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Mapping of Neamine Resistance: Identification of Two Genetic Loci, *nea* A and *nea* B

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Summary. Mutants of *Escherichia coli* resistant to the antibiotic neamine have been isolated. The mutants have been mapped and two separate loci for neamine resistance, *nea* A and *nea* B, have been identified on the genome. *Nea* A maps very closely to *spc* A and *nea* B is near to the *str* A locus. Both neamine resistance markers are situated, therefore, in the so-called ribosomal operon.

Introduction

Many antibiotics are inhibitors of protein synthesis with the ribosome as their primary site of action (for a review see Davies and Nomura, 1972). The aminoglycoside group of antibiotics is represented by some interesting and in some cases well-studied drugs. Streptomycin and spectinomycin block some function(s) of the 30 S subunit of the bacterial ribosome although the precise modes of action of these compounds, particularly *in vivo*, remain to be elucidated (for a review see Cundliffe, 1972). Other compounds in the group have been studied less extensively because of the difficulty found both in obtaining mutants resistant to the relevant drug and in characterization of the modes of action of the drugs *in vitro*. It is known, however, that neamine—an aminoglycoside related to neomycin—blocks protein synthesis in bacteria both *in vitro* and *in vivo* and the compound resembles streptomycin in that during protein synthesis *in vitro* it causes misreading of mRNA. Furthermore, when tested at sublethal concentrations against sensitive strains, neamine suppresses phenotypically nonsense and missense mutations, and mutants dependent for growth on either neamine or streptomycin display cross dependence between these drugs (Weisblum and Davies, 1968). It should be noted, however, that cross resistance between nea^R and str^R strains is not a general phenomenon (Davies, J., personal communication). A similar situation applies to some of the strains studied here.

In the present work mutants of *Escherichia coli* resistant to neamine have been selected. The mutations leading to resistance have been mapped and two separate loci have been identified on the genome. Since one locus maps near to the locus determined previously for spectinomycin resistance (*spc* A) and the other locus close to that which determines streptomycin resistance (*str* A), both genes—designated *nea* A and *nea* B respectively—are presumably located in the "operon" which controls the synthesis of ribosomal proteins (Nomura and Engbaek, 1972; Cabezon, Bollen and Faelen, 1974).

Materials and Methods

Bacterial Strains

E. coli K12-S———parental strain (sensitive to neamine, spectinomycin and streptomycin)
nea 301, *nea* 302 } nea^{R}_{50}—resistant to 50 μg/ml neamine

E. coli CA 244.1———*lac*$^{-}$, *try*$^{-}$———
nea 103, *nea* 104 } nea^{R}_{50}—resistant to 50 μg/ml neamine

E. coli AB 2834-280 (J. Davies)———*aro*E^{-}, *spc*^{R}, *mal*$^{-}$

E. coli AB 2834-272 (A. Bollen)———*aro*E^{-}, *str*^{R}, *mal*$^{-}$

E. coli AB 2834-M1 (J. Davies)———*aro*$^{-}$, B_1^{-}, *mal*$^{-}$, F^{-}———parental strain
nea UK1, *nea* UK2 } nea^{R}_{50}—resistant to 50 μg/ml neamine

E. coli AB 2847 (J. Davies)———*aro*B^{-}, B_1^{-}, *mal*$^{-}$, F^{-}———parental strain
nea UK3 nea^{R}_{50}—resistant to 50 μg/ml neamine

E. coli AB 2847-M1 (J. Davies)———*aro*B^{-}, B_1^{-}, *mal*$^{-}$, F^{-}, *str*^{R}———parental strain
nea UK4 nea^{R}_{50}—resistant to 50 μg/ml neamine

E. coli K12-3000 (W. Hayes)

Table 1. Transduction map-

Donor	Recipient	Selected markers		
		*aro*E^{+}	*spc*	*aro*B^{+}
nea301—*aro*E^{+} *spc*$^{+}$ *nea*	AB2834—*aro*E *spc nea*$^{+}$	177	—	—
AB2834—*aro*E *spc nea*$^{+}$	nea301—*aro*E^{+} *nea spc*$^{+}$	—	119	—
nea302—*aro*E^{+} *spc*$^{+}$ *nea*	AB2834—*aro*E *spc nea*$^{+}$	179	—	—
nea103—*aro*E^{+} *spc*$^{+}$ *nea*	AB2834—*aro*E *spc nea*$^{+}$	120	—	—
nea103—*aro*E^{+} *nea str*$^{+}$	AB2834-272—*aro*E *nea*$^{+}$ *str*	120	—	—
nea104—*aro*E^{+} *spc*$^{+}$ *nea*	AB2834—*aro*E *spc nea*$^{+}$	120	—	—
nea104—*aro*E^{+} *nea str*$^{+}$	AB2834-272—*aro*E *nea*$^{+}$ *str*	120	—	—
K12-3000—*aro*E^{+} *nea*$^{+}$	neaUK1—*aro*E *nea*	128	—	—
K12-3000—*aro*E^{+} *nea*$^{+}$	neaUK2—*aro*E *nea*	128	—	—
K12-3000—*nea*$^{+}$ *aro*B^{+}	neaUK3—*aro*B *nea*	—	—	128
K12-3000—*nea*$^{+}$ *aro*B^{+} *str*$^{+}$	neaUK4—*aro*B *nea str*	—	—	128

Isolation of Mutants

Cultures of *E. coli* were grown overnight at 37° by shaking in rich medium. Cells were sedimented by centrifugation and, after the medium had been decanted cells were resuspended in the remaining small volume of liquid. Cells were then spread on to nutrient agar plates containing 50 μg/ml of neamine, and the plates were incubated for 48 hr at 37°. Mutants, resistant to neamine, arose spontaneously. Colonies were selected and then re-streaked on nutrient agar plates containing neamine.

Transductions

The results presented in Table 1 were obtained by transduction of all strains using the bacteriophage P1 vir. P1 stocks grown on donor strains contained generally 10^{10} to 10^{11} phages per ml. Receptor strains grown to 10^9 bact/ml were centrifuged and resuspended in the same volume of 10 mM $MgSO_4$ and 5 mM $CaCl_2$. The multiplicity of infection was 0.2. After 20′ adsorption at 37°C with the phage, the bacteria were spreaded on selective medium and transductants selected either for *aro*E$^+$, *aro*B$^+$ or *spc*^{R}. No citrate was added to the plates since in the experimental conditions we used (low multiplicity of infection, P1 vir) there was no risk to obtain P1 lysogens or multiple adsorptions in the transductants.

The direct selection for *spc*^{R} transductants was done as described above up to the adsorption step. Then, 0.1 ml of the mixture was poured together with 5 ml agar (minimum medium plus requirements) onto a plate in the same medium. After 4 to 5 hours incubation at 37°C, spectinomycin mixed with 2.5 ml soft agar was poured on the plate in order to obtain a final concentration of 100 μg/ml. Incubation was then continued for 48 hrs at 37°C.

ping of neamine resistance

Unselected markers								Cotransduction (%)		Locus
*aro*E$^+$	*aro*E	*spc*$^+$	*spc*	*nea*$^+$	*nea*	*str*$^+$	*str*			
		139	38	46	131			*aro*E *spcA* *aro*E *nea* *spc*A *nea*	78 74 91	*nea*A
63	56			114	5			*spc*A *nea* *spcA aroE* *nea aroE*	96 47 43	*nea*A
		125	54	47	129			*aro*E *spcA* *aroE nea* *spcA nea*	71 72 97	*nea*A
		86	34	32	78			*aroE spcA* *aro*E *nea*	72 65	*nea*A
				43	77	35		*aroE strA* *aro*E *nea*	29 64	*nea*A
		93	27	42	78			*aro*E *spc*A *aro*E *nea*	78 65	*nea*A
				30	90	36		*aro*E *strA* *aro*E *nea*	30 75	*nea*A
				113	15			*aro*E *nea*	88	*nea*A
				31	97			*aro*E *nea*	24	*nea*B
				58	70			*aro*B *nea*	45	*nea*B
				45	83	27	101	*aro*B *nea* *aroB strA*	35 21	*nea*B

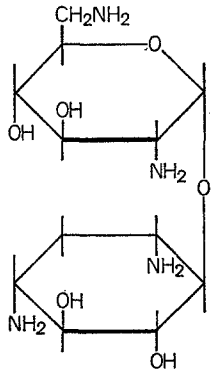

Fig. 1. Structure of neamine

Results and Discussion

The aminoglycoside antibiotic neamine has the structure depicted in Fig. 1. Mutants resistant to the drug (50 μg/ml) arose spontaneously with a frequency of about 10^{-7} and were easily selected and isolated. Cell-free extracts from some of these mutants have been used to study protein synthesis *in vitro* although so far we have been unable to demonstrate resistance to neamine in these experiments. Further work in this area is in progress.

Two independent neamine resistant mutants from each of the strains *E. coli* K12-S and CA 244.1 were selected and the mutations mapped using the *aro*E, *spc*A and *str*A loci as markers. The results presented in the upper part of Table 1 indicate that a locus for neamine resistance, designated *nea*A, maps very closely to *spc*A. Thus, in the mutants *nea* 301 and *nea* 302, *spc*A and *nea*A cotransduce with an average frequency of 95%. We think that the *nea*A locus maps to the right of *spc*A although we realize that before the precise location of *nea*A on the genome can be described it will be necessary to analyze many more transductants. However, although the data provided by the mutant *nea* 302 do not permit the *nea*A and *spc*A loci to be separated, the mutants *nea* 301, 103 and 104 all provide independent analyses which suggest that *nea*A maps further away from *aro*E than does *spc*A (see Fig. 2).

Mapping data for strains *nea* UK1-4 are presented in the lower part of Table 1. In *nea* UK1 the mutation to neamine resistance maps very closely to *aro*E and although we have no figures for the percentage cotransduction between *aro*E and *spc*A in this particular strain we suggest nevertheless that the mutation is at the *nea*A locus previously postulated from the results shown in the top portion of Table 1. Although *nea* UK1 and *nea* UK2 both derive from the same wild-type strain, *nea* UK2 clearly carries a different locus for neamine resistance than does *nea* UK1. Thus, in the latter mutant *aro*E and *nea*A cotransduce with a frequency of 88%. In contrast *aro*E and the gene designated *nea*B, controlling neamine resistance in *nea* UK2, cotransduce with a frequency of only 24%.

Finally, we have mapped two further mutations to neamine resistance in separate strains, one of which is coresistant to streptomycin, using the *aro*B locus as marker. Our data indicate that the mutation in both cases is the same and

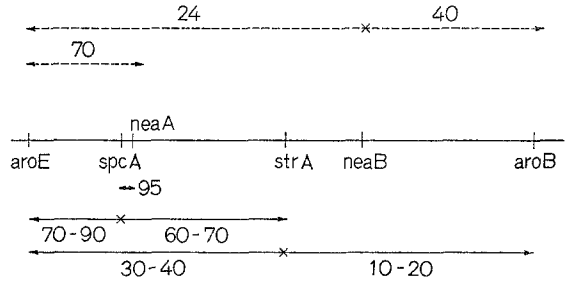

Fig. 2. Proposed linkage map showing the positions of the *nea* A and *nea* B loci. (——) Approximate P1 cotransduction frequencies as compiled from the results of Anderson, Gorini, Pittard and Davies (personal communication) and the present studies. (------) Proposed positions of the *nea* A and *nea* B genes on the map. Figures on the map refer to % cotransduction

we propose that it maps between *aro*B and *str*A. Furthermore, we feel that this mutation is at the *nea*B locus described above. Certainly our data provide convincing support for the presence of two separate genes controlling neamine resistance and the relative positions of these two genes *nea*A and *nea*B on the genetic linkage map are indicated in Fig. 2.

All the mutants we tested were independent from each other. They were all sensitive to neomycin and kanamycin, indicating that the *nea*A and *nea*B loci are clearly distinct from the *nek* locus reported by Apirion and Schlessinger (1968). There seems to be no interaction between the nea^R and spc^R mutations as judged by the cotransduction frequencies of these markers with *aro*E when they are present together in the same strain. We feel thus that we are dealing with different ribosomal constituants than the one(s) controlled by the *nek* gene. It should be pointed out also that our mutants were obtained spontaneoulsy whereas the nek^R ones showing interaction with the spc^R mutation (Apirion and Schlessinger, 1968) were selected after treatment with nitrosoguanidine which could have produced several neighbouring mutations (Guerola, Ingraham and Cerda-Olmedo, 1971).

Although we have no experimental evidence which would indicate that resistance to neamine, *in vivo*, can be correlated with an alteration in (a) specific ribosomal component(s) it is tempting to speculate that such a correlation could exist. The loci for *nea*A and *spc*A are closely adjacent on the genome and *nea*B maps near to *str*A. The ribosomal target sites for both streptomycin and spectinomycin have been determined as proteins S12 and S5 respectively (Ozaki, Mizushima and Nomura, 1969; Bollen, Davies, Ozaki and Mizushima, 1969) although the precise steps in protein synthesis inhibited by these antibiotics have not been unequivocally identified. Spectinomycin resistance has been examined in some detail and has been shown in two separate mutants to involve single amino acid substitutions in a specific region of protein S5 (Funatsu, Schiltz and Wittmann, 1972). More recent work (Burns and Cundliffe, 1973) has suggested that spectinomycin added to bacterial cells *in vivo* inhibits translocation of peptidyl-tRNA from the ribosomal A site to the P site. It may be coincidental that *nea*A and *nea*B are so closely associated on the genetic linkage map with *spc*A and *str*A respectively.

However, there is evidence that this region of the *E. coli* chromosome contains the genes which code for the proteins of both the 50 S and 30 S ribosomal subunits (Nomura and Engbaek, 1972). This cluster occupies only a small part of the map but still contains resistance markers to the antibiotics erythromycin and fusidic acid in addition to streptomycin and spectinomycin. Since all four inhibitors undoubtedly affect ribosomal function the implications following from the location of *nea*A and *nea*B in this gene cluster are obvious. We feel it is of particular interest that resistance to neamine is governed by one of two distinct mutations within this highly specific area of the bacterial genome.

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