative of strain AB301 it was isolated from AB301 after two successive treatments with heavy doses of nitrosoguanidine. There are that least five known mutations by which strain Q13 differs from strain AB301 (see Weather-

ford *et al.*, 1972). Moreover, strain Q13 is more sensitive to erythromycin than strain AB301 and this sensitivity is probably due to a mutation that maps in the *str* region of the *E. coli* chromosome (Immken and Apirion, unpublished observations). Therefore, it is possible that in the genetic background of strain Q13 only alterations in protein L4 could lead to high levels of resistance to erythromycin while in the more wild type like genetic background of strain AB301 and N314 (these are two different K12 strains) there are more ways by which a strain can become resistant to erythromycin.

The Order of the str spc ery Genes. The experiments presented here suggest that *ery* mutants are closely linked to *str* and *spc* genes and much closer to the *spc* than to the *str* gene. However, while all the data at present agree on the approximate location of the *ery* genes, there is some conflict: Some experiments using non-isogenic strains suggest that the order is *str spc ery* (Dekio, 1971), while others suggest that the order is *str ery spc*. The data presented here (Tables 1 and 2) suggests the latter order. These experiments were carried out in St. Louis. Similar results were obtained in Hiroshima, when the same strains (N281 and N54) were used in transduction experiments. However, when strains WH107, WH108 or JCSPCO2 were used as donors and strain N282 as recipient the order found was *str spc ery*. A similar order was also indicated by the experiments of Nomura and Engbaek (1972). In all cases consistent results with regard to classes of recombinants were obtained whether Str-R or Spc-R transductants were selected (see Tables 1 and 2) and the segregation of the other two unselected markers was analyzed. Since in all these cases the four possible classes of transductants with respect to the unselected markers were found, it is unlikely that some trivial reason can explain this difference.

One possibility is that this region of the chromosome is not identical in all strains and the results reflect a genuine difference in gene order in different *E. coli* K12 strains. On the other hand these results could be due to the peculiarities of the genetic organization of the ribosomal genes about which we still do not know enough. A third problem is the extensive interactions of various ribosomal mutations which can result in masked genotypes as was also shown in this paper. Of course a combination of these three factors could be effective and further studies should hopefully clarify this problem.

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