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Alteration of Ribosomal Protein L6 in Mutants of *Escherichia coli* Resistant to Gentamicin

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Summary. Spontaneous and ethylmethane-sulfonate induced mutants of *Escherichia coli* resistant to gentamicin sulfate were isolated and investigated for alterations in the ribosomal protein pattern. It was found by two-dimensional polyacrylamide gel electrophoresis that three independently isolated strains did not show any spot for ribosomal protein L6. On cochromatography of radioactively labelled mutant and wild-type ribosomal proteins on carboxymethyl-cellulose columns a shift of the elution position of protein L6 was observed, the new elution positions being characteristic for the individual mutants analyzed which indicates that they possess different alterations in the L6 primary structure.

Genetic analysis showed that the gentamicin resistant strains contain at least two mutations. One of them correlates with the altered L6 protein and causes an increased minimal inhibitory concentration of the drug by about 5 to 10-fold. The other mutation is not yet biochemically characterized. Its presence is connected with an about 10 to 20-fold increase in the resistance. Both mutations, when put together, confer resistance to 50 to 100 μg/ml of the antibiotic in a low salt rich medium and to 1 mg/ml in a defined medium with a high concentration of phosphate. Cross-resistance analysis demonstrated that the three gentamicin-resistant (double-mutant) strains with the altered L6 protein are resistant to 50–100 μg per ml of all other aminoglycoside antibiotics tested. This forms a sharp contrast to the streptomycin resistance mutations present in *strA1*, *strA40* or *strA60* mutants which do not confer markedly increased levels of resistance to most of the other aminoglycosides.

Introduction

The investigation of bacterial mutants with non-enzymatic resistance to aminoglycoside antibiotics has contributed much to the knowledge of the site and the mode of action of these antibiotics and, in addition, has provided information on many details of the catalytic functions of the bacterial ribosome in general. Most of these studies have been carried out with the mutants which are resistant to streptomycin because stable and single-step mutants are easily obtainable and because a clear correlation between in vitro and in vivo resistance could be established for this antibiotic (for review see Gorini, 1974; Benveniste and Davies, 1973; Wittmann and Wittmann-Liebold, 1974; Pestka, 1977).

The isolation of mutants which are intrinsically resistant to other members of the aminoglycoside group has been reported frequently but only in a very few cases could the resistance be traced down to the defined alteration of a ribosomal component. This difficulty was ascribed in part to a proposed multi-site action of this antibiotic group (Davies and Davis, 1968) in contrast to the apparent single-site action of streptomycin. One of those antibiotics to which no one-step mutants with a defined high-level resistance are described is gentamicin, a product of *Micromonospora* species which is of medical importance because of its bactericidal activity against gram-negative bacteria. It was the purpose of this investigation to isolate and to biochemically characterize gentamicin resistant mutants of *Escherichia coli* and to thereby possibly gain information on the mechanism of action of the non-streptomycin group of aminoglycosides.

Materials and Methods

Bacteria, Media and Growth Conditions. The *E. coli* K12 strains used in this work are listed in Table 1. Gene designations are adopted from Bachmann et al. (1976). The rich and minimal media employed and the growth conditions were as described previously (Piepersberg et al., 1975a, b).

Electrophoretic and Chromatographic Analysis of Ribosomal Proteins. The procedures for preparation of 70S ribosomes and of the 30S and 50S subunits were as described in Wittmann et

Table 1. List of bacterial strains used

Strain	Genotype	Reference or Source
A19	*thi⁻ met⁻ RNaseI⁻ λ⁺*	Gesteland (1966)
Sm1	*thi⁻ met⁻ RNaseI⁻ λ⁺ strA40*	Funatsu et al. (1972)
Sm5	*thi⁻ met⁻ RNaseI⁻ λ⁺ strA60*	Funatsu et al. (1972)
Sm10	*thi⁻ met⁻ RNaseI⁻ λ⁺ strA1*	Funatsu et al. (1972)
AB2834	*thi-21 mal-352 aroE353*	Piepersberg et al. (1975b)
AB2834 strA	*thi-21 mal-352 aroE353 strA*	Piepersberg et al. (1975b)
PR11	*F⁻ ara leu lacY purE gal trp his argG malA xyl mtl ilv metA thi spc-13 strA aroE353*	laboratory stock
GE20-6	like A19, gentamicin resistant, *rpsS6*	this work
GE20-8	like A19, gentamicin resistant, *rplF8*	this work
GS20-10	like A19, gentamicin resistant, *rplF10*	this work
GS50-15	like A19, gentamicin resistant, *rplF15*	this work
TD3[a]	*rplF⁺ strA^R*	Transduction: AB2834 strA → GS20-10
TD6[a]	*rplF10 strA^R*	Transduction: AB2834 strA → GS20-10
33R	*thi-21 mal-352 rplF8*	Transduction: GE20-8 → AB2834
52R	*thi-21 mal-352 rplF8*	Transduction: GE20-8 → AB2834

[a] Strains TD3 and TD6 still contain the second (non-ribosomal) mutation conferring low level gentamicin resistance

al. (1974). The ribosomal proteins were extracted with acetic acid and analyzed on two-dimensional polyacrylamide gels according to Kaltschmidt and Wittmann (1970). Carboxymethylcellulose chromatography of radioactively double-labelled ribosomal proteins was carried out according to Bollen and Herzog (1970). The identification of relevant 50S ribosomal proteins present in the elution peaks was performed as follows: Peak fractions were first mixed with 3 mg of unlabelled 70S carrier ribosomal proteins, concentrated by acetone precipitation (5 volumes) and the resulting precipitate (after drying) dissolved in sample gel and subjected to two-dimensional polyacrylamide gel electrophoresis. The 50S protein spots were cut out from the gels, oxidized in a Packard sample oxidizer and the separated ^3H and ^{14}C radioactivity determined in a liquid scintillation spectrometer. Monophase 40 (for ^3H) and Carbosorb II and Permafluor (for ^{14}C) were used as scintillation fluids.

Genetic Procedures, Cross-Resistance Analysis. Transductions, selection and screening of recombinants were carried out as described by Piepersberg et al (1975a, b). For the cross-resistance tests, a loopful (about 10^{-3} ml) of suspensions of the cells to be tested (about 5×10^8 cells per ml) was streaked on plates containing 30 ml of rich medium (1% tryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.2% glucose, 1.5% agar (no extra Mg^{2+} or Ca^{2+} supplements)] with the antibiotics at one of the following concentrations: 0, 1, 2, 5, 10, 25, 50, 100 μg per ml. Incubation was at 37° C. That concentration of the antibiotic completely preventing visible growth was recorded as the plate minimal inhibitory concentration. The same procedure was followed when the minimal inhibitory concentration was to be determined on defined medium plates (Piepersberg et al., 1975a, b) except that the antibiotic was supplemented at the following concentrations: 0, 2, 5, 10, 20, 50, 100, 200, 400, 750 and 1000 μg per ml. Ca^{2+} and Mg^{2+} concentrations in this medium were 1 mM and 10 mM, respectively.

Chemicals. The antibiotics used were from the following sources: Gentamicin sulfate (Sigma), Streptomycin sulfate and Neomycin sulfate (Serva), Kanamycin sulfate (Boehringer Mannheim), Kasugamycin hydrochloride (Calbiochem), Spectinomycin (Mycofarmdelft), Tobramycin and Hygromycin B (The Lilly Laboratories) and Bluensomycin (The Upjohn Company).

Results

Mutant Isolation. When 0.1 ml of an overnight culture of *E. coli* strain A19 is plated on rich medium (TGYE) containing 10 μg gentamicin sulfate per ml the frequency of the appearence of resistant colonies is about 1 per 10^8 cells plated. Spontaneous mutation to resistance to 20 μg/ml of the antibiotic, however, is very scarce and most of the mutants obtained by a one-step selection to resistance against this concentration are unstable. However, stable strains resistant to gentamicin sulfate concentrations of 20 μg/ml can be obtained either by mutagenesis e.g. with ethylmethanesulfonate (frequency: 1 per 10^6 cells plated) or by first isolating mutants spontaneously resistant to 10 μg/ml and subsequently exposing them to higher concentrations.

Analysis of Ribosomal Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis. A total of 35 independent gentamicin-resistant mutants were isolated; 11 of them were one-step spontaneous mutants resistant to 10 μg/ml (class I), 11 were two-step spontaneous mutants resistant to 20 μg/ml (class II), 12 were obtained after ethylmethane-sulfonate mutagenesis and direct plating on 20 μg/ml (class III), and one mutant was isolated by a three-step isolation, first at 10 μg/ml and then at 20 and finally at 50 μg/ml (class IV). The ribosomes of these strains were prepared and analyzed for alterations in the protein pattern by two-dimensional polyacrylamide gel electrophoresis.

Changes were found in the electropherograms of four strains: Three mutants, strains GS20-10 (class II), GE20-8 (class III) and GS50-15 (class IV) did

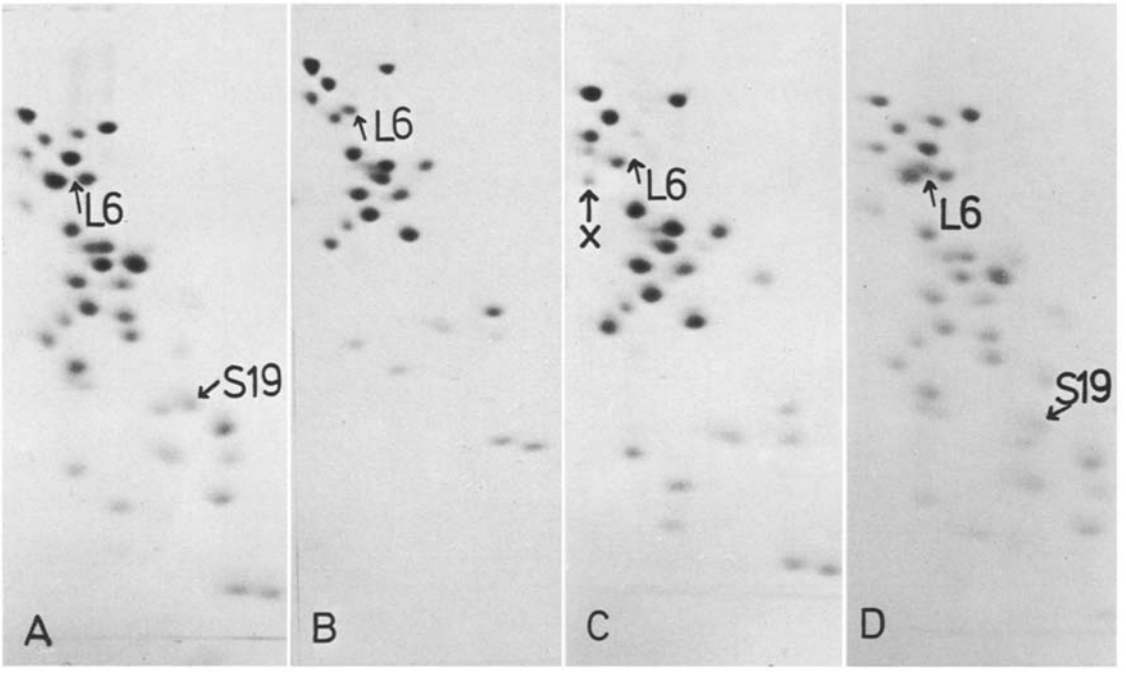

Fig. 1A–D. Sections of two-dimensional gel electropherograms of **A** 70S ribosomal proteins from strain GS50-15; **B** 50S ribosomal proteins from strain A19; **C** 50S ribosomal proteins from strain GE20-8; **D** 70S ribosomal proteins from strain GE20-6. The positions of proteins S19 and L6 are indicated by arrows

not show any spot of ribosomal protein L6 on the two-dimensional electropherograms of 70S ribosomal proteins. Fig. 1A illustrates this for the total 70S proteins of strain GS50-15. When 30S and 50S subunits of strains GE20-8 and GS50-15 were prepared and electrophoretically analyzed for their protein composition, it was found that the protein pattern of 30S particles was indistinguishable from that of the wild-type strain (not shown) whereas the 50S subunit electropherograms differ from that of the wild-type (Fig. 1B and 1C) in the complete apparent absence of protein L6. A second striking difference was the appearance in the 50S subunit electropherograms of a protein migrating in the electrophoretic position of protein S8 (marked "x" in Fig. 1C). Electrophoresis of acetic acid extracts of whole cells did not reveal protein L6, either; this argues against the possibility that the mutant L6 might not be assembled into ribosomes due to a structural alteration and therefore be solely present in the cytosol.

In addition to these strains with apparently altered L6 proteins one gentamicin resistant mutant (strain GE20-6; class III) was found to possess an alteration in ribosomal protein S19 (Fig. 1D).

Carboxymethylcellulose Column Chromatography of Ribosomal Proteins from Strains GE20-8, GS20-10 and GS50-15. Chromatography on carboxymethylcellulose (CMC) columns was performed in order to investigate whether protein L6 in strains GE20-8, GS20-10 and GS50-15 is mutationally altered or not present on the ribosome at all. For this purpose ribosomes of ^{3}H-labelled wild-type cells and of ^{14}C-labelled mutants were prepared, subunits were separated and, after mixing wild-type and mutant subunits, ribosomal proteins were extracted and chromatographed on CMC columns. For all three gentamicin-resistant strains (GE20-8; GS20-10; GS50-15) the elution profile of the 30S subunit proteins was indistinguishable from that of the wild-type proteins. Figure 2 illustrates the pattern for strain GE20-8.

In contrast, the three mutants show a striking difference in the 50S subunit protein elution (Fig. 3).: Some material from the peak which contains proteins L1, L6 and L29 is lacking and is apparently shifted to another position which is different for the strains GE20-8 and GS20-10. The elution profile of 50S proteins from strain GS50-15 (not shown) closely resembles that of strain GE20-8. These results present strong evidence that protein L6 in the three gentamicin-resistant strains is altered.

It was further investigated whether the mutant L6 proteins co-migrate with any other ribosomal protein on the two-dimensional gel or whether they do not enter the gel under our standard conditions. To this end, the proteins present in peaks I, II and

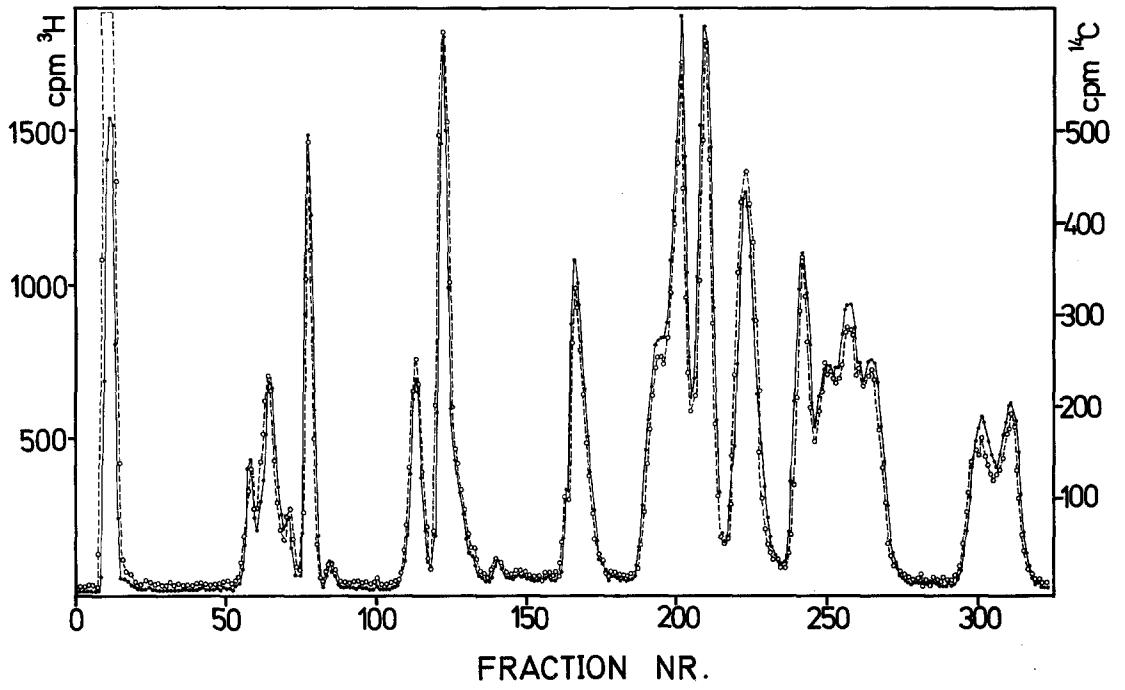

Fig. 2. CMC column chromatography of a mixture of ^{3}H-labelled 30S ribosomal proteins from strain A19 (—) and of ^{14}C-labelled 30S ribosomal proteins (– – –) from strain GE20-8)

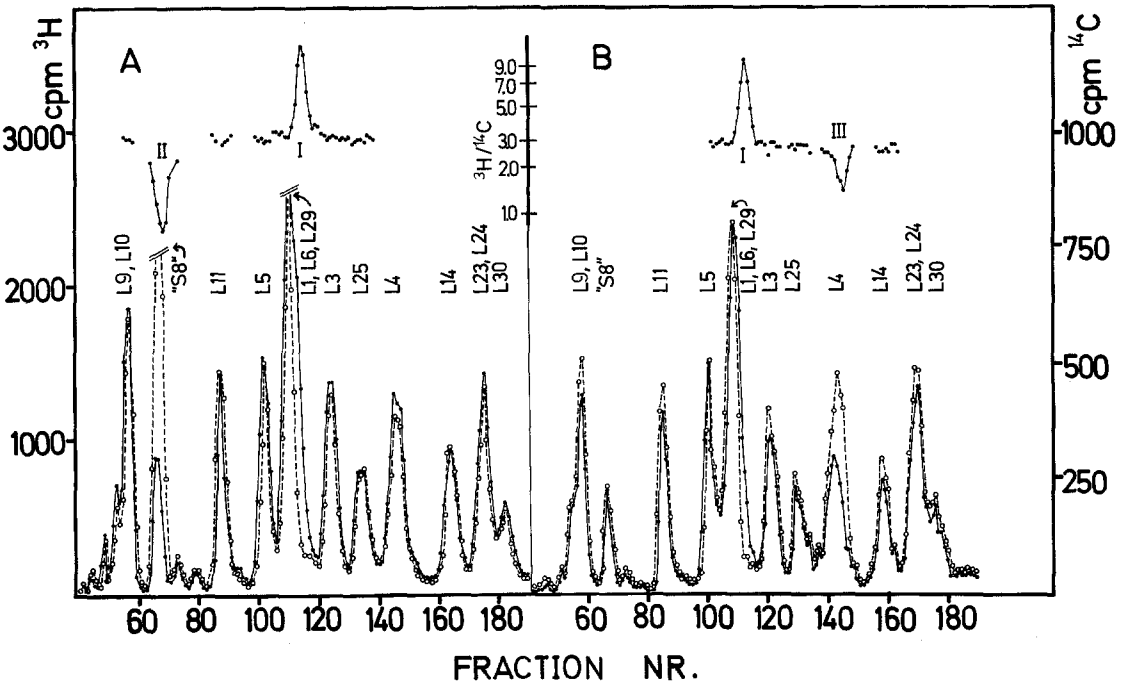

Fig. 3A and B. Sections of the elution profile of CMC column chromatography of a mixture of ^{3}H-labelled 50S ribosomal proteins from strain A19 (—) and ^{14}C-labelled 50S ribosomal proteins (– – –) from **A** strain GE20-8, **B** GS20-10

III of Fig. 3A and 3B were mixed with unlabelled wild-type carrier ribosomal proteins and separated by two-dimensional polyacrylamide gel electrophoresis. The protein spots were cut out from this gel, the slices were burnt in an oxidizer and their radioactivity was determined. It was found that the decrease of ^{14}C-counts in peak I of Figure 3A and 3B is due to the absence of protein L6: proteins

Table 2. ^{3}H and ^{14}C radioactivity present in several ribosomal proteins[a] upon gel electrophoresis of the material present in Peaks I, II and III from the experiment of Figure 3

cpm ^{3}H/cpm ^{14}C in ribosomal proteins from peaks			
I (Fig. 3A)	I (Fig. 3B)	II (Fig. 3A)	III (Fig. 3B)
L1: 1041/416	L1: 1056/469	"S8": 391/165	L3: 92/48
L6: 921/40	L6: 906/49	L10: 35/51	L4: 329/147
L29: 306/101	L29: 274/127	L12: 37/42	L5: 52/40
L11/S5: 44/37	L11/S5: 159/58		L14: 52/43

[a] Proteins not listed in this table did not contain any significant radioactivity

Table 3. Transductional analysis of gentamicin resistance

Donor	Recipient	Marker selected	Cotransduction frequency (%)		
			SPC[a]	STR[a]	GMC[a]
GE20-8	PR11	*aroE*$^{+}$	67.5	35.9	55.0
GS50-15	AB2834*strA*	*aroE*$^{+}$	–	36.0	50.0
GE20-6	AB2834	*aroE*$^{+}$	–	–	59.7

[a] SPC, STR, GMC denote spectinomycin sensitivity, streptomycin sensitivity and low level gentamicin resistance, respectively

L1 and L29 still possess the normal $^{3}H/^{14}C$ ratio of label whereas the spot for protein L6 predominantly carries ^{3}H radioactivity (Table 2). Electropherograms of peak II material (Fig. 3A) revealed radioactivity only in the protein which migrates identically to ribosomal protein S8. However, as the $^{3}H/^{14}C$ ratio of this material was equal to that of other ribosomal proteins (Table 2) one has to conclude that the mutant L6 of strain GE20-8 does not migrate into the S8 position and that the "S8" present in 50S subunits of the mutants is either authentic S8 or some other protein which is able to bind to 50S subunits when L6 is altered.

Similarly, electrophoresis of the peak III material (Fig. 3B) revealed that the only protein spot containing radioactivity is that of protein L4 (Table 2); again, however, the $^{3}H/^{14}C$-ratio was normal and the surplus of ^{14}C-radioactivity could not be found on the two-dimensional gels.

In conclusion, therefore, these experiments point out that protein L6 in the gentamicin resistant strains is altered and that the inability to detect the altered form on the gel electropherograms is probably due to the fact that either it cannot enter or it migrates out of the polyacrylamide gel or that it is in a position which is not cut out. However, the latter possibility is not very likely since in this case a new spot should have been seen on the two-dimensional electropherograms of the mutant proteins (Fig. 1). The presence of an altered L6-protein in the 50S subunits of strains GE20-8 and GS20-10 has been demonstrated by immunological techniques (G. Stöffler, unpublished).

Correlation Between Gentamicin-Resistance and the Alteraltion of Ribosomal Proteins. Genetic studies were carried out in order to find out whether any correlation exists between the alterations in ribosomal proteins L6 and S19 and gentamicin resistance. First attempts to transduce resistance to 20 μg per ml (on rich medium) into recipient strains failed: even after extensive phenotypic expression periods no transductants emerged. This result and also the necessity of a step-wise selection procedure indicate that the resistance level present in strains GE20-8, GS20-10, GS50-15 (L6 mutants) and GE20-6 (S19 mutant) might be due to the additive or cooperative effects of two or more mutations. To test this hypothesis the transduction of resistance to lower than 20 μg/ml of gentamicin was attempted and it was found that it is possible to cotransduce with *aroE* a "low level resistance" phenotype from all four strains into a sensitive recipient. Table 3 gives the co-transduction frequencies; they indicate a location of the mutation for the "low level resistance" between the *spc* (*rpsE*) and the *strA* (*rpsL*) cistrons at a site which is in accordance with the approximate location of the structural genes for ribosomal proteins L6 (*rplF*) and S19 (*rpsS*) (Nomura and Jaskunas, 1976).

To test whether this low level resistance mutation correlates with the L6 alteration, we analyzed gentamicin sensitive and resistant recombinants for the alteration of L6 or S19 by gel electrophoresis. Transductants from the cross GE20-6, GE20-8, GS20-10 and GS50-15 into strain AB2834 were used for this purpose in order to avoid any possible interference with the spectinomycin or streptomycin resistance markers present in strains AB2834*strA* or PR11 (see Table 3). From the crosses with the three L6 mutants 29 sensitive and 25 resistant transductants were chosen and it was found that those which had inherited resistance to low concentrations of gentamicin sulfate also had acquired the altered L6 protein whereas those remaining sensitive had retained the wild-type form of L6.

In the case of mutant GE20-6 it was not possible to demonstrate any direct correlation between the alteration in protein S19 and gentamicin resistance. It was found that low level resistance could be cotransduced with *aroE* (Table 3) but the gel electrophoretic analysis of 12 transductants showed that the resistance is due to a mutation located more proximal to *aroE* than the S19 structural gene. The possibility

remains open that the S19 alteration could have arisen as a secondary mutation stabilising the primary resistance mutation.

In order to provide further evidence for a positive correlation between the alteration of L6 and gentamicin resistance use was made of the fact that the low level resistant transductants obtained by the transfer of the L6 mutation into another genetic background are extremely unstable and frequently segregate faster growing derivatives. It was found that part of these segregants had concomitantly become gentamicin sensitive. As these might constitute L6 revertants we analyzed a total of 12 sensitive and 10 resistant derivatives whether they had lost the mutant form of L6 or not. It was found that all the resistant segregants maintained the mutant L6 protein whereas from the 12 sensitive segregants 7 had obtained the apparent wild-type form of L6 and the residual ones were still mutant with respect to L6. This indicates that one way of reverting to normal growth is the reversion of the L6 mutation and this event is always connected with the acquisition of the gentamicin-sensitive phenotype. Besides that, fast growth may also be caused by suppressor mutations outside the L6 cistron which may or may not "mask" gentamicin resistance.

Relation Between the L6 Mutations and strA Mutations. As ribosomal mutations leading to resistance to aminoglycoside antibiotics have until now only been connected with the alteration of a 30S subunit protein (Gorini, 1974; Wittmann and Wittmann-Liebold, 1974; Bollen et al., 1975) it was of importance to demonstrate, that the L6 mutation does not through some conformational interaction render the 30S subunit more resistant to the binding or the action of aminoglycosides. We have tested this possibility by a transductional introduction of the *strA* marker of strain AB2834*strA* (see Table 1) into strain GS20-10; selection was for streptomycin resistance.

Two classes of recombinants were obtained, one being resistant to gentamicin *and* streptomycin in the same way as the parents, and the other being fully resistant to streptomycin but having lost the high level gentamicin resistance. Two transductants from each class were analyzed for the status of protein L6 and it was found that those being resistant to both aminoglycosides have maintained the L6 mutation (e.g. strain TD6) and the other ones (e.g. strain TD3) have lost it (see Tables 1 and 4). This demonstrates that the L6 mutation alone can confer low level gentamicin resistance per se and not by any hypothetical interaction with protein S12 of the 30S subunit.

In conclusion, therefore, the results of the genetic analysis demonstrate that gentamicin resistance in our original isolates is due to at least two mutations which are non-cotransducible by phage P1. One of them is the alteration in ribosomal protein L6.

Table 4. Minimal inhibitory concentration for gentamicin sulfate of wild-type and mutant strains on minimal medium plates

Strain	Growth on Gentamicin sulfat plates (μg/ml)									
	2	5	10	20	50	100	200	400	750	1000
A19	+	+	±	−	−	−	−	−	−	−
AB2834	+	+	−	−	−	−	−	−	−	−
AB2834*strA*	+	+	−	−	−	−	−	−	−	−
GE20-8	+	+	+	+	+	+	+	+	+	+
GS20-10	+	+	+	+	+	+	+	+	+	+
GS50-15	+	+	+	+	+	+	+	+	+	+
TD3	+	+	+	+	+	+	−	−	−	−
TD6	+	+	+	+	+	+	+	+	+	+
33R	+	+	+	+	±	−	−	−	−	−
52R	+	+	+	+	±	−	−	−	−	−

+: full growth; ±: growth only at the beginning of the streak; −: no growth at all

Minimal Inhibitory Concentrations of Gentamicin for the Wild-Strains and for Gentamicin-Resistant Strains. In order to investigate the contribution of the L6 alteration and of the second, still unidentified mutation(s) to gentamicin resistance, the minimal inhibitory concentrations (MIC) of this drug for the original isolates and for various derivatives thereof were determined (Table 4). Minimal medium plates containing high phosphate concentration were used for this purpose since it was found that on this medium survival and stability of the L6 mutants is optimal whereas on rich medium plates the strains show a lowered efficiency of plating and also inhomogeneous growth.

It is evident that presence of the L6 mutation alone (strains 33R and 52R) increases the MIC value 5 to 10-fold in comparison to the wild-type strain (AB2834). On the other hand, removal of the L6 mutation from the original isolates (strain TD3) causes the MIC to drop about 10-fold that means that the additional mutation(s) present cause(s) an about 10 to 20-fold increased resistance level. Both mutations, when combined together, confer resistance up to 1 mg/ml on this medium (strain TD6).

Cross-Resistance Analysis. The "sensitivity profile" to different aminoglycosides of the gentamicin resistant mutants containing the altered L6 (together with a second unidentified mutation) was compared with that of the wild-type and of genetically and biochemically defined streptomycin resistant mutants. Rich medium plates (TGYE) were used in this experiment

Table 5. Aminoglycoside cross-resistance of streptomycin- and gentamicin-resistant strains on rich medium plates

Antibiotic	Minimal inhibitory concentrations (μg/ml) of strains						
	A19	SM1	SM5	Sm10	GE20-8	GS20-10	GS50-15
Streptomycin	<1	>100	>100	>100	50	50	100
Bluensomycin	2	>100	>100	>100	>100	>100	>100
Tobramycin	<1	<1	<1	<1	50	50	50
Hygromycin B	2	5	10	10	>100	>100	>100
Kanamycin	<1	<1	<1	1	100	100	100
Gentamicin	<1	<1	<1	1	50	50	100
Neomycin	1	1	1	5	100	100	100
Kasugamycin	50	50	50	50	100	100	50
Spectinomycin	5	5	5	5	5	5	5

since several of the antibiotics tested were only available in small amounts and sensitivity is generally much more enhanced under this growth condition.

As shown in Table 5 the *strA* mutations tested (strains SM1, SM5 and SM10) increase the resistance level (besides to streptomycin) only to bluensomycin and partially also to hygromycin B. On the other hand, the mutations in the gentamicin-resistant isolates including the one caused by the L6 alteration increase the resistance to all of the aminoglycosides tested except spectinomycin and kasugamycin.

Discussion

The results described here confirm the earlier reports that it is not possible to obtain one-step mutants with non-enzymatic high-level resistance to aminoglycosides like gentamicin (Milanesi and Ciferri, 1966; Davies and Davis, 1968). Possible explanations could be (i) that there are several targets where gentamicin can act, (ii) that mutation to high-level resistance could introduce a lethal effect or (iii) that several mutations are necessary to desensitize one single target. As the gentamicin sulfate used in this work consists of a mixture of several though closely related substances (gentamicins C_1, C_{1A} and C_2) and as it is possible to "train" mutants to higher resistance levels by the step-wise exposure to increasing concentrations of the drug the possibilities (i) or (iii) mentioned above seem most probable.

The genetic results point out that at least two mutations are necessary to obtain strains which – on rich medium – are resistant to 50 to 100 μg per ml of the antibiotic. One of them is identified as an alteration in protein L6, but the other one is not identified yet. On different media the former (ribosomal) mutation causes a 5 to 10-fold, the second, unidentified mutation a 10 to 20-fold increase of the resistance level; both together act cooperatively which indicates that different target sites might be involved.

Alteration of a 50S subunit protein in connection with aminoglycoside resistance is unique as only 30S proteins have been implicated until now in the establishment of resistance (for ref. see Wittmann and Wittmann-Liebold, 1974; Vazquez, 1974; Pestka, 1977). Although the 30S subunits of all three L6 mutants have been analyzed by gel electrophoresis and CMC column chromatography no other ribosomal alteration could be detected (Fig. 2). The appearance in the 50S subunit of a new protein which on two-dimensional electropherograms migrates into the position of protein S8 is not specific for gentamicin-resistant mutants since it has also been observed in other ribosomal mutants (E. Dabbs and H.G. Wittmann, unpublished). Chemical and immunological characterization of this protein after its isolation has shown that it is not related to protein S8 or any other of the standard proteins of the *E. coli* ribosome (J. Brosius, G. Stöffler and H.G. Wittmann, unpublished).

Evidence for the correlation between partial gentamicin resistance and the alteration of protein L6 is provided by the genetic experiments since it could be shown that the gentamicin resistance mutation is located in a chromosomal region where the structural gene for ribosomal protein L6 has been localized (Nomura and Jaskunas, 1976; Bachmann et al., 1976) and that gentamicin resistance is expressed irrespective of whether or not the strains contain a mutation in protein S12 conferring resistance to streptomycin. Also, revertant analysis has shown that reversion of L6 from the mutant to the wild-type form is correlated with the loss of gentamicin resistance. Finally, the fact that three L6 mutants were obtained with different isolation procedures in itself indicates the existence of a correlation between the altered L6 and antibiotic resistance.

Preliminary biochemical analysis revealed that the partial gentamicin resistance of the L6 mutants is an intrinsic property of the ribosomes per se. (Kühberger et al., unpublished results). It is not yet known,

whether, in addition, some interplay exists between the ribosomal (L6) mutation and the cytoplasmic membrane as in the case of certain spectinomycin-resistant strains (Mizuno et al., 1976).

Finally, it is interesting to note that with the exception of spectinomycin and kasugamycin which have a mode of action different from that of the other antibiotics tested (Vazques, 1974; Pestka, 1977) the gentamicin resistance mutations confer resistance against all the other aminoglycosides tested. The finding that spectinomycin and kasugamycin sensitivity is not changed in our isolates indicates that the mutations confer specific resistance against aminoglycosides and not a generalized (e.g. permeability) defect for all antibiotics.

Both mutations in our isolates – the L6 and the second, unidentified mutation – contribute to this cross-resistance since strain TD3 which acquired the wild-type L6 but still possesses the second mutation shows reduced cross-resistance but not complete wild-type like sensitivity. On the other hand, the *strA1*, *strA40* and *strA60* mutations only induce higher resistance to bluensomycin and partially to hygromycin B. These results indicate, therefore, that the aminoglycosides possess different target sites on the ribosomes.

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