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Mutations Determining Generalized Resistance to Aminoglycoside Antibiotics in *Escherichia coli*

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Summary. Mutations conferring resistance to low levels of kanamycin in *Escherichia coli* have been mapped at 3 locations: the *unc* locus (min. 83), a locus we have designated *kanA* (min. 72), close to *strA* (*rpsL*), and a locus at min. 86.5 previously discovered by Plate (1976) that we have designated *ecfB*. The *unc* and *ecfB* mutations are associated with defects in energy metabolism, while mutations at *kanA* may be in the gene coding for ribosomal protein S12 (*rpsL*). The three types of mutations cause cross resistance to a number of different aminoglycoside antibiotics and the effects of the mutations are cumulative in combination.

Introduction

Aminoglycoside antibiotics are extremely useful in the treatment of infections caused by Gram-negative bacteria. In clinical practice, bacterial strains resistant to one or more aminoglycosides are frequently encountered. Many of these strains harbor R-factors (plasmids) that code for aminoglycoside modifying enzymes, which confer resistance to one or several closely related aminoglycosides (Benveniste and Davies, 1973). Recently, clinical isolates of *Pseudomonas* have been reported which are resistant to all commonly used aminoglycosides (Bryan et al., 1976) not by virtue of the known R-factor coded modifying enzymes but believed due to mutation, which may affect the active transport of the drugs (Bryan and Van Den Elzen, 1975).

In the laboratory strain *E. coli* K12, mutations to aminoglycoside-aminocyclitol resistance have been reported for (a) streptomycin, (b) spectinomycin, (c) kasugamycin, (d) neomycin-kanamycin (for a review of a–d see Jaskunas et al., 1975), (e) neamine (Cannon et al., 1974), (f) the group amikacin, gentamicin, neomycin and kanamycin (Hull et al., 1976), and (g) kanamycin (Tanaka et al., 1964). (For information on the structure and chemistry of these antibiotics see Umezawa, 1975). Mutations to spectinomycin and kasugamycin resistance are specific, but types a, d, e and f exhibit varying degrees of cross resistance to structurally related antibiotics, e.g. mutations to streptomycin resistance often confer resistance to neamine and/or myomycin (J. Davies, pers. comm.).

These mutations fall into two major categories: (1) those which map in the cluster of ribosomal genes at min. 72 on the genetic map and probably give rise to altered structural components of the ribosome; (2) those which affect energy metabolism in the bacterial cell and map outside the known clusters of ribosomal genes.

The first category includes *nea* mutations found by Bollen and collaborators. Three types of *nea* mutants were described: *neaA* mutants with an altered ribosomal protein S17 (Bollen et al., 1975), *neaB* mutants with an unidentified alteration mapping close to the *rpsL* (*strA*) locus (Cannon et al., 1974), and mutants altered in both S12 and S5 (De Wilde et al., 1975). The *nek* mutations map close to the *rpsE* (*spcA*) locus and appear to affect ribosomal function although they have not been characterized in detail (Apirion and Schlessinger, 1968a and b, 1969; Brown and Apirion, 1974). Tanaka et al. (1964) isolated an *E. coli* strain resistant to 2000 μg/ml of kanamycin. The strain has not been subjected to genetic analysis but may have an altered S12 ribosomal protein (Masukawa, 1969). Recently, a mutation conferring resistance to amikacin, gentamicin, neomycin and kanamycin was mapped close to the *rpsL* locus (Hull et al., 1976).

The second category of mutants usually has relatively low levels of resistance and is deficient in energy

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metabolism. The first such mutants to be studied were deficient in the synthesis of hemin (Săsarmăn et al., 1968). More recently, neomycin resistant (Neo-R) mutants have been described which are defective in membrane energization mediated by the Mg^{++}-Ca^{++}-ATPase complex or are unable to maintain a normal proton gradient across the cell membrane (*unc*-mutants) (Kanner and Gutnik, 1972; Rosen, 1973; Adler and Rosen, 1976; Simoni and Postma, 1975; Haddock and Jones, 1977). These mutants are unable to metabolize Krebs cycle intermediates such as succinate and malate as sole sources of carbon and energy, but are able to grow on glucose. They have a reduced ability to accumulate amino acids, and Bryan and Van Den Elzen (1977) reported that they are also defective in the ability to accumulate ^{3}H-labeled gentamicin. *Unc* mutants map at min. 83.

A Neo-R mutation with different properties has been mapped at min. 64 and is thought to be defective in coupling metabolic energy to active transport (Lieberman and Hong, 1974; Lieberman et al., 1977). Plate (1976) has also used selection for neomycin resistance to isolate mutants resistant to colicin K. The latter are defective in the respiration linked transport of proline but appear normal for the ATP dependent transport of glutamine. The Neo-R/colicin K resistant mutations have been mapped between min. 86 and 87 on the *E. coli* map (Plate, pers. comm.). Bryan and Van Den Elzen (1977) have shown that a mutant of this type fails to accumulate ^{3}H-gentamicin and ^{3}H-dihydrostreptomycin.

In this paper we report the isolation and characterization of spontaneous mutations which confer resistance to low levels of kanamycin and several other aminoglycoside antibiotics. In strains carrying more than one of these mutations a cumulative effect on resistance is observed. The resistance mutations have been mapped in 3 different regions of the chromosome: in the cluster of ribosomal protein genes at min. 72, in the *unc* region near min. 83, and at a locus between min. 86 and 87.

Materials and Methods

Bacterial Strains. All strains used are derived from *E. coli* K-12. Mutant allele designations are registered in the *E. coli* Genetic Stock Center, Yale University. The strains are listed in Table 1.

Bacteriophage. Plkc was used for transduction. λ*cI*857 *b*221 *rex*::Tn5 *Pam*201 (constructed by D. Berg).

Media. The minimal medium used is a half-strength preparation of medium 56 (Monod et al., 1951) supplemented with glucose (2 mg/ml) and, when required, with amino acids at a final concentration of 20–40 μg/ml and thiamine at a conc. of 1 μg/ml. Glucose was substituted by rhamnose (2 mg/ml), glycerol (5 mg/ml), malate or succinate (1 mg/ml) as indicated.

L-broth (Luria and Burrous, 1957) was used for preparation of bacterial cultures; for agar plates this medium was supplemented with 1.5% Bacto- or Oxoid agar No. 1. For the purpose of selecting resistant mutants or scoring transductants for resistance, spectinomycin and streptomycin were, unless otherwise stated, added at a final concentration of 150 μg/ml and 200 μg/ml, respectively. Kanamycin was used at the concentrations indicated. Streptomycin, kanamycin and neomycin were obtained from Schwarz-Mann, gentamicin from Schering, amikacin from Bristol, spectinomycin from Upjohn, ampicillin from Wyeth Laboratories and chloramphenicol from Sigma.

Isolation of Mutants Resistant to Kanamycin. Spontaneous kanamycin resistant mutants of strain AB663 and its derivatives were obtained by plating L-broth cultures on L-agar supplemented with varying concentrations of the antibiotic. Strain AB663 does not grow on L-agar containing more than 3–4 μg/ml of kanamycin. Primary mutants were obtained on 6–7 μg/ml of kanamycin; resistant colonies were purified on L-agar without antibiotic. Secondary mutants resistant to higher levels of kanamycin were obtained by plating primary mutants on 30 μg/ml of the antibiotic. Such mutants were purified in the same way as the primary mutants and were used to provide tertiary mutants by selection on plates containing 90 μg/ml of kanamycin. In all mutant selections plates were incubated for 2–3 days at 37° C.

Strains carrying different combinations of resistance mutations were constructed by P1 transduction (see Table 1).

Tn5 Insertion in the ecfB Region. Bacteriophage λ*cI*857 *b*221 *rex*::Tn5 *Pam*201 (Berg et al., 1975) was grown lytically on the *SuII*$^{+}$ strain KH802. The transposon-containing phage was used to infect strain MO grown in tryptone +0.2% maltose and adjusted to a concentration of 10^{10} cells/ml. Infections were carried out at a m.o.i of 1 and phage were adsorbed for 30 min at room temperature, the culture diluted to 10^{8}/ml and grown at 32° C for 5 h. It was then spread on L-agar plates containing 50 mg/ml of neomycin and incubated at 37° C to select cells having Tn5 inserted into the chromosome ($f \sim 10^{-4}$). About 10^{4} Neo-R colonies were pooled and dilutions spread on L-plates containing 8 μg/ml (two fold minimal inhibitory concentration) of amikacin. Amk-R colonies ($f \sim 10^{-4}$) were patched on L-agar containing 50 μg/ml of neomycin and purified on L-agar with 8 μg/ml of amikacin.

Transduction Procedures. Stationary phase cultures of the recipient strain were concentrated ten-fold and suspended in a solution of 7.5×10^{-3} M $CaCl_2$ and 2.5×10^{-1} M $MgSO_4$. The suspension was maintained at room temperature for 15 min before mixing with an equal volume of a Plkc lysate [Lennox (1955)] containing at least 10^{9} p.f.u./ml. The mixture was maintained at room temperature for 5 min and spread on selective agar plates. Following incubation for 2–3 days at 37°, transductants were transferred to master plates of the selective medium, incubated for 1–2 days, and replica plated onto media appropriate for scoring unselected markers and antibiotic resistance.

Minimal Inhibitory Concentration (MIC). Minimal inhibitory concentrations of antibiotics were determined by spotting 20 μl of an overnight bacterial culture on a series of two fold dilutions of the antibiotics in 10 ml of neomycin assay agar, pH 7.9.

Antibiotic Discs. Discs containing ampicillin, tetracycline and chloramphenicol were obtained from Baltimore Biological Laboratories. Other antibiotics were dispensed onto Schleicher and Schuell discs No. 740-E (6.35 mm). Inhibition zone diameters were determined according to Ericsson and Sherris (1971).

Table 1. Bacterial strains

Strain no.	Sex	Genotype	Source
AB663	Hfr	*argH1, purF1, xyl-7, supE44*	Eggertsson and Adelberg, 1965
AT753	F$^-$	*rha-1, metB1, argH1, ilv-1, gal-6, lacY1, or Z4, rpsL8, 9 or 17 (str*^{r}*), supE44?*	A.L. Taylor strain, from B. Bachmann
AT2455	Hfr	*thi-1, cysG44, mal-18, rel-1*	A.L. Taylor strain, from B. Bachmann
GE314	Hfr	*thi-1, aroE24, rel-2, arg-291*	Arg$^-$ mutant of AT2472 induced by EMS
GE440	F$^+$	*ecfB1.* Other markers as in AB663	Spontaneous Kan-R mutant of AB663 selected on 7.5 μg/ml of kanamycin
GE441	F$^+$	*ecfB1, rpsE19 (spc*^{r}*).* Other markers as in AB663	Spont. Spc-R mutant of GE440
GE442	F$^+$	*ecfB1, kanA10, rpsE19.* Other markers as in AB663	Spont. Kan-R mutant of GE441 selected on 30 μg/ml of kanamycin
GE443	F$^+$	*ecfB1, kanA10, rpsE19, rpsL187 (str*^{r}*).* Other markers as in AB663	Spont. Str-R mutant of GE442
GE444	F$^+$	*ecfB1, kanA10, unc-5.* Other markers as in AB663	Spont. Kan-R mutant of GE442 selected on 90 μ/ml of kanamycin
GE445	F$^+$	*ecfB1, kanA10, kanA12, rpsE19.* Other markers as in AB663	Derived by same method as GE444
GE446	F$^+$	*ecfB1, kanA10, kanA13, rpsE19.* Other markers as in AB663	Derived by same method as GE444
GE447	Hfr	*unc-2.* Other markers as in AB663	Derived by same method as GE440
GE448	Hfr	*ecfB3.* Other markers as in AB663	Derived by same method as GE440
GE450	Hfr	*rpsE20 (spc*^{r}*).* Other markers as in GE314	Spont. Spc-R mutant of GE314
GE451	F$^-$	*argH*$^+$*, glpK1.* Other markers as in AT753	AT753 made *argH*$^+$*, glpK1* by Plkc grown on Lin 154 (Cozzarelli and Lin, 1966)
GE452	F$^-$	*rpsE20, str*^{s}*, aroE24.* Other markers as in GE451	Plkc (GE450)→GE451 *rpsE20, str*^{s}*, aroE24.* Selection for Spc-R
GE453	F$^-$	*rpsE20, str*^{s}. Other markers as in GE451	Plkc (GE450)→GE451 *aroE*$^+$*, rpsE20, str*^{s}. Selection for Spc-R
GE454	F$^-$	*rpsE20, str*^{s}*, ecfB3, metB*$^+$*, glpK*$^+$ *rha*$^+$. Other markers as in GE451	Plkc (GE448)→GE453 *metB*$^+$*, glpK*$^+$*, rha*$^+$*, ecfB3.* Selection for MetB$^+$
GE455	F$^-$	*rpsE20, str*^{s}*, unc-2, ilv*$^+$. Other markers as in GE451	Plkc (GE447)→GE453 *ilv*$^+$*, unc-2.* Selection for Ilv$^+$
GE456	F$^-$	*rpsE20, str*^{s}*, ecfB3, unc-2, metB*$^+$*, glpK*$^+$*, rha*$^+$*, ilv*$^+$. Others markers as in GE451	Plkc (GE447)→GE454 *ilv*$^+$*, unc-2.* Selection for Ilv$^+$
GE457	F$^-$	*rpsE19 or 20, atr*^{s}*, kanA10.* Other markers as in GE451	Plkc (GE442)→GE452 *aroE*$^+$*, kanA10, rpsE19?* Selection for AroE$^+$
GE458	F$^-$	*rpsE19 or 20, str*^{s}*, ecfB3, kanA10, metB*$^+$*, glpK*$^+$*, rha*$^+$. Other markers as in GE451	Plkc (GE448)→GE457 *rha*$^+$*, ecfB3, metB*$^+$*, glpK*$^+$. Selection for Rha$^+$
GE459	F$^-$	*rpsE19 or 20, str*^{s}*, ecfB3, kanA10, unc-2, metB*$^+$*, glpK*$^+$*, rha*$^+$*, ilv*$^+$. Other markers as in GE451	Plkc (GE447)→GE458 *ilv*$^+$*, unc-2.* Selection for Ilv$^+$
GE465	F$^-$	Relevant markers: *ilv-594, metE46*	Ilv$^-$ mutant of AB1976 (Eggertsson and Adelberg, 1965) induced by EMS
GE466	Hfr	*kanA8.* Other markers as in AB663	Derived by same method as GE440
GE467	Hfr	*kanA9.* Other markers as in AB663	Derived by same method as GE440
GE489	Hfr	*kanA8, rpsL188.* Other markers as in AB663	Spont. Str-R mutant of GE466
GE490	Hfr	*kanA9, rpsL189.* Other markers as in AB663	Spont. Str-R mutant of GE467
MO	F$^-$	*strA*	W.S. Reznikoff
OSA50	F$^-$	*strA, ksgA*::Tn5	Ó.S. Andrésson, Tn5 insertion in MO selected on kasugamycin
OSA52	F$^-$	*strA, ecfB*::Tn5	Tn5 insertion in MO (see Materials and Methods)
K802	F$^-$	*supE, hsr, hsm, met*	J.E. Davies

Accumulation of Labeled Antibiotic. ^{3}H-gentamicin and ^{3}H-dihydrostreptomycin (Amersham) were added to shaking cultures of exponential phase cells (2.5×10^8 cells/ml) in nutrient broth. The concentration of gentamicin was 2.8×10^{-6} M, specific activity 637 Ci/mole and the concentration of dihydrostreptomycin was 8.2×10^{-7} M, specific activity 3000 Ci/mole. Samples (0.5 ml) were removed, immediately collected on nitrocellulose filters (Millipore), washed with 10 volumes of 3% NaCl and counted in toluene based scintillation fluid.

Nomenclature. The symbols Spc-R, Str-R, Kan-R and Amk-R are used to denote resistance to spectinomycin, streptomycin, kanamycin and amikacin, respectively. The symbol *kan* is used for genes of unknown function having mutations giving rise to kanamycin resistance. The wild type forms of the *rpsE* (*spcA*), *rpsL* (*strA*) and *kan* loci are occasionally denoted by the symbols *spc*^{s}, , *str*^{s}, and *kan*^{s} while the symbols *spc*^{r}, *str*^{r}, *kan*^{r} are used for alleles conferring resistance to spectinomycin, streptomycin and kanamycin, respectively. The symbols *ecfB* and *kanA* are used as described in Results. Other symbols are as described by Bachmann et al., 1976.

Results

Isolation of Primary Kan-R Mutants of AB663. Spontaneous Kan-R mutants (primary mutants) of strain AB663 were obtained as described in Materials and Methods. They occurred at a frequency of 7×10^{-6} (Table 2). However, the majority (70–80%) of the Kan-R mutants grew extremely poorly with or without kanamycin and were discarded; among the remaining mutant strains several were unstable with regard to the Kan-R phenotype. Nine independently isolated stable Kan-R mutants (nr. 1–9) were selected for further study. With the exception of mutant nr. 3 (strain GE448) these mutants grow slowly and/or irregularly on agar media. Two of these mutants, nr. 2 (GE447) and nr. 4, were unable to grow on malate or succinate as a sole carbon source.

Table 2. Frequency of *kan*^{r} mutations in strain AB663 and its derivatives

Strain	Resistance markers	Conc. of kan. in selective medium (µg/ml)	Frequency of Kan-R mutants	Number of mutants analyzed	Map positions of *kan*^{r} mutations (Minutes)
AB663	*kan*^{s}, *str*^{s}, *spc*^{s}	6.0–7.5	7.1×10^{-6}	9	86.5, 83, 72
		15	$\sim 10^{-9}$		
		30	$< 10^{-10}$		
		90	$< 3 \times 10^{-11}$		
GE440	*ecfB1*, *str*^{s}, *spc*^{s}	30	1×10^{-9}	2	72
		90	$< 10^{-10}$		
GE441	*ecfB1*, *str*^{s}, *spc*^{r}	15	5×10^{-8}		
		30	3×10^{-9}	1	72
GE442	*ecfB1*, *kanA10*, *str*^{s}, *spc*^{r}	90	$\sim 10^{-9}$	3	72, 83

It should be noted that the number of Kan-R colonies dropped sharply when the concentration of kanamycin was raised above 8 µg/ml (Table 2).

Genetic Analysis of Primary Kan-R Mutants. Approximate mapping of mutations causing kanamycin resistance in primary mutants nr. 2 and 3 was carried out by conjugation using the mutant strains GE447 and GE448 as donors, and strain GE264 as recipient. The results indicated (a) that kanamycin resistance of GE447 is due to a mutation (called *kan-2*) which is closely linked to the *ilv* locus near minute 83, (b) that resistance of GE448 is due to a mutation (called *kan-3*) mapping close to the *metB* locus near minute 87.

The results of mapping the *kan-2* mutation by transduction are shown in Table 3. *Kan-2* was cotransduced with *ilv* at a frequency of 61% and with *metE* at a frequency of 11%, the order of these loci being *kan–ilv–metE*. These results place the *kan-2* mutation in the region of the *uncA* and *B* loci (Bachmann et al., 1976). Strain GE447 and *kan-2* transductants fail to grow on succinate and GE447 has reduced Mg^{++}-stimulated ATPase activity (data not shown). Therefore we shall refer to the *kan-2* mutation as *unc-2*.

Results of transduction mapping of the *kan-3* mutation are shown in Table 4. This mutation is cotransduced with the *metB*, *glpK* and *rha* loci and maps between *glpK* and *rha*. Cotransduction frequencies are shown in Figure 1. Strain GE448 (*kan-3*) has properties similar to those of the mutant described by Plate (1976) and maps in the same region (min 86.5). We will refer to this locus as *ecfB* (energy coupling factor B, cf. mutant described by Lieberman et al., 1977).

The remaining 7 primary mutants of AB663 have all been tested for the presence of resistance mutations

Table 3. Map position of *kan-2 (kan*^{r}*)* determined by transduction with P1

Selection	Number of transductants tested	Distribution of unselected markers (%)	
metE$^+$	150	*ilv*$^+$ *kan*^{r}	10.0
		ilv-594 kan^r	1.3
		ilv$^+$ kan^s	46.7
		ilv-594 kan^s	42.0
ilv$^+$	156	*metE*$^+$ *kan*^{r}	5.1
		metE46 kan^r	55.8
		metE$^+$ *kan*^{s}	16.7
		metE46 kan^s	22.4

Recipient: GE465 (*kan*^{s}, *ilv-594, metE46*); donor: GE447 (*kan*^{r}, *ilv*$^+$, *metE*$^+$)

Table 4. Map position of the *kan-3* (kan^r) mutation determined by transduction with P1

Selection	Number of transductants scored	Distribution of unselected markers (%)			
$metB^+$	300	kan^r $glpK^+$ rha^+	21.2	kan^s $glpK^+$ rha^+	0
		kan^r $glpK^+$ *rha-1*	6.7	kan^s $glpK^+$ *rha-1*	22.3
		kan^r *glpK1* rha^+	0.3	kan^s *glpK1* rha^+	1.7
		kan^r *glpK1 rha-1*	1.0	kan^s *glpK1* rha^+	46.7
rha^+	200	kan^r $glpK^+$ $metB^+$	27.5	kan^s $glpK^+$ $metB^+$	0
		kan^r $glpK^+$ *metB1*	16.5	kan^s $glpK^+$ *metB1*	3.0
		kan^r *glpK1* $metB^+$	2.5	kan^s *glpK1* $metB^+$	1.0
		kan^r *glpK1 metB1*	16.0	kan^s *glpK1 metB1*	33.5
glp^+	200	kan^r rha^+ $metB^+$	16.5	kan^s rha^+ $metB^+$	0
		kan^r rha^+ *metB1*	11.5	kan^s rha^+ *metB1*	1
		kan^r *rha-1* $metB^+$	6.0	kan^s *rha-1* $metB^+$	23.5
		kan^r *rha-1 metB1*	5.0	kan^s *rha-1 metB1*	36.5

Recipient: GE451 (*rha-1*, kan^s, *glpK1*, *metB1*); donor: GE448 (rha^+, kan^r, $glpK^+$, $metB^+$)

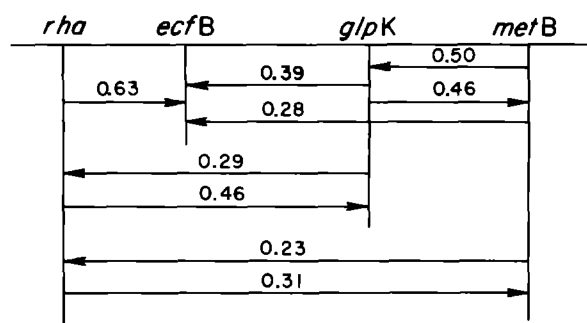

Fig. 1. Cotransduction of *rha-1*, *ecfB3*, *glpK1* and *metB1*

cotransducible with *ilv*, *rha* or *aroE*. *Kan-4* cotransduced with *ilv* at a frequency similar to *unc-2*; we assume it is an *unc* mutation (*unc-4*). In four of the mutants (nr. 1, 5, 6 and 7) resistance was cotransducible with *rha* and *glpK* at frequencies comparable to that of *kan-3* (*ecfB3*). These mutations are assumed to represent the *ecfB* locus. Mutants nr. 8 and 9 (GE466 and GE467) carry mutations located in the cluster of ribosomal genes at min. 72. These mutations, *kan-8* in GE466 and *kan-9* in GE467, were cotransduced at frequencies of 50–60% with the *aroE* locus. Further mapping was carried out using strains GE489 and GE490 which are Str-R (*rpsL*) derivatives of GE466 and GE467, respectively. Close linkage of the *kan-8* and *kan-9* mutations to the *rpsL* locus was observed (Table 5) and they may be mutations of the *rpsL* locus itself. Results of mapping primary *Kan-R* mutations of AB663 are summarized in Table 6.

When selection of primary Kan-R mutations was carried out at 13–15 μg/ml (see Table 2), the class of *ecfB* mutations was excluded. Most mutations obtained this way were cotransducible with *aroE* (Thorbjarnardóttir, unpublished observations).

Table 5. Map position of *kanA8* (kan^r) and *kanA9* (kan^r) determined by transduction with P1

Recipient	Donor	Selection	Number of trans-ductants tested	Distribution of unselected markers (%)	
GE314	GE489	$aroE^+$	200	kan^r str^r	67.5
				kan^r str^s	0
				kan^s str^r	1.0
				kan^s str^s	31.5
GE314	GE490	$aroE^+$	100	kan^r str^r	46.0
				kan^r str^s	0
				kan^s str^r	1.0
				kan^s str^s	53.0
AT2455	GE489	$cysG^+$	175	kan^r str^r	12.6
				kan^r str^s	0
				kan^s str^r	0.6
				kan^s str^s	86.8
AT2455	GE490	$cysG^+$	200	kan^r str^r	7.5
				kan^r str^s	0
				kan^s str^r	0
				kan^s str^s	92.5

The recipient strains are kan^s, str^s. The donor strains GE489 and GE490 carry the *kanA8*, *strA148* and *kanA9*, *strA149* mutations, respectively

The symbol *kanA* will be used for mutations such as *kan-8* and *kan-9* which are located in the cluster of ribosomal genes at min. 72. However, this use of the *kanA* symbol is not intended to suggest that all such mutations are in the same gene.

Isolation and Characterization of Secondary Kan-R Mutants. Spontaneous mutants of strain GE440 resistant to 30 μg/ml of kanamycin were obtained at a frequency of 10^{-9} (Table 2). Mapping of two such derivatives demonstrated the presence of a second

Table 6. Classification of primary Kan-R mutants of AB663

Original designation	Map location, minute	New designation
kan-1	86.5	*ecfB1*
kan-2	83	*unc-2*
kan-3	86.5	*ecfB3*
kan-4	83	*unc-4*
kan-5	86.5	*ecfB5*
kan-6	86.5	*ecfB6*
kan-7	86.5	*ecfB7*
kan-8	72	*kanA8*
kan-9	72	*kanA9*

resistance mutation, cotransducible with the *aroE* locus at a frequency of 50–70%.

To facilitate mapping in the *aroE* region, similar mutations were obtained in strain GE441 which is a Spc-R (*rpsE19*) derivative of GE440 (Table 2). One mutant of this strain, GE442, was studied in more detail. It harbors the mutation *kanA10* which is cotransducible with *aroE*, *rpsE* (*spcA*) and *cysG*, the order of these loci being *aroE–rpsE–kanA10–cysG* (Table 7). Mapping of this mutation was carried out using strain GE443 which is a Str-R derivative of strain GE442. As shown in Table 7, *kanA10* is closely linked to the *rpsL* (*strA*) locus. The data of Table 7 suggest the order *rpsE–kanA10–rpsL187*. Like *kanA8* and *kanA9*, the *kanA10* mutation may be a mutation of the *rpsL* locus.

Isolation and Characterization of Tertiary Kan-R Mutants. Spontaneous mutants resistant to 90 μg/ml of kanamycin were derived from strain GE442 which

Table 7. Mapping of *kanA10* (*kan*^{r}) by P1 transduction

Recipient	Donor	Selection	Number of transductants tested	Distribution of unselected markers (%)	
GE314 (*kan*^{s}, *str*^{s}, *spc*^{s})	GE442 (*kan*^{r}, *str*^{s}, *spc*^{r})	*aroE*$^+$	385	*kan*^{r} *spc*^{r}	69.1
				kan^r *spc*^{s}	1.0
				kan^s *spc*^{r}	21.0
				kan^s *spc*^{s}	8.9
GE314	GE443 (*kan*^{r}, *str*^{r}, *spc*^{r})	*aroE*$^+$	974	*kan*^{r} *str*^{r}	66.9
				kan^r *str*^{s}	0.8
				kan^s *str*^{r}	0.4
				kan^s *str*^{s}	31.9
AT2455 (*kan*^{s}, *str*^{s}, *spc*^{s})	GE442	*cysG*$^+$	150	*kan*^{r} *spc*^{r}	4.0
				kan^r *spc*^{s}	12.0
				kan^s *spc*^{r}	0
				kan^s *spc*^{s}	84.0
AT2455	GE443	*cysG*$^+$	850	*kan*^{r} *str*^{r}	17.6
				kan^r *str*^{s}	0
				kan^s *str*^{r}	1.2
				kan^s *str*^{s}	81.2

carries the *ecfB1* and *kanA10* mutations. The frequency of such mutations in this strain is $\sim 10^{-9}$ (Table 2).

Three such tertiary Kan-R mutants, GE444, GE445 and GE446, were studied. Strain GE444 was unable to grow on succinate, and the third resistance mutation of this strain mapped at the *unc* locus. Strains GE445 and GE446 carry additional mutations cotransducible with *kanA10*.

Resistance Mutations Induced by Tn5. The isolation of Tn5 induced amikacin resistant (Amk-R) mutants of strain MO is described in Materials and Methods. Use was made of the fact that Tn5 confers high level resistance to neomycin and kanamycin but not to amikacin[1]. P_1 lysates were made of 4 such mutants and tested for cotransduction of Amk-R and Neo-R with *glpK*. One strain (OSA50) was characterized further; when *rha*$^+$, *glpK*$^+$ or *metB*$^+$ was used as the selective marker, Neo-R and Amk-R were 100% cotransducible and mapped in approximately the same location as *ecfB1* and *ecfB3*. When Neo-R was used for selection only about 12% of the transductants were Amk-R, and none of the Neo-R, Amk-S transductants were Rha$^+$, GlpK$^+$ or MetB$^+$. We attribute this abnormality to spontaneous transposition of Tn5 from the segment of transducing DNA to various sites in the chromosome. Our interpretation is strengthened by the frequent occurrence (1–2%) of auxotrophic mutants among Neo-R, Amk-S transductants, presumably due to insertion of Tn5.

OSA50 grows slowly on succinate, a characteristic which we have been unable to separate from Amk-R. Together with the mapping data this leads us to believe that in OSA50 the transposon Tn5 is inserted in the *ecfB* locus.

Minimal Inhibitory Concentrations (MIC's) and Antibiotic Disc Sensitivities. We have determined the MIC's to seven antibiotics and have also examined the relative sensitivities to nine antibiotics by disc sensitivity tests. The results obtained with the isogenic series GE453 through GE459 are presented in Table 8.

In general the results of the MIC and disc tests agree. In all cases the three mutations exhibit a cumulative increase in resistance when combined. Of the three mutations the *kanA10* mutation is the most specific. As judged by disc tests, it confers high levels of resistance to kanamycin and none to streptomycin. Sensitivity tests with OSA50 (*ecfB*::Tn5) and control strains revealed that the Tn5 insertion mutation has effects similar to the *ecfB1* and *ecfB3* mutations.

[1] Amikacin is a semisynthetic derivative of kanamycin that is not modified by the phosphotransferase coded by Tn5

Table 8. MIC's and antibiotic discs

	GE453	GE454	GE455	GE456	GE457	GE458	GE459
Genotype							
unc	+	+	2	2	+	+	2
ecfB	+	3	+	3	+	3	3
kanA	+	+	+	+	10	10	10
Antibiotic inhibition zone (mm)							
amp	19	13	18	18	19	14	13
tet	24	25	27	26	26	21	20
cam	10	9	14	14	11	9	9
spc	–	–	–	–	11	10	8
str	21	17	16	13	21	17	13
kan	17	12	9	–	8	–	–
gen	20	16	12	10	16	11	–
amk	23	20	17	15	20	17	9
neo	18	15	15	12	16	13	–
Minimal inhibitory concentration (μg/ml)							
cam	8	8	2	2	4	4	4
spc	1024	256	1024	1024	128	128	64
str	1	2	4	8	1	2	8
kan	0.25	0.5	2	4	0.5	4	16
gen	0.5	0.5	1	4	0.5	1	8
amk	0.5	2	4	4	2	4	16
neo	0.25	1	1	4	1	4	8

Amp: ampicillin, tet: tetracycline, cam: chloramphenicol, spc: spectinomycin, str: streptomycin, kan: kanamycin, gen: gentamicin, amk: amikacin, neo: neomycin. –: indicates no observable inhibition zone

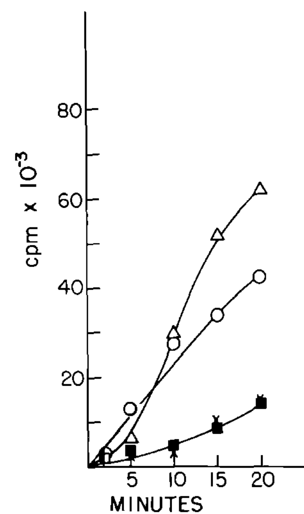

Fig. 2. Accumulation of ^{3}H-gentamicin. ○ GE453, △ GE454 (*ecfB3*), × GE455 (*unc-2*), ■ GE457 (*kanA10*)

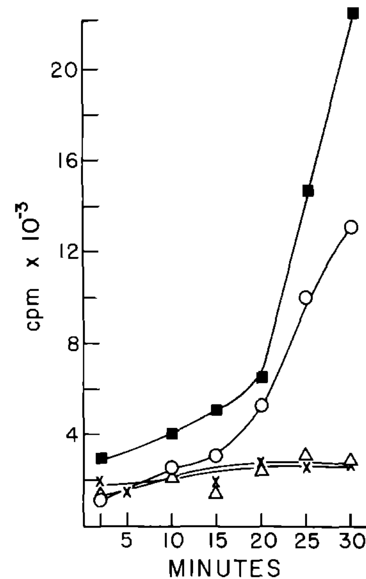

Fig. 3. Accumulation of ^{3}H-dihydrostreptomycin. ○ GE453, △ GE454 (*ecfB3*), × GE455 (*unc-2*), ■ GE457 (*kanA10*)

The series isogenic with AB663 gave results identical to the strains in Table 8. In addition we observed: 1) the presence of a *strA* mutation increased resistance to other aminoglycosides but increased the sensitivity to chloramphenicol and tetracycline slightly (data not shown); 2) the tertiary Kan-R mutations in GE445 and GE446 (*kanA12* and *kanA13*) drastically decreased the sensitivity to kanamycin but concomitantly increased sensitivity to tetracycline and chloramphenicol, and the strains became hypersensitive to kasugamycin.

Preliminary studies of protein synthesis in vitro indicate that the ribosomes of *kanA10* strains have decreased aminoglycoside induced misreading and that the *kanA12* and *kanA13* mutations further decrease this misreading (Andrésson, unpublished).

MIC's and disc tests indicate that the *kanA10* mutation phenotypically suppresses the *spcA* mutation (i.e. increases sensitivity to spectinomycin) as has been described for the *nek* (Apirion and Schlessinger, 1969) and *mod* (Berg and Kang, 1975) mutations.

Accumulation of Gentamicin and Dihydrostreptomycin. An isogenic series of four strains, GE453, GE454 (*ecfB3*), GE455 (*unc-2*) and GE457 (*kanA10*) was used to study accumulation of ^{3}H-gentamicin (Fig. 2) and ^{3}H-dihydrostreptomycin (Fig. 3). With GE453 and derivatives the rate of antibiotic accumulation correlated with sensitivity disc and MIC results, with one exception (GE457).

Accumulation of ^{3}H-gentamicin and ^{3}H-dihydrostreptomycin was also tested with derivatives of AB663 (Figs. 4 and 5). Two derivatives of AB663 did not exhibit a parallel decrease of antibiotic accumulation and antibiotic sensitivity: 1) despite an 8-fold higher

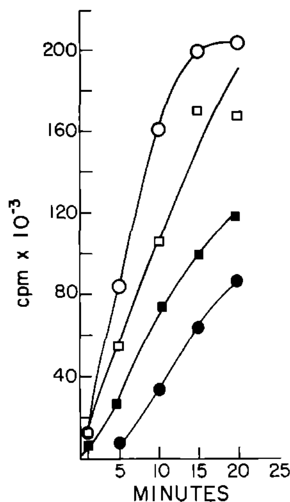

Fig. 4. Accumulation of ^{3}H-gentamicin. ○ AB663, □ GE447 (*unc-2*), ● GE440 (*ecfB1*), ■ GE448 (*ecfB3*)

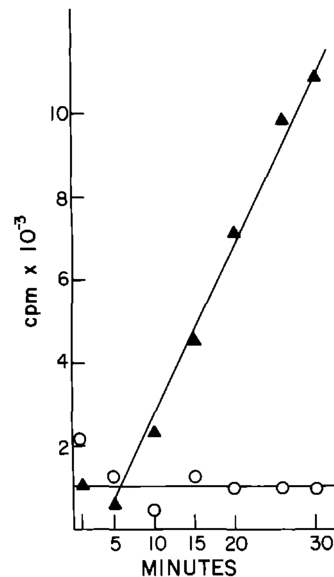

Fig. 6. Accumulation of ^{3}H-gentamicin. ▲ OSA52 (*ksgA*::Tn5), ○ OSA50 (*ecfB*::Tn5)

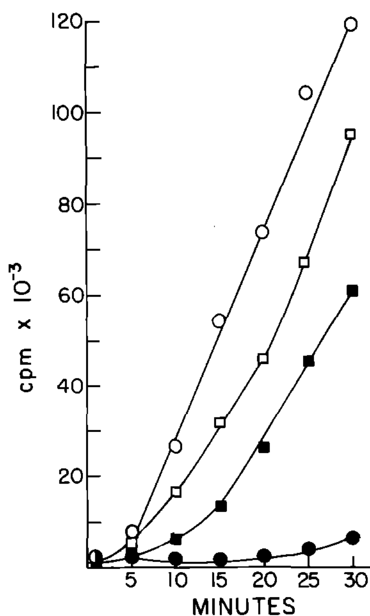

Fig. 5. Accumulation of ^{3}H-dihydrostreptomycin. ○ AB663, □ GE447 (*unc-2*), ● GE440 (*ecfB1*), ■ GE448 (*ecfB3*)

MIC, antibiotic accumulation of GE447 (Figs. 4 and 5) was not appreciably affected; 2) although GE440 (*ecfB1*) and GE448 (*ecfB3*) were equally sensitive to gentamicin, GE440 accumulated markedly less gentamicin (Fig. 4). That the product of *ecfB* is, in fact, important for gentamicin accumulation is shown by data in Figure 6, since the Tn5 insertion in *ecfB* (OSA50) causes a drastic decrease in gentamicin accumulation.

Discussion

In this paper we show that resistance to relatively high levels of kanamycin and related aminoglycoside antibiotics can be obtained as the result of the cumulative effect of mutations which separately confer resistance to low levels of antibiotic. The mutations have been mapped within three different regions of the chromosome: 1) in the cluster of ribosomal protein genes at min. 72; 2) at the *unc* locus near min. 83; 3) at a locus, *ecfB*, between min. 86 and 87.

Of the resistance mutations near minute 72, three (*kanA8, kanA9* and *kanA10*) were found to map close to the *rpsL* (*strA*) locus. Preliminary results with other mutations of this region (*kanA12, kanA13*) indicate that they are much closer to *rpsL* than to *rpsE* (*spcA*). The same was found to be true for two mutations which jointly cause resistance to 30–40 µg/ml of kanamycin in a strain of *E. coli* (NF119) not used in this study (R.Á. Magnúsdóttir and J. Kristjánsson, unpublished experiments). Studies of protein synthesis in vitro confirm the suggestion that the *kanA10, kanA12* and *kanA13* mutations alter the properties of the ribosomes (Andrésson, unpublished).

There is only one previous report of a mutation to kanamycin resistance which maps close to *rpsL* (Hull et al., 1976); in other cases such mutations have been mapped close to *rpsE* (Apirion and Schlessinger, 1968a and b; Brown and Apirion, 1974; Berg and Kang, 1975). The *rpsE* linked *nek* mutations studied by Apirion and Schlessinger confer resistance to 100 µg/ml of kanamycin and to 150 µg/ml of neomycin. We did not find spontaneous single step mutants resistant to 90 µg/ml of kanamycin (frequency

$<3\times10^{-11}$, see Table 2). This is in agreement with the results of Apirion and Schlessinger (1968a); the high level *nek* mutations described by these authors were obtained at low frequencies after extensive mutagenesis with nitrosoguanidine. We therefore suggest that the *nek* mutants harbor multiple mutations and that resistance to high levels ($\sim$100 μg/ml) of kanamycin cannot be attained by point mutation.

A possible explanation is as follows: the ribosome may have multiple kanamycin binding sites with different affinities. By mutating the highest affinity site, the ribosome would become resistant to low levels of drug. A higher drug concentration would be needed to bind to the lower affinity site(s). Thus *kanA8*, *kanA9* and *kanA10*, may be mutations of the primary site and *kanA12* and *kanA13* may be mutations of a second site or mutations further decreasing binding to the first site.

Apirion and Schlessinger (1969) showed that the phenotypic suppresion of Spc-R mediated by *nek* mutations is a ribosomal property. These authors explained phenotypic suppression on the basis of interdependence of ribosomal components and suggested that the ribosomal binding sites for spectinomycin and neomycin are partially interdependent.

Since mutations to resistance to aminoglycosides that induce misreading make the ribosome more "restrictive" (i.e. decrease the rate of amino acid misincorporation), an alternative explanation for phenotypic suppression is possible. Based on models such as those of Ninio (1974) and Hopfield (1974), one might expect ribosomes of *kan* and *nek* mutants to show a higher degree of discrimination towards incorrectly paired aminoacyl-tRNAs. This would be achieved by lengthening the discrimination period before irreversible binding of the aminoacyl-tRNA (i.e. increasing the severity of the "kinetic proofreading"). By increasing the length of the discrimination period, the ribosome may become more susceptible to antibiotics that bind at that stage. Sensitivity to chloramphenicol and tetracycline, both of which are thought to act by blocking the A site of the ribosome, is increased by the *kanA10*, *kanA12*, and *kanA13* mutations, which cause decreased kanamycin induced misreading.

Although the product of *ecfB* may be directly involved in accumulation of gentamicin (Fig. 6), it is also possible that the *ecfB* mutations alter the Ca^{++}-Mg^{++} ATPase acitvity and thus indirectly the proton-motive force. Cox et al. (1973) found a mutant containing a lesion that maps at or near the *ecfB* locus having increased ATPase activity. The mutant studied by Plate (1976) also has an increased Mg^{++}-ATPase activity, and strain GE448 was found to have increased Mg^{++}-ATPase activity, whereas GE440 showed levels comparable to wild type (data not shown).

It is well established that mutations affecting the binding of dihydrostreptomycin to the ribosome alter the accumulation of dihydrostreptomycin and streptomycin by whole cells (Szybalski and Mashima, 1959; Chang and Flaks, 1972; Bryan et al., 1976). In this paper we have shown that the *kanA10* mutation that maps close to *strA* (*rpsL*), and may be a ribosomal alteration, affects the accumulation of gentamicin (Fig. 2). Buckel et al. (1977) have isolated gentamicin resistant mutants exhibiting properties similar to the kanamycin resistant mutants described in this paper. Several of the mutants described by Buckel and collaborators have an altered ribosomal protein L6. We have evidence that a wide variety of such ribosomal mutants can be obtained; these mutants may prove useful in studying the interaction of aminoglycoside antibiotics with their primary target, the ribosome.

In summary, at least three types of mutations increase resistance to kanamycin and related aminoglycosides in *E. coli*. Two of them, *unc* and *ecfB*, apparently affect the rate limiting step of accumulation by decreasing the electrochemical gradient or by altering the cell membrane, and the third type alters the ribosome.

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