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Coresistance to Neomycin and Kanamycin by Mutations in an *Escherichia coli* Locus that Affects Ribosomes

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Mutant strains resistant to neomycin or to kanamycin sulfate were isolated from *Escherichia coli* K-12. Nine mutants were analyzed; all were resistant to both antibiotics (about 150 and 100 μg/ml, respectively), and were designated *nek*. In the mutant strains, the ribosomes are changed from those of the parental strain; for when they were used in assays for polypeptide formation directed by polyadenylic acid or polycytidylic acid, coding fidelity in presence of the drugs was increased and inhibition of synthesis by the drugs was lessened. Mating experiments and transduction tests showed that all of the nine *nek* mutants are either closely linked or allelic, and the *nek* locus is closely linked to two genes—*str* (streptomycin) and *spc* (spectinomycin)—known to affect the 30*S* ribosome. The two *nek* mutants tested were recessive to the sensitive, wild-type allele. When the *nek* mutants were compared to the parental strain, pleiotropic effects of the *nek* mutations were observed. Resistance to low levels of streptomycin and spectinomycin was increased, whereas resistance to chloramphenicol was decreased. Also, the mutants were less able to adapt to high concentrations of lincomycin, and could no longer show phenotypic suppression of an arginine requirement by neomycin or kanamycin. Such pleiotropic effects are suggested to be the rule for mutations in genes that participate in the biosynthesis of a cellular organelle.

Because there appear to be at least 50 ribosomal proteins in *Escherichia coli* (9, 20), there is probably a comparable number of structural genes. Though it is not now clear whether any known mutation falls in a ribosomal structural gene (4), several approaches have permitted the isolation of mutants with altered ribosomes. One approach, exemplified here, involves the selection of mutants that are resistant to antibiotics that block protein synthesis.

Neomycin and kanamycin, like streptomycin, are aminoglycoside antibiotics. They share with streptomycin the ability to "cure" (i.e., phenotypically suppress) certain mutations in streptomycin-sensitive strains (10, 22); again, like streptomycin, they lose this ability in streptomycin-resistant strains (5). These observations, as well as a study of an *E. coli* mutant (very likely a multistep mutant) resistant to kanamycin (17), suggested that mutants resistant to these antibiotics have modified ribosomes.

Strains resistant to neomycin or to kanamycin have been isolated; this paper presents the analysis of nine such mutants. All were resistant to both neomycin and kanamycin, and were mapped near the streptomycin locus. The strains tested had ribosomes that differed from those of the parental wild-type strains. These mutants have been designated *nek*.

MATERIALS AND METHODS

Genetic techniques and incorporation tests were as described previously (3, 4), except that the ammonium acetate wash of ribosomes was omitted.

In crosses between two *nek* mutants (*see* Results), the selective markers were chosen in such a way that the portions of the chromosome likely to bear the *nek* mutations would necessarily be included in the zygotes.

The levels of resistance to antibiotics were gauged by replicating colonies from broth-agar onto the same medium containing the relevant antibiotics. Selection of mutants was also carried out on broth-agar plates supplemented with antibiotics.

All antibiotics were commerically obtained, except for spectinomycin sulfate, which was a gift from G. B. Witfield, The Upjohn Co., Kalamazoo, Mich. Streptomycin sulfate was obtained from Eli Lilly & Co., Indianapolis, Ind., neomycin sulfate and lincomycin hydrochloride, from The Upjohn Co.; kanamycin

sulfate, from Bristol Laboratories, Inc., Syracuse, N.Y.; and chloramphenicol, from Parke, Davis & Co., Detroit, Mich. Different lots of chloramphenicol show bacteriostatic potency that varies by as much as a factor of 4; in general, the pure product, containing no additives, gives more reproducible results. For all lots, the relative resistance of various strains is the same. For all the other drugs tested, results were invariable from batch to batch.

RESULTS

Isolation and characteristics of mutants. E. coli strains N141 or AB312 (*see* Table 1) were grown in broth, treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine), regrown in fresh medium for 4.5 hr, and plated on broth-agar containing different levels of neomycin sulfate or kanamycin sulfate or, as a control of the efficiency of mutagenesis, on broth-agar containing 200 μg of streptomycin per ml. Wild-type strains, tested by replication, are resistant to about 5 μg of streptomycin per ml, 5 μg of kanamycin per ml, and 20 μg of neomycin per ml. The highest concentrations of kanamycin and neomycin on which single-step mutants have been isolable are 100 and 150 μg/ml, respectively. When either antibiotic was present at a concentration of 200 μg/ml, no colonies were produced by a number of cells adequate to yield about 800 streptomycin-resistant mutants. In several trials in which either 80 μg of kanamycin per ml or 150 μg of neomycin per ml was used, the spontaneous frequency of mutants resistant to kanamycin or neomycin was found to be very low, of the order of 10^{-11}. This value is equivalent to about one-tenth the frequency of mutation to high-level streptomycin resistance, as determined by plating samples of the same culture on broth-agar plates containing 200 μg of streptomycin per ml (*see also* 15).

All mutants isolated on either drug are resistant to both drugs, and have been therefore designated *nek* (for neomycin and kanamycin). The strains used in this work are listed and described in Tables 1–3.

Pleiotropic effects. Comparison of the *nek* mutants with the parent strain revealed three effects of the *nek* mutations.

(i) *Changes in resistance to other antibiotics.* Eight *nek* mutants were tested by replication for resistance to streptomycin and spectinomycin; all showed an increased resistance to streptomycin, and most also showed an increased resistance to spectinomycin (Table 3). However, the levels of resistance were much lower than those usually observed for mutants isolated as *str*-HR or *spc*^{r} (5, 8, 15; also, cf. 17).

The resistance of *nek* mutants to several other antibiotics that affect ribosome function was also checked. The *nek* mutants were no more resistant to lincomycin or erythromycin than the parental strains. However, they were appreciably more sensitive to chloramphenicol: wild-type strains withstand about 10 μg of chloramphenicol per ml, but *nek* strains are able to resist less than 10 μg/ml (Table 3). This characteristic of the *nek* mutants enables one to select a few *nek*$^+$ cells from among a large population of *nek* cells. This was successfully done with all three *nek* mutants tried; the results with one of them, N733, are given in Table 4. The selection pressure is sufficient to permit the isolation of rare Nek-S (neomycin-kanamycin sensitive) recombinants from a cross of two *nek* strains; the results obtained in one such cross are given in Table 5. In this cross of *nek* strains N743 and N751, portions of the mating mixture were plated either on medium that permitted growth of *ad*$^+$ *his*$^+$ recombinants, or in the same medium supplemented with chloramphenicol. (Comparable numbers of the two strains were plated independently, but no revertants were found.) Colonies that grew on the medium containing chloramphenicol were streaked out; five colonies from each streak were tested for their characteristics. Most of the streaks gave rise to *nek*$^+$ colonies, identified by their wild-type sensitivity to neomycin and kanamycin. The frequency of recombination between the two unselected markers *mal* and *argG* was measured among a sample of *ad*$^+$ *his*$^+$ recombinants (all Nek-R) and among an equivalent sample of *ad*$^+$ *his*$^+$ *nek*$^+$ recombinants (50 colonies in each case). In the first instance, the recombination frequency was 28%; in the second, it was 37%. It therefore seems very likely that the *nek*$^+$ colonies isolated in this cross are true recombinants.

(ii) *Abolition of phenotypic suppression.* In the parental strain N141, the arginine requirement can be replaced if the medium is supplemented by low, nonlethal levels of streptomycin, kanamycin, or neomycin (2 μg/ml; these tests were carried out as described in reference 5). This phenotypic suppression (curability) by kanamycin and neomycin was lost in four *nek* mutants (N711, N712, N733, and N735), and was partially retained in the fifth mutant examined (N734). Phenotypic suppression by streptomycin was retained in three of these five *nek* strains, partially retained in N712, and abolished in N735 (*see* Discussion).

(iii) *Lessened adaptability to lincomycin.* Another difference between the response of *nek* strains and wild-type *E. coli* was observed in their response to phenotypic adaptation to high levels of lincomycin (4). Whereas the parental strain N141 adapted, growing up after about 24 to 48 hr, the *nek* mutants adapted only after a longer

TABLE 1. *Genotypes of neomycin- and kanamycin-sensitive strains*[a]

Strain	*lac*	*xyl*	*mal*	*ara*	*gal*	*ad*	*thi*	*thr*	*leu*	*met*	*arg*	*his*	*ilv*	*pro*	*rec*	*str*	*spc*	Episomes	Mating type	Direction of transfer	Source
N53	4	+	+	+	+	+	−	−	−	+	+	+	+	+	+	r	r		Hfr	← *str xyl met*	Sp[b] from AB312
N141	1	−	141	+	2	+	−	+	+	+	3	4	158	2	+	s	s		F⁻	—	Sp from AB774
AB258	−	−	3	−	−	+	−	−	−	+	+	+	+	+	+	s	s		F⁻	—	E. A. Adelberg (Yale Univ.)
AB301	+	+	+	+	+	+	+	+	+	1	+	+	+	+	+	s	s	λ	Hfr	← *thr pro his*	E. A. Adelberg
AB301-1	+	+	+	+	+	+	+	+	+	1	+	+	+	+	+	s	s		Hfr	← *thr pro his*	Sp from AB301
N302	−	−	3	−	−	+	−	−	−	+	+	+	+	+	+	s	r		F⁻	—	Sp from AB258
N304	1	−	+	+	2	+	−	+	+	+	3	4	158	2	+	s	r		F⁻	—	NG from AB774
N307	1	−	+	+	2	+	−	+	+	+	3	4	158	2	+	r	r		F⁻	—	Sp from N315
AB312	4	+	+	+	+	+	−	−	−	+	+	+	+	+	+	r	s		Hfr	← *str xyl met*	L. Gorini (Harvard Med. Sch.)
N315	1	−	+	+	2	+	−	+	+	+	3	4	158	2	+	r	s		F⁻	—	Ac from AB774
AB774	1	−	+	+	2	+	−	+	+	+	3	4	158	2	+	s	s		F⁻	—	E. A. Adelberg
KLF41	−	−	−	+	+	+	+	+	−	−	G	−	+	+	−	r	s	F′	F′	← *argG str mal*	B. Low (New York Univ.)
JC12	−	−	+	+	−	−	+	+	+	−	+	+	+	+	+	s	s		Hfr	← *argG str mal*	B. Low (New York Univ.)
N2	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	s	s		Hfr	← *argG str mal*	JC12 transduced by AB301-1
N1304	−	−	−	+	−	+	+	+	−	−	G	−	+	+	+	s	s		F⁻	—	B. Low (New York Univ.)

[a] Markers are designated by gene letter or allele number where known; otherwise, + for wild type and − for mutant; *lac*, *xyl*, *mal*, *ara*, and *gal* designate the ability (+) or inability (−) to ferment lactose, xylose, maltose, arabinose, and galactose, respectively. The other markers indicate prototrophy (+) or auxotrophy (−) for adenine, thiamine, L-threonine, L-leucine, L-methionine, L-arginine, L-histidine, L-isoleucine, L-valine, and L-proline as indicated; rec + or − indicates a deficiency in the capacity to recombine. With respect to *str* (streptomycin) and *spc* (spectinomycin), r indicates resistance and s indicates sensitivity. The extrachromosomal fragment in strain KLF41 starts before *argG* and covers clockwise the segment that ends between *mal* and *xyl* (for *argG* location, see 14; for other markers, see 18).

[b] Sp, arose spontaneously; NG, arose after treatment with nitrosoguanidine; Ac, arose after treatment with acridine half mustard ICR 191 (16).

TABLE 2. *Genotypes of (nek) neomycin- and kanamycin-resistant strains*[a]

Strain	nek allele	*lac*	*xyl*	*mal*	*gal*	*ad*	*thi*	*thr*	*leu*	*arg*	*his*	*ilv*	*pro*	*str*	*spc*	Mating type	Source[b]
N709	741	4	+	+	+	+	−	−	−	+	+	+	+	r	s	Hfr	Kan from AB312
N710	709	−	−	+	2	+	−	+	+	3	4	+	2	r	r	F⁻	From cross N709 × N304
N711	711	1	−	141	2	+	−	+	+	3	4	158	2	s	s	F⁻	Kan from N141
N712	712	1	−	141	2	+	−	+	+	3	4	158	2	s	s	F⁻	Kan from N141
N733	733	1	−	141	2	+	−	+	+	3	4	158	2	s	s	F⁻	Neo from N141
N734	734	1	−	141	2	+	−	+	+	3	4	158	2	s	s	F⁻	Neo from N141
N735	735	1	−	141	2	+	−	+	+	3	4	158	2	s	s	F⁻	Neo from N141
N737	737	4	+	+	+	+	−	−	−	+	+	+	+	r	s	Hfr	Neo from AB312
N741	741	−	−	+	−	−	+	+	+	+	+	+	+	s	s	Hfr	Kan from N2
N743	743	−	−	+	−	−	+	+	+	+	+	+	+	s	s	Hfr	Kan from N2
N751	741	−	−	−	−	+	+	+	−	G	−	+	+	s	s	F⁻	From cross N741 × N1304

[a] For symbols and parental strains, see Table 1.

[b] The parental strains resist about 5 μg of kanamycin per ml and 20 μg of neomycin per ml. All of the mutants are resistant, in replication on broth-agar plates, to about 100 μg of kanamycin (Kan) per ml and 150 μg of neomycin (Neo) per ml, and were initially isolated, after nitrosoguanidine treatment of the relevant parental strain, as colonies that grew on plates containing one or the other drug, as indicated.

TABLE 3. *Response to antibiotics of nek strains*[a]

Strain	nek allele	Streptomycin	Spectinomycin	Chloramphenicol
N141	nek⁺	2–5	30–50	10
N709	nek_{709}	>5,000[b]	30–50	<10
N711	nek_{711}	30–40	30–50	<10
N712	nek_{712}	30–40	>200	<10
N733	nek_{733}	30–40	>200	<10
N734	nek_{734}	30–40	50–100	<10
N735	nek_{735}	30–40	50–100	<10
N737	nek_{737}	>5,000[b]	50–100	<10
N741	nek_{741}	30–40	50–100	<10

[a] Tests were performed by replicating colonies from nutrient broth-Casamino Acids-agar onto the same medium supplemented with various concentrations of the antibiotics; plates were scored after 1 and 2 days. The numbers listed are the range of antibiotic concentration (μg/ml) at which colony formation is blocked.

[b] These two strains carry a str^r allele.

TABLE 4. *Reconstruction experiment for selection of nek⁺ cells from among nek cells*[a]

No. of cells per plate		Total cells plated		No. of Cap-R colonies observed	Found among Cap-R colonies tested	
AB774 (*nek*⁺)	N733 (*nek*)	AB774	N733		AB774	N733
108	10^7	324	3×10^7	284	70	0

[a] Two strains were separately titered on minimal medium agar supplemented with all the requirements necessary for growth. Cells of the two strains were then mixed and plated together on the same medium supplemented with 20 μg of chloramphenicol per ml, for the selection of *nek*⁺ cells. Colonies growing on the plates supplemented with chloramphenicol were isolated (Cap-R) and streaked out, and some individual colonies from each streak were assayed for their genotype. AB774 was characterized by being Mal⁺ and Nek-S; N733 was characterized by being Mal⁻ and Nek-R.

lag of 3 to 5 days, or, in the case of one strain (N734), could not adapt at all (Table 6).

The mutation frequency of N734 to *lin* (high resistance to lincomycin; 4) was found to be about 10^{-9}, reduced by a factor of 10^3 in comparison with the parental strain N141. This incompatibility of a *nek* mutation and *lin* mutations may be related to the failure of N734 to adapt to high levels of lincomycin.

Occurrence of altered ribosomes in nek mutants. Ribosomes from *nek* strains N711 and N733 were compared with those from the parental *nek*⁺ strain N141 in their response to artificial messenger ribonucleic acids (mRNA), including polyuridylic acid (poly U), polyadenylic acid (poly A), and polycytidylic acid (poly C). The results are summarized in Table 7.

All the experiments were run in duplicate and were repeated three to five times. The mean of each set of experiments with ribosomes from a mutant strain was compared to the relevant mean obtained with the ribosomes from the parental strain, in the *t* (Student) test, in order to gauge the

TABLE 5. *Selection of nek⁺ recombinants in a cross between two nek mutants*[a]

Cross	nek alleles	Recombinants/ml		No. of Cap-R colonies analyzed	No. of Nek-S colonies
		Ad⁺ His⁺	Ad⁺ His⁺ Cap-R		
N743 × N751	nek_{743} × nek_{741}	0.51 × 10⁶	2,390	64	49

[a] The cross was performed by "prolonged mating" (4). Portions were withdrawn, diluted, and plated on minimal medium plates supplemented with L-arginine and L-leucine (Ad⁺ His⁺ selection), or in the same medium with the addition of 10 μg of chloramphenicol per ml (Ad⁺ His⁺ Cap-R selection). Colonies that grew up on plates containing chloramphenicol were streaked out on broth-agar and retested for their other characteristics.

TABLE 6. *Adaptation of nek mutants to growth in presence of 1,000 μg of lincomycin per ml*[a]

Strain	nek allele	Lag before growth (days)
N141	nek⁺	2
N711	nek_{711}	3
N712	nek_{712}	3
N733	nek_{733}	5
N734	nek_{734}	—[b]
N735	nek_{735}	3

[a] From cultures grown exponentially in broth, about 500 cells were inoculated into 10 ml of broth containing 1,000 μg of lincomycin per ml; growth was recorded as positive when cultures showed turbidity corresponding to about 5 × 10⁸ cells/ml. Colonies from the adapted cultures, if grown on medium lacking lincomycin, are once more indistinguishable from the original strains.

[b] Incapable of adapting; after 7 days, even the input cells were no longer recoverable.

probability that the two means represented determinations on samples of the same population (Table 7). When this probability was less than 0.05, it was concluded that the two means are significantly different. The primary conclusion from the results presented in Table 7 is that the ribosomes of the two *nek* strains tested are different from those of the parental strain. (In all experiments, changing the source of the supernatant enzymes did not change the results; one such set of experiments is presented in Table 7.)

The results given in Table 7 also indicate that, when poly U is the mRNA employed, mutant and wild-type ribosomes function with no significant difference. They are equally inhibited by drugs (Table 7), and also miscode for isoleucine to the same extent (*unpublished data*). When poly A is the messenger used, the ribosomes from *nek* mutants are significantly more resistant to the inhibition of lysine incorporation induced by neomycin, by kanamycin, or by streptomycin. When poly C is the mRNA, miscoded incorporation of histidine, threonine, or serine induced by neomycin or kanamycin is reduced in the *nek* strains.

Formal genetics of mutants. The *nek* mutations occur at a chromosomal site very closely linked to the well-known *str* and *spc* loci. This location was inferred from crosses of four *nek* mutants. For example, in the cross N709 × N302 (*str*ʳ *nek* × *spc*ʳ), of 54 *spc*ʳ *str*ʳ recombinants, 38 were *nek*. Similarly, in the cross N737 × N302 (*str*ʳ *nek* × *spc*ʳ), among 161 *str*ʳ *spc*ʳ recombinants, 147 were *nek*, and of 145 *spc*ʳ *nek* colonies isolated, all were *str*ʳ. In the cross N53 × N711 (*str*ʳ *spc*ʳ × *nek*), 100 *ilv*⁺ *thr*⁺ *leu*⁺ recombinants were isolated, and among them the various classes of recombinants with respect to drug markers showed 7% recombination between the *str* and *spc* markers, 1% between *str* and *nek* markers, and 8% between the *spc* and *nek* markers. A similar result was obtained in the cross N53 × N735 (*str*ʳ *spc*ʳ × *nek*).

Close linkage of *nek* to the *str* and *spc* loci has been also confirmed in a series of transduction experiments. The results of one such experiment were as follows.

When N710 (*nek str*ʳ *spc*ʳ *mal*⁺) was the donor, and N141 (*nek*⁺ *str*ˢ *spc*ˢ *mal*⁻) was the recipient, of 30 *nek* transductants, all were *mal*⁻, 20 were *spc*ˢ *str*ˢ, 4 were *spc*ʳ *str*ʳ, 4 were *spc*ˢ *str*ʳ, and 2 were *spc*ʳ *str*ˢ.

The close linkage of various *nek* mutations with the *str* and *spc* loci suggests that they all fall within a very small chromosomal region. Corroboration was obtained by direct tests in mating experiments. Each of the two *nek* Hfr strains (N709 and N737) was crossed with three different *nek* F⁻ strains (N711, N733, N735). In each case, 224 *thr*⁺ *leu*⁺ *ilv*⁺ *arg*⁺ recombinants were selected; among all of these, only a single *nek*⁺ recombinant was found (in the cross N709 × N735). A similar result was obtained with two other *nek* F⁻ strains, in crosses of N709 × N712 and N737 × N734; in each case, 100 recombinants were analyzed, and only in the first cross was a single *nek*⁺ recombinant colony detected. Thus, all

TABLE 7. *Inhibition and stimulation of polypeptide synthesis in vitro by streptomycin, kanamycin, and neomycin*[a]

Source of ribosomes	Incorporation system																			
	Poly U phenylalanine				Poly A lysine				Poly C											
									Histidine				Threonine				Serine			
	0	S	K	N	0	S	K	N	0	S	K	N	0	S	K	N	0	S	K	N
Strain nek allele																				
N141 *nek*$^+$	5,725	46	46	17	6,391	36	23	9	49	166	875	923	120	140	304	350	95	449	925	554
N711 nek_{711}	4,270	55	54	22	2,205	58	41	28	80	209	439	450	155	193	173	202	195	377	446	259
N733 nek_{733}	3,295	51	43	16	1,670	51	48	29	51	269	410	579	113	221	161	171	164	324	349	167
N141 *nek*$^+$					4,371	33	21	11												
N711 nek_{711}					2,645	54	46	25												
P values																				
N141/N711		>.100	>.100	>.200		<.050	<.050	<.050		>.100	<.050	<.025		>.200	<.050	<.050		>.200	<.025	<.040
N141/N733		>.200	>.200	>.200		<.050	<.025	<.050		>.150	<.050	<.050		>.150	<.025	<.060		>.100	<.025	<.025

[a] The incorporation of each amino acid in presence of 20 μg of streptomycin (S), kanamycin (K), or neomycin (N) per ml is expressed as percentage of the control incorporation without the drug (0) (mean values). Column 0 is given in counts per minute; the other columns list percentages. Experiments were repeated with different preparations of ribosomes. Most experiments were repeated three to five times, with the exception of poly C-directed serine incorporation for all the three strains, and poly U phenylalanine for strain N733, which were carried out only twice. To test for the significance of the differences, the means obtained with ribosomes of strains N711 and N733 were each compared with the corresponding mean for ribosomes of the parental strain N141, in a *t* test. The probability that the two means represent samples from the same population is listed in the last two lines. *P* values smaller than 0.05 are accepted to indicate significant differences between mutant and parental strain. Incorporation of phenylalanine was directed by poly U; polylysine formation was directed by poly A; histidine, threonine, and serine incorporation were directed by poly C. The specific activity of the amino acids used were as follows: L-histidine, 164; L-isoleucine, 160; L-lysine, 187; L-phenylalanine, 330; L-serine, 87; and L-threonine, 160 (expressed in millicuries per millimole). The blanks, in counts per minute, for these amino acids were 55, 85, 95, 640, 63, and 104, respectively. These values were obtained by incubating mixtures identical to the experimental incubation mixtures, but lacking ribosomes. Although the results obtained depended on the source of the ribosomes, they were independent of the source of the supernatant factors used in the incorporation reaction. For example, two sets of results are given for poly A-directed lysine incorporation for strains N141 and N711; in the experiments reported in the first two lines of the table, supernatant fluid from strain N141 was used; in the experiments reported in the fourth and fifth lines, supernatant fluid from strain N711 was used.

seven *nek* mutations analyzed fall in a single locus. Two more *nek* mutants, N741 and N743, were subsequently analyzed and shown to be allelic (by the same criteria) with the seven *nek* mutants mentioned here.

Test for dominance. To see whether nek^+ or *nek* is dominant, they were put into the same cytoplasm in a partial diploid. The nek^+ allele was introduced from strain KLF41 into N711 (nek_{711}) in one experiment, and into N735 (nek_{735}) in a second. In each case, 2×10^7 cells per ml of each parental strain were slowly agitated for 5 min at 37 C, and then left without further agitation for 25 min at 37 C. The mixture was then plated on basal medium containing maltose, L-isoleucine, L-valine, L-proline, L-arginine, L-histidine, and thiamine to select for colonies with the phenotype Mal^+ Leu^+ Met^+. These recombinant colonies include both haploid cells and merodiploids.

Among the Mal^+ Leu^+ Met^+ recombinants, colonies sensitive to neomycin and kanamycin were observed; a number were repurified by streaking on rich medium, and were then grown up in broth and restreaked on eosin methylene blue-maltose plates. Some of the colonies tested yielded Mal^- segregants. A number of Mal^+ and Mal^- colonies were then tested for resistance to neomycin and kanamycin, and colonies resembling the female parental strain were found. From a merozygote isolated from the cross KLF41 × N711, 23 Mal^+ Nek-R, 73 Mal^- Nek-R, 14 Mal^+ Nek-S, and 3 Mal^- Nek-S segregants were observed. Similarly, from a merozygote isolated from cross KLF41 × N735, segregants included 9 Mal^+ Nek-R, 57 Mal^- Nek-R, 6 Mal^+ Nek-S, and 1 Mal^- Nek-S colonies. Since both *nek* and nek^+ alleles could be recovered in segregants of Nek-S partial diploids, it is concluded that the nek^+ allele is dominant.

DISCUSSION

The new locus described is located near the *str* locus of *E. coli* and affects ribosomes. The phenotypic characteristics of mutants in this locus suggest a general feature for mutations that affect ribosomes: pleiotropy. Here we discuss some of the effects of the mutations.

Effects of neomycin and kanamycin on protein synthesis in the mutants and the parental strain. As with most of the other known mutations that affect *E. coli* ribosomes, the in vitro difference between nek^+ and *nek* strains in amino acid incorporation studies are relatively small, whereas in vivo differences between these strains are striking (cf. 4). Also, as observed for mutants to streptomycin resistance, in vitro tests with homopolymers exhibit sharper differences in miscoding, rather than in susceptibility to inhibition by the drugs (Table 7). The binding site for streptomycin is probably distinct from that for neomycin and kanamycin, since the mutations to resistance to the drugs fall in genetically distinct loci. However, the two sites must either overlap physically, or else a change in one site must distort the other, since, for example, mutations to streptomycin resistance abolish phenotypic suppression by all three drugs. Also, *nek* mutations confer limited resistance to streptomycin as well (Table 3).

In some instances, the effects in vitro parallel those in vivo. For example, streptomycin only partially inhibits polyphenylalanine synthesis on ribosome from a sensitive strain, even at very high drug concentrations, and this inhibition is totally alleviated in resistant strains; cultures of *str*-HR mutants withstand any measurable level of streptomycin. In contrast, comparable levels of neomycin and kanamycin block polymerization of phenylalanine and lysine nearly totally both in extracts of nek^+ and of nek^- strains (Table 7); even the small remaining incorporation can be abolished by higher levels of drug (*unpublished data*), and *nek* strains acquire only limited resistance to either neomycin or kanamycin. Another example of parallelism in vitro and in vivo is provided by the miscoding induced by antibiotics when the messenger is poly C. While this miscoding is significantly reduced in the *nek* strains N711 and N733 in the presence of neomycin or kanamycin, the miscoding is not significantly reduced in the presence of streptomycin (Table 7). In a comparable way, in vivo in these two strains, neomycin and kanamycin cannot give phenotypic suppression, but streptomycin does (*see* Results). However, the parallels in vivo and in vitro may be fortuitous, as it is not clear to what extent inhibition or miscoding in protein synthesis directed by a homopolymer is a relevant index for the sensitivity of cells to drugs.

The effects of neomycin and kanamycin are probably exerted through an effect on 30*S* ribosomes, for there is (i) tight linkage of the *nek* locus to other genes which are known to affect the 30*S* ribosomes, the *str* and *spc* loci (6–8); (ii) cross-resistance of *nek* mutants to streptomycin; and (iii) similar behavior of neomycin, kanamycin, and streptomycin in phenotypic suppression.

Genetic analysis. The crosses and transduction data support the claim that all of the nine *nek* mutations tested are closely linked, and that the *nek* locus is closely linked to the *str* and *spc* loci. We conclude that it is less than 1 min from these loci, assuming that *1* time unit = 20 recombination units = 10% contransduction frequency. This correlation is constructed from data pub-

lished previously (12, 19). The relationship is definitely adequate, at least to a first approximation, since all of the data obtained in experiments with other markers in this chromosomal region are consistent with it. The linkage data presented in this paper concerning the *str*, *nek*, and *spc*, loci suggest that they are located in this order. However, the data are not conclusive.

Pleiotropic effects. Pleiotropy should be the rule rather than the exception for mutations that affect an organelle such as the ribosome, both because a ribosomal protein must interact with a variety of other proteins and with ribosomal RNA to form the structure, and because the resultant structure has to function in the precise and elaborate way required for accurate protein synthesis. Thus, the better-known cause for pleiotropy in bacteria, polar mutations in operons, is not a prerequisite for pleiotropy caused by mutations in genes that affect ribosomes.

In the other reported case in which mutants in the same locus confer a drastically changed response to two antibiotics, the *lir* locus, not all the mutants shared the property [sensitivity to either lincomycin or erythromycin (4)]. In the present case, however, all of the *nek* mutant strains tested resist both of the antibiotics. This might result if the ribosomal binding sites of these two antibiotics overlap completely, in contrast to those of lincomycin and erythromycin which do not. Alternatively, the *nek* mutations occur only at the overlap of the ribosomal binding sites for neomycin and kanamycin. The rarity of the *nek* mutations might result from such a limitation. In agreement with the second alternative is the fact that lower-level resistant mutants have been isolated that show increased resistance only to one drug or the other (*unpublished data*).

The fact that *nek* strains, unlike the parental strain, adapt very poorly to high concentrations of lincomycin is of particular interest. It has been unclear whether adaptation to lincomycin occurs through a change in the ribosome, or through some other process, such as a decrease in cell permeability to the drug. Since *nek* mutations have no obvious direct effect on cellular components other than the ribosome, the loss of adaptability in *nek* mutants supports the idea that adaptation involves a modification in the ribosome, possibly comparable to the one observed in ribosomes isolated from anaerobic cultures (12). Another possible case of adaptation through a change in the ribosome is the "phenotypic masking" described by Gorini, Rosset, and Zimmermann (11).

Perhaps the most interesting and useful of the observed pleiotropic effects is the increased sensitivity of *nek* mutants to chloramphenicol. The *nek* mutants probably affect the 30*S* ribosome, but chloramphenicol binds specifically to the 50*S* ribosome (21; Monro and Staehelin, *personal communication*). Thus, a mutation in one ribosomal particle may indirectly affect the function of the other particle.

The increased sensitivity of the *nek* strains to chloramphenicol permitted a reconstruction experiment (Table 4), in which a few *nek*$^+$ (sensitive) cells were selected from among a large number of *nek* (resistant) cells. This finding opened the way for the fine genetic analysis of the *nek* locus and made possible a "two-way selection" (1, 2) for a gene that affects the ribosome. Crosses between pairs of *nek* mutants have been performed, and indeed *nek*$^+$ recombinants have been obtained (Table 5).

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