

## Isolation and Characterization of Mutants of *Escherichia coli* K12 Resistant to the New Aminoglycoside Antibiotic, Amikacin

By R. HULL, J. D. KLINGER AND E. E. M. MOODY

*Department of Microbiology, The University of Texas,  
Health Science Center at San Antonio, San Antonio, Texas 78284, U.S.A.*

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### SUMMARY

Spontaneous mutants of *Escherichia coli* K12 that are resistant to the new aminoglycoside antibiotic, amikacin, were isolated. These mutants have simultaneously acquired cross-resistance to kanamycin, gentamicin and neomycin but not to streptomycin or spectinomycin. Sensitivity of the mutant strains to the non-aminoglycoside antibiotics, ampicillin, tetracycline and polymyxin, was unaffected.

The mutation responsible for amikacin resistance was mapped by P1 transduction and found to be tightly linked to *strA*, distal with respect to *spcA* and *aroE*.

### INTRODUCTION

Since the onset of antibiotic chemotherapy, drug resistance among Gram-negative micro-organisms has become increasingly prevalent (Cooksey, Bannister & Farrar, 1975; Snelling *et al.*, 1971). Bacteria may become resistant to antibiotics in several ways: mutational alteration of the target site (Weisblum & Davies, 1968); alteration of cellular permeability to the drug (Yamado & Davies, 1971); or production of antibiotic inactivating enzymes under the direction of either chromosomal or plasmid genes (Benveniste & Davies, 1973; Bowman *et al.*, 1968). The molecular mechanisms involved in determining resistance to many commonly used drugs have been described in detail elsewhere (cf. Benveniste & Davies, 1973).

Most chromosomal mutations conferring resistance to the aminoglycoside antibiotics result in a change in the structure of the ribosomal subunits, decreasing their ability to interact with the antibiotic (Apirion & Schlessinger, 1969; Davies, 1971; Weisblum & Davies, 1968). In particular, mutations toward resistance to the aminoglycosides kanamycin and neomycin have been shown to affect the same site on the 30 S ribosomal subunit of *Escherichia coli*, i.e. mutations effecting kanamycin resistance also confer resistance to neomycin and have been mapped at the *nek* locus in the ribosomal cluster of the *E. coli* chromosome (Apirion & Schlessinger, 1968*a, b*).

Amikacin (BB-K8) is a new semi-synthetic aminoglycoside antibiotic structurally similar to kanamycin but refractory to the action of the kanamycin phosphotransferase enzyme produced by resistance (R) plasmids (Clark *et al.*, 1974; Kawaguchi *et al.*, 1972; Yu & Washington, 1974). Its broad spectrum of antimicrobial activity has made it particularly useful in the treatment of gentamicin- and kanamycin-resistant organisms (Howard & McCracken, 1975; Yourassowsky, Schoutens & Vanderlinden, 1975). However, the emergence of R plasmids cross-resistant to amikacin and kanamycin has already been reported

(Kawabe *et al.*, 1975; Kawabe, Naito & Mitsuhashi, 1975). Unstable chromosomal mutants of *Pseudomonas* with increased resistance to amikacin have also been observed (Holmes *et al.*, 1974).

In this communication, we report the isolation of stable chromosomal mutants of *E. coli* K12 which are resistant to inhibition by this derivative.

#### METHODS

*Strains.* *Escherichia coli* K12 strains used were DM506 (as AB1157, also *nal<sup>r</sup>*), KL14 (prototrophic, Hfr) and AB2828sp (*aroE<sup>-</sup>*, *spc<sup>r</sup>*). *Escherichia coli* derived from ATCC25922 and *Pseudomonas aeruginosa* derived from ATCC27853 were used as control organisms in antibiotic sensitivity testing.

*Media.* The minimal medium was that described by Davis & Mingioli (1950). Plate lysates of phage P1 for transductions were prepared on LC agar containing (per litre): 10 g tryptone; 5 g yeast extract; 5 g NaCl; 1 g glucose; and 25 mg thymidine (Willets & Achtman, 1972). Nutrient broth and nutrient agar plates were made from Wilson Nutrient Extract Broth no. 2. All media, except those used for sensitivity assays, were solidified using 1.25% (w/v) Davis New Zealand agar.

*Transductions.* Exponentially-growing recipient bacteria were concentrated 10-fold to  $2 \times 10^9$  bacteria  $\text{ml}^{-1}$  in nutrient broth containing 1 mM- $\text{CaCl}_2$  and 1 mM- $\text{MgSO}_4$ . These cultures were infected with P1 transducing phage at a multiplicity of 0.1. After 20 min static incubation at 37 °C, the bacteria were washed to remove unadsorbed phage and spread on appropriate minimal agar plates.

*Antimicrobial sensitivity tests.* These were performed using standard procedures for agar (Washington, 1974) and tube-dilution methods (Washington & Barry, 1974). Liquid cultures were prepared in Mueller-Hinton broth (MHB) (BBL, lot 209668). Calcium and magnesium ion concentrations were adjusted to 75 and 28 mg  $\text{l}^{-1}$ , respectively. After inoculation, cultures were incubated in a shaking water bath at 37 °C until a density of approximately  $10^8$  bacteria  $\text{ml}^{-1}$  was reached. Such suspensions were diluted 1:10 in MHB, and inoculated with a  $10^{-3}$  ml calibrated loop on to the surfaces of Mueller-Hinton agar (MHA) plates containing antibiotic dilutions. A mixture of two lots of MHA (Difco, lots 608887 and 603865), containing 0.405 parts and 0.595 parts respectively, was used as the agar base in all agar dilution procedures. The MHA mixture was analysed using a Perkin-Elmer 403 atomic absorption spectrophotometer and was found to contain 73.7 (mg  $\text{Ca}^{2+}$ )  $\text{l}^{-1}$  and 36.6 (mg  $\text{Mg}^{2+}$ )  $\text{l}^{-1}$ . Dilutions of reagent amikacin (Bristol Laboratories), gentamicin sulphate (Garamycin, Schering Corporation), kanamycin sulphate (Bristol Laboratories) and neomycin sulphate (Mycifradin sulphate, The Upjohn Company) were prepared in MHA. The minimum inhibitory concentration (m.i.c.) was taken as the lowest concentration of antibiotic at which no growth of the organism, or a barely visible haze on the agar surface, was seen (Ericsson & Sherris, 1971).

Disc sensitivity tests were done using the MHA mixture described above, and inocula were obtained from MHB cultures of approximately  $10^8$  bacteria  $\text{ml}^{-1}$ . Discs containing 10 µg ampicillin, 300 units polymyxin B, and 30 µg tetracycline were used.

Mueller-Hinton broth, as described above, was used to establish minimum bactericidal concentrations (m.b.c.); cultures were prepared by inoculating 0.5 ml of a  $10^{-3}$  dilution of a MHB culture (approximately  $10^7$  bacteria  $\text{ml}^{-1}$ ) into 0.5 ml of antibiotic serially diluted in MHB. Samples (0.1 ml) were taken from tubes showing no visible turbidity after 18 to 24 h incubation at 37 °C and spread on to the surfaces of nutrient or tryptic-soy agar

Table 1. *Minimum inhibitory concentrations of aminoglycoside antibiotics and minimum bactericidal concentrations of amikacin*

Species	Strain	m.i.c. ( $\mu\text{g ml}^{-1}$ )				m.b.c. ( $\mu\text{g ml}^{-1}$ )
		AMK*	GM	NEO	KAN	
<i>E. coli</i>	DM506	3.12	0.78	10	3.12	6.25
	KLI4	3.12	1.56	10	6.25	6.25
	KLI4r3	25	6.25	10	50	50
	DM506r1	50	25	62.5	> 50	75
	DM506r2	50	25	31.3	> 50	75
	ATCC25922	3.12	1.56	10	3.12	12.5
<i>P. aeruginosa</i>	ATCC27853	3.12	1.56	250	> 50	—

\* Abbreviations designate aminoglycoside antibiotics: AMK, amikacin; GM, gentamicin; NEO, neomycin; KAN, kanamycin.

plates. The m.b.c. was taken as that concentration of antibiotic from which no growth was obtained upon sub-culture.

*Isolation of mutants.* Spontaneous mutants resistant to amikacin were isolated in the following manner. Bacteria were spread on a nutrient agar plate and grown overnight to confluence. A (2 mm)<sup>3</sup> sample was scraped from the agar surface, inoculated into 500 ml nutrient broth containing 5  $\mu\text{g amikacin ml}^{-1}$  and incubated with aeration at 37 °C overnight. Survivors were isolated on nutrient agar plates containing 5  $\mu\text{g amikacin ml}^{-1}$  and verified as having nutritional requirements identical with the parent strain. Two mutants derived from DM506 and once from KLI4 were selected for further study.

## RESULTS

The level of antibiotic resistance was measured by determining the m.b.c. of amikacin and the m.i.c. of amikacin and the related aminoglycoside antibiotics kanamycin, neomycin and gentamicin for each isolate (Table 1). Though the effects of media inconsistencies on aminoglycoside sensitivity results are most pronounced in strains of *P. aeruginosa* (Price *et al.*, 1972; Reller *et al.*, 1974), a similar variation, attributed to cation content, has been noted among other Gram-negative organisms, including *E. coli* (Martin *et al.*, 1971; Sanders & Sanders, 1975). The  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contents of the MHA employed are similar to the values suggested by Reller *et al.* (1974), and the mixture was found to be reliable in disc-sensitivity testing using 10  $\mu\text{g amikacin discs}$  and a control strain of *P. aeruginosa*. The mean of several measurements of amikacin inhibition zones for the control organism on this mixture was  $16.3 \text{ mm} \pm 0.833$  (S.D.).

The results show that mutants isolated because of their resistance to amikacin have simultaneously acquired resistance to kanamycin, neomycin and gentamicin suggesting that the molecular basis of resistance to each is the same. In order to exclude the possibility that these mutants were multiply resistant as a result of a non-specific alteration in cellular permeability, their sensitivity to the structurally unrelated antibiotics tetracycline, ampicillin and polymyxin was tested by measuring the size of the zone of inhibition around sensitivity discs. In each case, the mutants were found to be as sensitive to the drug as the parent strain confirming that the mutations are specific for aminoglycoside antibiotics and not a generalized effect (unpublished results). In addition, sensitivity or resistance to the aminoglycosides, streptomycin and spectinomycin, was unaffected as shown below.

Table 2. Ordering of *amk<sup>r</sup>* with respect to adjacent loci by three-point analysis

*Escherichia coli* K12 strain DM506 (*amk<sup>r</sup>*, *str<sup>r</sup>*) was used as the donor and strain AB2828sp (*str<sup>s</sup>*, *amk<sup>s</sup>*, *spc<sup>r</sup>*) as the recipient. The selected marker was *aroE<sup>+</sup>*. The number of recombinants scored was 497.

Recombinant class	Number
<i>str<sup>r</sup></i> , <i>amk<sup>r</sup></i>	192 (39 %)
<i>str<sup>s</sup></i> , <i>amk<sup>s</sup></i>	279 (56 %)
<i>str<sup>r</sup></i> , <i>amk<sup>s</sup></i>	20 (4 %)
<i>str<sup>s</sup></i> , <i>amk<sup>r</sup></i>	6 (1 %)
<i>spc<sup>r</sup></i> , <i>amk<sup>s</sup></i>	125 (25 %)
<i>spc<sup>s</sup></i> , <i>amk<sup>r</sup></i>	180 (36 %)
<i>spc<sup>s</sup></i> , <i>amk<sup>s</sup></i>	174 (35 %)
<i>spc<sup>r</sup></i> , <i>amk<sup>r</sup></i>	18 (4 %)

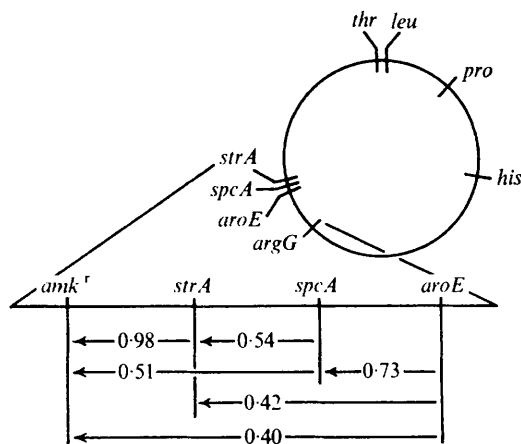


Fig. 1. Location of the amikacin locus on the circular linkage map of *E. coli* K12. Reference loci are placed as described by Taylor & Trotter (1972). Arrows point to the unselected donor markers. Distances are not drawn to scale.

A genetic analysis was undertaken to determine the map position of the mutation responsible for amikacin resistance. *Escherichia coli* K12 strain AB2828sp was transduced to *aroE<sup>+</sup>* using transducing phage grown on one of the amikacin-resistant DM506 derivatives. Recombinants were then scored for inheritance of the *strA*, *spcA* and amikacin alleles. These results are shown in Table 2 and Fig. 1. The recombinant classes listed for the three point analysis described in Table 2 are arranged so that, given the gene order shown in Fig. 1, the class of recombinants that requires four crossover events, and is thus least frequent, is shown in the last row. These results indicate that the genetic locus for ribosomal resistance to amikacin is tightly linked to the *strA* gene and is located distal to *strA* with respect to *aroE*.

#### DISCUSSION

Cross-resistance to related aminoglycoside antibiotics after mutation to resistance to one of them is frequently observed in both clinical and laboratory strains (Kawaguchi, 1975; Price *et al.*, 1974; Weinstein *et al.*, 1971). Price *et al.* (1974) have shown that isolates resistant to kanamycin are often cross-resistant to gentamicin, neomycin, butirosin and other aminoglycoside antibiotics. Apirion & Schlessinger (1968a) were able to isolate *E. coli*

mutants resistant to neomycin with cross-resistance to kanamycin. The affected genetic locus was designated *nek* and tentatively positioned near the ribosomal cistrons on the *E. coli* genome. However, attempts to position the *nek* locus more accurately were frustrated by phenotypic suppression of the spectinomycin resistance phenotype by some *nek*<sup>-</sup> alleles (Apirion & Schlessinger, 1969). They observed that when *spc*<sup>r</sup> *E. coli* derivatives were mutagenically altered to neomycin resistance (*nek*<sup>-</sup>), they became sensitive to spectinomycin. The continued presence of the *spc*<sup>r</sup> allele was verified genetically. As a consequence, recombinants of the *nek*<sup>-</sup> *spc*<sup>s</sup> class and the *nek*<sup>-</sup> *spc*<sup>r</sup> class became indistinguishable.

Since the amikacin-resistant mutants reported here have a cross-resistance spectrum similar to the mutants reported by Apirion & Schlessinger (1968*a*), we propose that amikacin resistance mutations are alleles of the *nek* gene. These *nek*<sup>-</sup> alleles do not seem to suppress *spc*<sup>r</sup> phenotypically since the observed linkage between *spcA* and *aroE* measured in the presence of this *nek*<sup>-</sup> allele is similar to that reported by Epstein & Kim (1971) for *nek*<sup>+</sup> strains. If the *spc*<sup>r</sup> mutations were phenotypically suppressed, the *spcA* locus would have appeared to be more closely linked to *aroE*.

We conclude that chromosomal mutations which affect the sensitivity of *E. coli* K12 to amikacin also confer cross-resistance to related aminoglycoside antibiotics. These mutations are phenotypically similar to those associated with the *nek* locus and are shown to be tightly linked to *strA*, distal to *spcA*.

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