Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 755-762

MUTATIONAL ALTERATIONS OF EITHER LARGE OR SMALL RIBOSOMAL SUBUNIT FOR THE KANAMYCIN RESISTANCE

Eung Chil Choi, Toshio Nishimura, and Nobuo Tanaka
Institute of Applied Microbiology, University of Tokyo, Tokyo 113

Received April 25,1980

SUMMARY

By the treatment of E. coli Q13 with a mutagen, 76 kanamycin-resistant mutants were isolated. Of them, five mutants, showing various degrees of cross-resistance to neomycin, gentamicin or streptomycin, were studied in detail. The degree of inhibition by kanamycin of polyphenylalanine synthesis was less on the ribosomes derived from the resistant mutants than on the parental ribosomes. The mutant ribosomes exhibited less affinity for [^{3}H]kanamycin than the sensitive ribosomes. The effect of the antibiotic on polypeptide synthesis with the hybrid ribosomes, consisting of the resistant and parental subunits, and the binding of [^{3}H]kanamycir to the subunits suggested that the resistance is attributed to the 30S ribosomal subunits in the three mutants, and to the 50S subunits in the other two mutants.

The ribosomal changes, associated with the resistance to aminoglycoside antibiotics, have been analyzed by a number of investigators in E. coli mutants. Thus, the streptomycin resistance is attributed to S12 ribosomal protein (1-4). Protein S5 is responsible for the spectinomycin resistance (5,6). In a kanamycin-resistant mutant, the resistance is located in S12 protein (7). The mutational alteration for the neamine resistance is linked to protein S17 (8). The kasugamycin resistance is associated with 16S RNA of the 30S ribosomal subunit (9,10). These investigations indicate that the resistance to various aminoglycosides are linked to alterations of the small ribosomal subunit but not to the large subunit. On the other hand, the mutational change of protein L6 of the 50S ribosomal subunit has been found in gentamicin-resistant mutants (11).

We have recently demonstrated that kanamycin, gentamicin and neomycin bind to both large and small ribosomal subunits, and inhibit translocation of peptidyl-tRNA and other ribosomal functions (12). For the purpose of confirming the interaction of aminoglycoside antibiotics with both ribosomal subunits, we have systematically examined a number of kanamycin-resistant mutants of E. coli, and found that the resistance is due to alterations of either 30S or 50S ribosomal subunit. The results are presented in this communication.

0006-291X/80/110755-08$01.00/0

MATERIALS AND METHODS

[^{14}C]Phenylalanine (513 mCi/mmol) was supplied by the Radiochemical Centre, Amersham, England. Kanamycin, streptomycin and neomycin were products of Meiji Seika Kaisha, Ltd., Tokyo, and gentamicin of Schering Corporation, Bloomfield, New Jersey. Phosphoenolpyruvate, ATP, GTP, pyruvate kinase, poly[U] and E. coli tRNA were purchased from Boehringer, Mannheim, Germany. Bacto-penassay broth and agar, and yeast extracts were obtained from Difco Lab., Detroit, Michigan, and heart infusion broth from Nisui, Tokyo.

[^{3}H]Kanamycin (3.2 Ci/mol) was prepared by the method described previously (12). It gave a single spot by paper chromatography, using a solvent system (acetone : 10 % CH_3COONH_4 : 10 % NH_4OH = 30:9:1, v/v), and was microbiologically active.

Kanamycin-resistant mutants were isolated, following the procedure of Adelberg et al. (13). The culture of E. coli Q13 at logarithmic phase of growth was treated with 200 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine at 37°C for 30 min., and spread on heart infusion agar plates containing gradient concentrations of 0 - 500 μg/ml of kanamycin. The minimal growth-inhibitory concentrations of antibiotics for the parental and resistant strains were determined by a two-fold dilution method on heart infusion agar.

The preparation of S100 fractions, washed ribosomes and ribosomal subunits, and synthesis of [^{14}C]polyphenylalanine with poly[U] followed the procedures described previously (14).

The binding of [^{3}H]kanamycin to ribosomal particles was assayed by nitrocellulose (Millipore) filter method. The reaction mixture, in 0.1 ml, contained: 1 μM ribosomes or ribosomal subunits and 0.2 - 80 μM [^{3}H]kanamycin in a buffer (10 mM Tris-HCl, pH 7.8, 10 mM $Mg(OAc)_2$, 80 mM NH_4Cl and 6 mM 2-mercaptoethanol). The mixture was incubated at 30°C for five min., and cooled in an ice-bath; and then one ml of the cold buffer was added to the reaction mixture. It was collected on Millipore filters, and washed with 2.5 ml of the cold buffer twice. The radioactivity on filters was determined with correction for the values in a parallel mixture without ribosomal particles.

RESULTS

The inhibition by antibiotics of growth of E. coli strains.

Kanamycin-resistant mutants were isolated, and five of them were studied in detail. The minimal growth-inhibitory concentrations (MIC) of antibiotics were determined by an agar dilution method, and the results are presented in Table 1. MIC of kanamycin was 3.1 μg/ml for the parental strain (Q13), and 50 - 100 μg/ml for the resistant mutants: R1-4, R2-1, R2-2, R3-3 and R3-5. The difference was ca. 15 - 30 fold. The kanamycin-resistant mutants showed various degrees of cross-resistance to gentamicin, neomycin and streptomycin.

Effects of kanamycin on polyphenylalanine synthesis in extracts of the drug-sensitive and -resistant organisms.

Kanamycin was observed to block poly[U]-directed polypeptide synthesis in a cell-free system obtained from the parental strain.

Table 1. Minimal growth-inhibitory concentrations (MIC) of aminoglycoside antibiotics for kanamycin-resistant mutants of E. coli, in comparison with the parental strain Q13.

Antibiotics \ Strains	Parent	R1-4	R2-1	R2-2	R3-3	R3-5
Kanamycin	3.1	100	50	100	100	100
Gentamicin	1.6	50	25	25	25	25
Neomycin	3.1	200	50	50	50	50
Streptomycin	3.1	50	6.3	25	50	25

The number represents MIC (μg/ml).

The relative incorporation of [^{14}C]phenylalanine was shown in Table 2. Approximately 50 % inhibition was found at drug concentration of 20 μM. Much less effect of the antibiotic on polypeptide synthesis was demonstrated with the ribosomes derived from all the five drug-resistant mutants and the S100 fraction from the parental strain. The results indicated that the drug resistance is attributed to alterations of ribosomes in the mutant cells.

Effects of kanamycin on polypeptide synthesis on hybrid ribosomes.

The 30S or 50S ribosomal subunit from the resistant mutants was combined with the pair subunit from the parental strain Q13, and the effects of kanamycin on [^{14}C]phenylalanine uptake with poly[U] were examined. The results are illustrated in Fig. 1. The poly-

Table 2. Effects of kanamycin on poly[U]-dependent [^{14}C]polyphenylalanine synthesis on the ribosomes derived from the parental and kanamycin-resistant strains of E. coli.

Kanamycin \ Strains	Q13	R1-4	R2-1	R2-2	R3-3	R3-5
0 μM	100 (53,900)	100 (41,200)	100 (51,300)	100 (48,400)	100 (73,000)	100 (89,100)
3	80	99	88	96	101	106
30	43	80	86	85	94	100
100	28	68	80	83	91	103

The number represents relative incorporation of [^{14}C]phenylalanine, and that in the bracket cpm.

The reaction mixture, in 0.2 ml, contained: 50 mM Tris-HCl, pH 7.8, 8 mM $Mg(OAc)_2$, 80 mM NH_4Cl, 6 mM 2-mercaptoethanol, 2 mM ATP, 5 mM phosphoenolpyruvate, 4 μg pyruvate kinase, 0.2 mM GTP, 20 μg E. coli tRNA, 20 μg poly[U], 0.06 μCi [^{14}C]phenylalanine, 25.7 pmoles washed ribosomes of each strain, 160 μg protein of the parental (Q13) S100 fraction, and the antibiotic. The mixture was incubated at 37°C for 20 min., and the hot TCA-insoluble radioactivity was determined in a liquid scintillation counter.

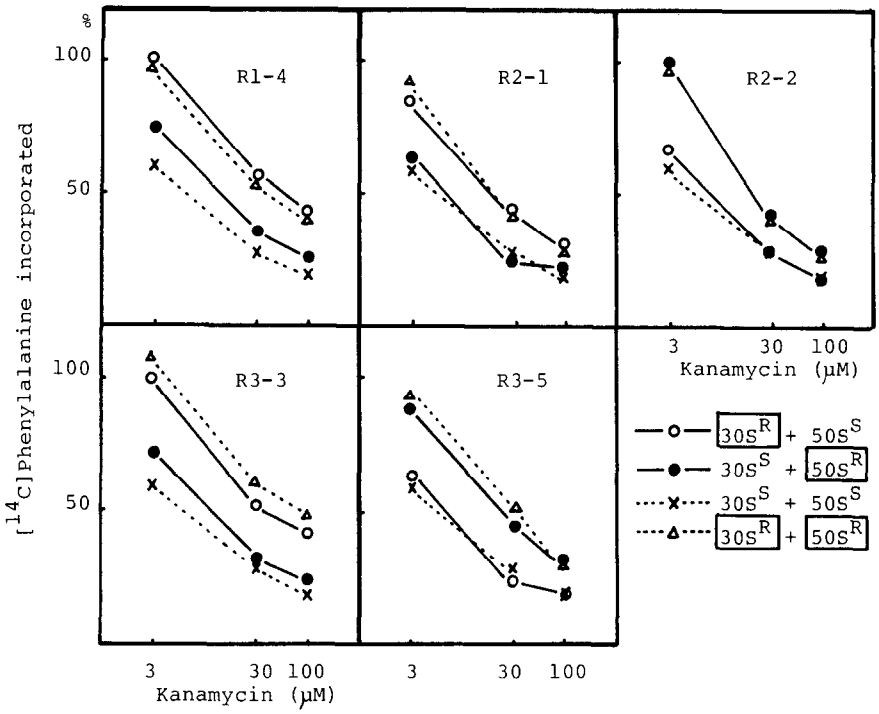

Fig. 1. Effects of kanamycin on poly[U]-directed polyphenylalanine synthesis on reconstituted ribosomes --- Localization of kanamycin resistance in the ribosomal subunits.
The procedure is described in the legend of Table 2. The ribosomes were replaced by 19.3 pmoles of the 30S subunits and 19.3 pmoles of the 50S subunits in the reaction mixture.

phenylalanine synthesis on the hybrid ribosome of R1-4 small subunit and Q13 large subunit was inhibited by the antibiotic at the same level as that on the resistant (R1-4) reconstituted ribosome; and the hybrid ribosome of Q13 small subunit and R1-4 large subunit was sensitive to kanamycin at a similar degree to the Q13 reconstituted ribosome. The results suggested that the drug resistance is localized in the 30S subunit of R1-4 ribosomes. Similarly, the resistance of R2-1 or R3-3 may be located in the 30S subunit, and that of R2-2 or R3-5 in the 50S subunit. The reconstituted ribosomes seemed to be more sensitive to kanamycin than the native ones (cf. Table 2).

The binding of [^{3}H]kanamycin to the ribosomes and ribosomal subunits derived from the sensitive and resistant cells.

The interaction of [^{3}H]kanamycin with the ribosomal particles obtained from kanamycin-sensitive and -resistant strains was studied by Millipore filter method at antibiotic concentration range of 0.2 - 80 μM. As illustrated in Fig. 2, the 50S subunits of R1-4, R2-1 and R3-3 showed similar affinity for the drug to that of the parental (Q13) large subunit, but those of R2-2 and R3-5 less than

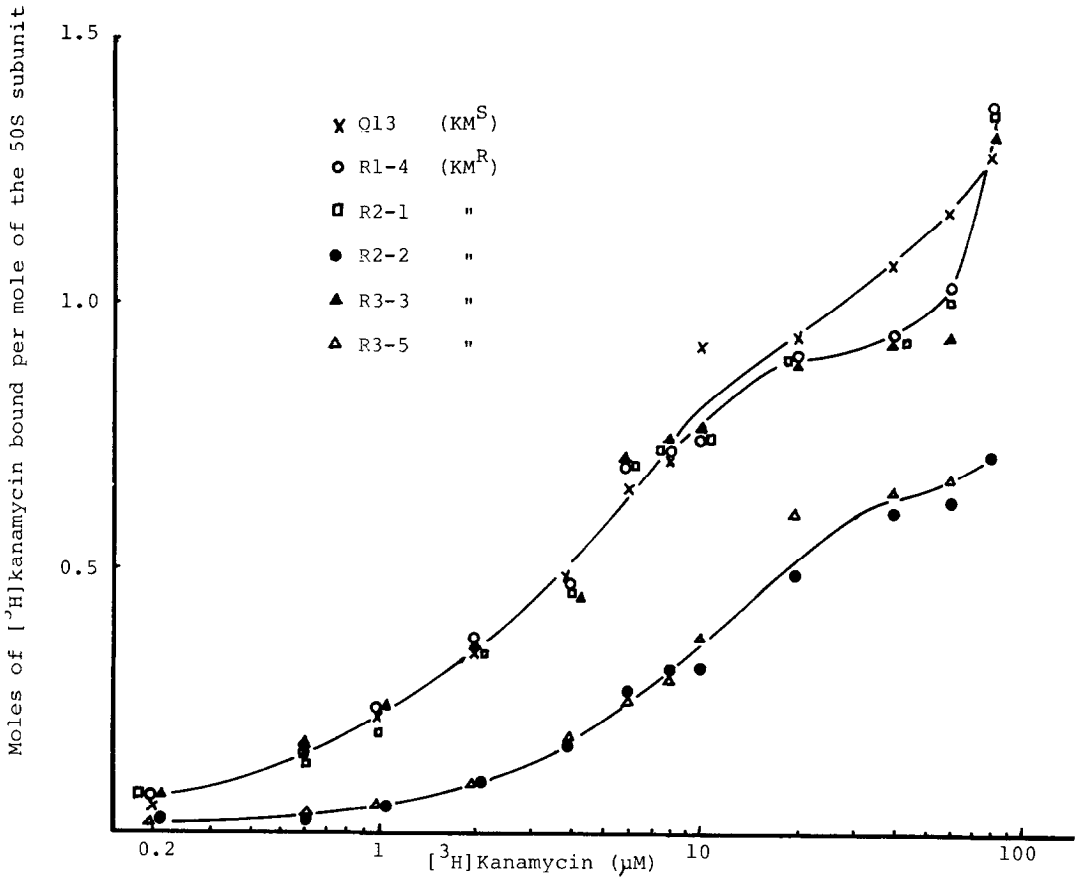

Fig. 2. The dependence on [^{3}H]kanamycin concentration for its binding to 50S ribosomal subunits derived from the sensitive and resistant strains of E. coli.

the sensitive subunit. The 30S subunits of R2-2 and R3-5 exhibited a similar level of binding to, but those of R1-4, R2-1 and R3-3 less than, that of the parental small subunit (Fig. 3).

The binding data were plotted according to the Scatchard equation for equlibrium binding, and the association constants of the ribosomal particles for the antibiotic were determined as previously described (12,14) (Table 3). The ribosomes from all the five mutants showed less affinity for kanamycin. The results were in accord with the finding that the cells are kanamycin-resistant. In the strains of R1-4, R2-1 and R3-3, the 30S ribosomal subunits exhibited weaker affinity for the antibiotic than the parental subunit, but the 50S subunits similar affinity to the sensitive subunit, indicating that the resistance is due to alterations of the small ribosomal subunits. In contrast, in the resistant strains of R2-2 and R3-5, the binding

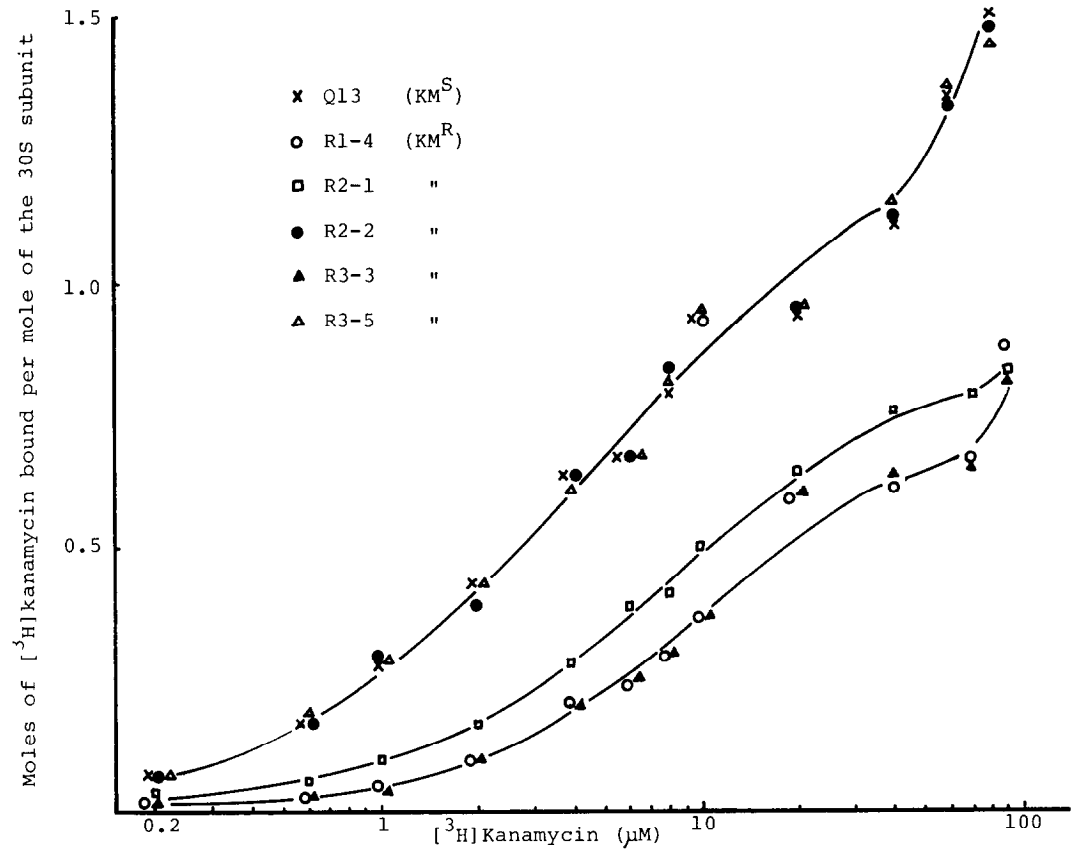

Fig. 3. The dependence on [^{3}H]kanamycin concentration for its binding to 30S ribosomal subunits derived from the parental and resistant strains of E. coli.

constants of the 30S subunits were similar to, but those of the 50S subunits less than those of the parental cells, indicating that the resistance is localized in the large subunits. Futhermore, in each mutant, the alteration of either ribosomal subunit with reduced

Table 3. Binding constants of [^{3}H]kanamycin to the ribosomes and ribosomal subunits.

E. coli strains	Association constants (Ka x 10^5 M^{-1})		
	70S ribosome	30S subunit	50S subunit
Q13 (KMS)	5.5	5.3	4.1
R1-4 (KMR)	1.3	0.62	3.9
R2-1 "	1.7	1.2	3.6
R2-2 "	0.95	5.7	0.51
R3-3 "	0.95	0.59	4.2
R3-5 "	1.5	5.4	0.56

Ka was calculated from the Scatchard plots for equilibrium binding of [^{3}H]kanamycin to the ribosomal particles, provided that the 70S ribosome possesses two strong binding sites and each ribosomal subunit one strong binding site (12).

affinity for the drug seemed to result in the reduced affinity of the entire 70S ribosome.

DISCUSSION

The current studies show that mutational alterations of either 50S or 30S ribosomal subunit result in reduced affinity for kanamycin and the antibiotic-resistant ability of synthesizing polypeptide. The results may be related to the drug resistance.

Of aminoglycoside antibiotics, the mechanism of action of streptomycin has been most extensively investigated [cf. a recent review by Wallace et al. (15)]. The mode of action of kanamycin was considered to be similar to that of streptomycin, because the two antibiotics, possessing similar chemical structures, interact with the bacterial ribosome and stimulate codon misreading. However, recent studies in our laboratory suggest that kanamycin exhibits a mechanism of action similar to those of gentamicin and neomycin, but distinctly different from that of streptomycin: i.e. kanamycin, gentamicin and neomycin bind to both ribosomal subunits and block translocation of peptidyl-tRNA, whereas streptomycin binds only to the 30S ribosomal subunit and does not significantly affect translocation (12). The inhibition of translocation by kanamycin and related aminoglycosides has been also observed by Cabañas et al. (16).

The results of kanamycin binding to ribosomal particles suggest that the alteration of either ribosomal subunit affect the binding of the antibiotic to the pair subunit and the entire ribosome becomes resistant to the drug. A similar phenomenon has been observed with viomycin resistance in Mycobacterium smegmatis (14).

The present experimental results seem to support the biological significance of the previous finding that kanamycin binds to or interacts with both large and small ribosomal subunits (12).

ACKNOWLEDGEMENTS

The authors express their deep thanks to Dr. Hamao Umezawa, Institute of Microbial Chemistry, Tokyo, for his kind advice and cooperation in the current study.

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