Genet. Res., Camb. (1970), 16, *pp.* 359–365
With 3 text-figures
Printed in Great Britain

Two mutations giving low-level streptomycin resistance in *Escherichia coli* K12

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(*Received* 2 *October* 1970)

SUMMARY

A mutation in *Escherichia coli* K12 giving resistance to about 5 μg/ml of streptomycin was found to be cotransducible by P1 with *proA* and *proB*, and is located at about 8·5 min on the chromosome map. The locus is named *strB*. A second mutation to the same resistance level was not cotransducible with either *proA* or *proB* and must be located elsewhere. Both mutations cause a marked increase in R-factor mediated streptomycin resistance, and significant decreases in resistance to several other antibiotics, both in the presence and absence of an R-factor determinant for the same antibiotic. The two mutations differ in their effects on bacterial sensitivity to crystal violet and EDTA.

1. INTRODUCTION

Selection of *Escherichia coli* B on nutrient agar plates containing 5 μg/ml of streptomycin gives resistant colonies at a frequency of about 5×10^{-7} of the cells plated (Demerec, 1948; Bryson & Demerec, 1950). A small proportion of these mutants results from mutation at the *strA* locus to give complete resistance or dependence, and the rest are mutations at other loci giving partial resistance to the drug. Partial resistance was difficult to locate or to transduce in *Salmonella typhimurium* (Watanabe & Watanabe, 1959) and appears to have received very little attention in *E. coli* since the early studies of Demerec. This paper describes mutations at two different loci in *E. coli* K12 which cause a low level of resistance to streptomycin, and gives the chromosomal location of one of them. Some phenotypic effects of each mutant are also described.

2. MATERIAL AND METHODS

(*i*) *Bacterial strains and R factors*

The following bacterial strains, all *strA*$^+$ derivatives of *E. coli* K12, were used. RE26 F$^-$ *proA trp his lac tsx*; RE82 HfrB11 *metB*; RE91 HfrH *thi*; RE174 HfrP4X *argA metB* and RE176 HfrP4X *argH metB* (both from Dr N. Glansdorff); RE260 HfrH *thi proB*. Relatives of RE26, e.g. J62, RC711 and D11, have been labelled *proB* or even *proC* in several papers, but are in fact *proA* (Dr E. Meynell, personal communication). We have confirmed that RE26 is *proA*. RE260 was obtained by mutagen treatment of RE91, and the *proB* mutation was confirmed by cross-feeding and cotransduction tests.

The R factors used had the following resistance characters. R1 is R1*drd*19 ACKSSu (from Dr E. Meynell); R46 is ASTSu and R57 is TSu (both from Dr N. Datta).

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(ii) *Media*

L-broth was 10 g Difco Bacto Tryptone, 5 g Difco Yeast Extract, 5 g NaCl, 1 g glucose and 0·2 g thymine/l. of distilled water. Nutrient broth was Difco Bacto Nutrient Broth with NaCl at 0·5 %. Minimal medium was that of Vogel & Bonner (1956). Amino acids at 50, vitamins at 10 μg/ml and sugars 0·2 % (w/v) were added to the minimal medium as required. Nutrient broth and the minimal medium were solidified with 1·5 % Difco Bacto Agar to make nutrient and minimal agar. Antibiotics were dissolved in distilled water, sterilized by filtration and added to melted agar media immediately before pouring. Buffer was the buffer of Clowes & Hayes (1968) page 187.

(iii) *Determination of streptomycin resistance level*

(*a*) *Streak test*: a fully grown L-broth culture of each strain to be tested was diluted 10^{-2} into normal saline and loopfuls were streaked across nutrient agar plates containing streptomycin at increasing concentrations. A template was used to keep the streak size constant. After overnight incubation at 37 °C the streaks were classified as + + (thick growth), + (thin confluent growth) or − (no growth or a few isolated colonies). The minimal inhibitory plate concentration (MIPC) was taken as the maximum level allowing + growth of the streak.

(*b*) *Viable count test*: 0·1 ml samples of a suitable dilution of a fully grown L-broth culture were plated on antibiotic plates as used for the streak test and on nutrient agar without antibiotic. Viable counts gave the percentage survival on each antibiotic concentration.

Otherwise, standard methods were used except as indicated in the text.

3. RESULTS

(i) *Selection of mutants and genetic location tests*

Two streptomycin-sensitive strains of K12, RE26 and RE91, were selected for resistance by plating 0·1 ml samples of fully grown L-Broth cultures on nutrient agar plates containing 5 μg/ml of streptomycin and incubating overnight at 37 °C. Both strains gave resistant colonies at about 10^{-6} times the number of cells plated, in good agreement with the frequency of 5×10^{-7} obtained by Demerec for *E. coli* B. 20 resistant colonies of each strain were purified on nutrient agar and streak-tested to determine their resistance levels (Table 1). The two parent strains had MIPCs of 1·4 and 1·0 μg/ml, three lines turned out to be streptomycin-dependent, and the 37 other resistant lines had MIPCs ranging

Table 1. *Resistance levels of mutants selected on 5 μg/ml streptomycin*

	Selection of RE26		Selection of RE91	
	MIPC (μg/ml)	No. of mutants	MIPC (μg/ml)	No. of mutants
Parent strain	1·4	—	1·0	—
Mutants	2·8	5	2·8	2
	5·0	14	4·0	6
	—	—	5·0	10
Str-dependent	—	1	—	2

MIPCs were obtained by the streak test described under Methods.

https://doi.org/10.1017/S0016672300002640 Published online by Cambridge University Press

from 2·8 to 5 μg/ml, indicating a consistently low level of resistance. Two mutants with MIPCs of 5 μg/ml (RE26–*str16* and RE91–*str26*) were selected for further study. Both mutants gave normal colony size and morphology on nutrient agar.

Mating tests with RE26–*str16* indicated that this mutation is located in the *proA–lac* region of the chromosome, and this was confirmed by cotransduction tests with P1. RE26 is *proA*$^-$, and when RE26–*str16* was transduced to Pro$^+$ with P1 grown on RE82, a Pro$^+$ Str-s strain, 37 % of the 867 transductants tested became Str-s. P1 grown on RE26–*str16* was then used to transduce RE260 (*proB* Lac$^+$ Str-s) to Pro$^+$, and the transductants were tested for the transfer of the *lac*$^-$ and *str16* alleles from the donor (Table 2). The cotransduction frequencies were 27 % for *proB–lac*, which agrees well with 23 % reported by Markovitz (1964), and 6·6 % for *proB–str16*, but no Pro$^+$ transductants received both *lac* and *str16*; so clearly *str16* lies between *proA* and *proB*, and not between *proB* and *lac*.

Table 2. *Transduction of RE260* proB lac$^+$ str-s *with P1 grown on RE26* proA proB$^+$ lac$^-$ str16

Pro$^+$ transductants tested	No. of cotransductants		
	lac$^-$	*str-r*	*lac*$^-$, *str-r*
396	106	28	0
474	—	28	—

Cotransduction frequencies (%)	
proB–lac	26·7
proB–str	6·6
proB–[*lac*, *str*]	0·0

Pro$^+$ transductants were selected on supplemented minimal agar, purified on plates of the same type, checked for the nutrient characters of the recipient strain, and streak-tested for ability to grow overnight on supplemented minimal agar containing 2 μg/ml streptomycin, which inhibited growth of Str-s lines.

P4X males have their origin of transfer between *proA* and *proB* and transfer *proA* very early and *proB* as a terminal marker (Curtiss, 1965). Two streptomycin-sensitive Pro$^+$ males of P4X type (RE174, RE176) were therefore mated to RE26–*str16*. Mating was mechanically interrupted at 5min and about 250 *proA*$^+$ recombinants from each mating were selected, purified and tested for level of streptomycin resistance. 22 and 26 % of these, in the two tests, carried the donor's *str-s* allele and the rest had the resistance level of the recipient *str16*. Neither donor was able to transfer *lac* early, confirming their P4X state. This result indicates that *str16* lies between *proA* and the F-factor location in P4X. Since P4X transfers *str16* before *proA* and close to it, one would expect a very high proportion of *proA*$^+$ recombinants to carry the donor's *str* allele instead of about 24 %. This discrepancy has been explained by Low (1965), who demonstrated that very early markers in Hfr mating are transferred less frequently than expected from the transfer gradient, because of the requirement for recombination to occur in the short region between the marker and the origin.

The *str16* locus will be named *strB*, and its probable location is given in Fig. 1. Taylor (1970) places *proA*, *proB* and *lac* at 7, 9·5 and 10 min, but our transduction data suggest that *proA* and *proB* are only about 1·5 min apart, on the basis of the correlations discussed by Taylor & Trotter (1967). We propose 8, 8·5 and 9·5 min for the locations of *proA*, *strB* and *proB*, with the P4X origin probably between 9 and 9·5 min.

The *str26* mutation selected in RE91 was tested for cotransduction with *proA* and *proB*

by transducing RE26 (*p.oA* Str-s) and RE260 (*proB* Str-s) to Pro+ with P1 grown on RE91-*str26*. None of the transductants received the *str26* allele (Table 3), so this mutation must be outside the *proA–proB* region and cannot be at the *strB* locus. Its location has not been tested further.

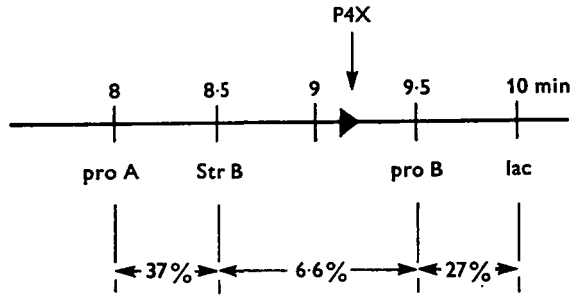

Fig. 1. Chromosomal location of *strB* mutation. Percentage cotransduction frequencies with P1 are shown below the gene symbols.

Table 3. *Cotransduction test on* str26

Donor	Recipient	No. of Pro+ transductants	
		Tested	*str-r*
RE91-*str26* Pro+	RE26 Str+ *proA*	160	0
RE91-*str26* Pro+	RE260 Str+ *proB*	225	0

Pro+ transductants were tested as in Table 2.

(*ii*) *Effects of* str16 *and* str26 *on Phenotype*

Fig. 2 shows the relative colony-forming abilities of the two parent and mutant strains on successive concentrations of streptomycin in nutrient agar. Both mutants require about twice as much antibiotic as the parent lines to give the same reduction in viable count, but RE26 and its mutant are a little more sensitive than RE91 and its mutant to the drug. This result is based on several tests and is likely to be more accurate than the streak test of Table 2.

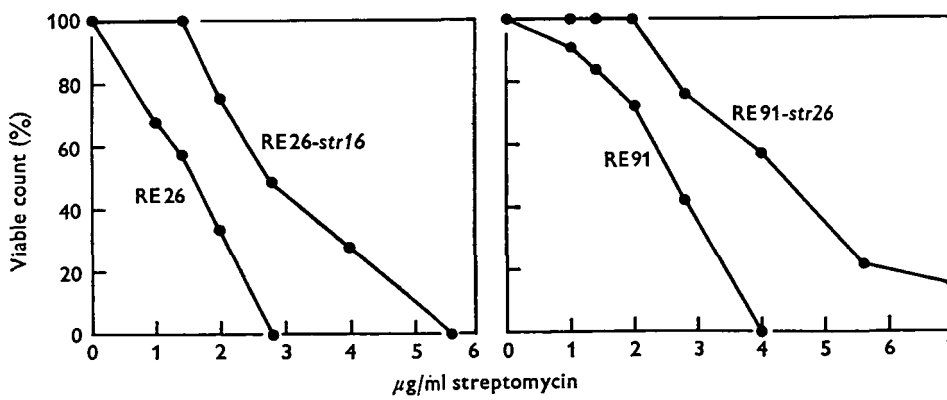

Fig. 2. Percentage survival of parent and mutant lines on streptomycin nutrient agar.

https://doi.org/10.1017/S0016672300002640 Published online by Cambridge University Press

Various interactions between chromosomal and R-factor mediated resistance have been reported by Reeve (1966), Pearce & Meynell (1968) and Normark *et al.* (1969); and several R-factors were therefore introduced into the sensitive and resistant lines. In the course of these experiments it became clear that the *str* mutations not only increased the level of resistance to streptomycin but also reduced the resistance to ampicillin, chloramphenicol and tetracycline produced by R-factor genes. The results of a number of experiments are summarized in Fig. 3, which gives the relative colony forming ability of the

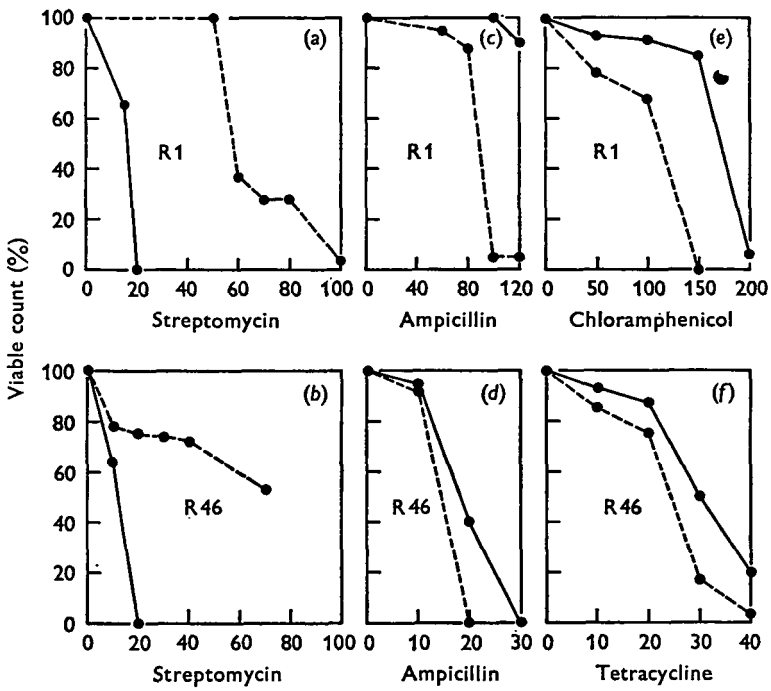

Fig. 3. Percentage survival of R-factor carrying lines on antibiotic nutrient agar. Antibiotic concs. in μg/ml. Solid lines: host strain RE26; broken lines: host strain RE26-*str16*. Graphs (*a–c*) show cells infected with R1, and graphs (*d–f*) cells infected with R46.

R-factor carrying strains on nutrient agar containing various antibiotics. In each graph the solid line refers to RE26 and the broken line to RE26*str16*. Graphs (*a*) and (*b*) show the streptomycin resistance profiles of bacteria carrying R1 and R46, respectively, and in both cases *str16* causes a marked increase in resistance, much greater than would be expected from the small effect of *str16* alone. Similar synergistic effects have been reported by Pearce & Meynell (1968).

In the case of ampicillin resistance, R1 (graph *c*) gives a much higher level than R46 (graph *d*), but in both cases the presence of *str16* causes an appreciable *decrease* in resistance. Likewise, the presence of *str16* reduces the effectiveness of R1-mediated chloramphenicol resistance (graph *e*) and of R46-mediated tetracycline resistance (graph *f*). There is no doubt about the statistical significance of these differences, since they were obtained consistently in repeated tests. When R46 was introduced into RE91 and its *str26* mutant, the same interactions were observed, so these are not restricted to the *strB* locus. Further tests then indicated that both *str* mutants are more sensitive than the parent strains to

ampicillin, chloramphenicol and tetracycline in the absence of an R-factor. These changes in sensitivity are difficult to measure accurately, but they suggest that the effects on R-factor mediated resistance are the result of changes in cell permeability to the various antibiotics caused by the *str* mutations.

Two antibacterial agents gave opposite reactions with the two mutants, as shown in Table 4. Sensitivity to crystal violet was strongly increased by *str16* but not by *str26*, while increased sensitivity to EDTA was given by *str26* alone. These differences should make it possible to sort out different classes of streptomycin-resistant mutant.

Table 4. *Resistance of mutant and parent strains to Crystal Violet and EDTA*

Strain	Crystal Violet LD_{50} (μg/ml)	Survival in EDTA (30 min) (%)
RE26	30	26
RE26*str16*	< 5	66
RE91	15	24
RE91*str26*	20	5

Resistance to Crystal violet was tested by plating a suitable dilution of a fully grown L-Broth culture of each strain on L-Broth agar containing successive concentrations of crystal violet. LD_{50} is the concentration of crystal violet allowing 50 % of the viable count on L-Broth agar alone. For the EDTA test, log-phase broth-grown cells were diluted 10^{-2} into 1 mM ethylenediaminetetra-acetic acid in distilled water and incubated 30 min at 37 °C. Dilutions were then plated for viable count on nutrient agar.

4. DISCUSSION

One possible explanation for the pleiotropic effects shown by the *str* mutations is that they cause minor modifications in enzymes concerned with the structure of the cell envelope, thus affecting the permeability of the cell to a number of substances. If these enzymes are essential for cell survival, as many of them must be, then such 'minor' mutations may provide the only means of studying the genes concerned. The high frequency of such mutants makes it likely that several different genes can give mutations of this type. Some other groups of mutations with varied pleiotropic effects have been attributed to genes concerned with cell envelope structure (cf. Nordström *et al.* 1970), but the types of pleiotropic effect obtained do not yet give any clue to the structural elements affected by the mutations.

L. R. was the holder of a S.R.C. Research Studentship during the course of this work.

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