A STUDY OF MUTATIONS AFFECTING ANTIBIOTIC RESISTANCE IN *ESCHERICHIA COLI*.

by

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1. INTRODUCTION

Antibiotic resistance in micro-organisms was shown by Demerec (1948) to be due to mutation rather than an interaction between the antibiotic and the bacterium. From a study of penicillin resistance in Staphlococcus aureus and streptomycin resistance in Escherichia coli he proposed that several genes were involved in resistance to both these antibiotics.

Penicillin resistance in S. aureus involves a stepwise increase in resistance to the drug. Streptomycin resistance in E. coli is much more variable. First step streptomycin resistant mutants vary from being only slightly more resistant than the sensitive parent strain to complete resistance.

The survival curve of E. coli with respect to streptomycin (Demerec 1950) reflects the variation observed in streptomycin resistance in this strain. Up to 1.5μg/ml streptomycin is not bacteriocidal; between 1.5 and 20 μg/ml the number of survivors decreases rapidly and above 20 μg/ml the number of survivors remains constant. This proportion of the bacterial population is regarded as being completely resistant to streptomycin.

Among these streptomycin resistant mutants it is possible to recognise two types of mutants: those which are merely resistant to the drug and those which are dependent upon it. Newcombe and Nyholm (1950) have shown that streptomycin resistance and streptomycin dependence are due to mutation at a single locus designated StrA. This mutation occurs rarely with a frequency of 5×10^{-9}.

Watanabe and Watanabe (1959a and b) have investigated streptomycin resistance in Salmonella typhimurium. By transduction analysis they have shown that high level streptomycin resistance, termed streptomycin indifference, and one step intermediate resistance are controlled by two separate loci. Further transduction analysis of several one step intermediate resistance lines suggests that more than one locus is responsible for the intermediate resistance.

In 1952 Cavalli and Macaccaro described a "penicillin pattern" of resistance to Chloramphenicol in E. coli K12. First step mutants were only slightly resistant to the antibiotic and high level resistance was obtained by successive transfers into increasing concentrations of the drug. More recently Reeve (1966) has also investigated Chloramphenicol resistance in E. coli K12. Six independent mutants have been shown to have three different resistance levels with respect to Chloramphenicol. The mutants also possess some degree of cross resistance to Tetracycline and Puromycin. Reeve and Suttie (1968) and Reeve (1968) have mapped two of these mutations in neighbouring but separate locations on the E. coli linkage map.

The common feature underlying the examples of antibiotic resistance outlined above is that each is due to mutation in one or more loci at different chromosomal locations. This concept of several genes being involved in one phenotypic character is reminiscent of the multiple factor hypothesis which was put forward by geneticists and biometricians at the beginning of the century to explain continuously varying characters such as seedweight in terms of dicrete genetic factors.

Essentially the multiple factor hypothesis states that the observed phenotypes are caused by a number of factors (genes) which have effects similar to each other but these effects are small in relation to the total variation (Mather 1949). These "polygenes", so called because of the polygenic variation they determine, behave in exactly the same manner as major genes with respect to segregation and linkage. Linkage between polygenes and to major genes was demonstrated by Mather in 1942 and Sax in 1923.

Contrary to the usual, though somewhat dangerous, practice of attempting to explain genetic mechanisms in higher organisms on the basis of results obtained from studies with micro-organisms we have here the reverse situation. The concept of polygenes is widely accepted in higher organisms and it seems likely that a similar situation is operating in micro-organisms with respect to resistance to the antibiotics penicillin, streptomycin and chloramphenicol.

Two antibiotic resistance "patterns" - the "penicillin pattern" and the "streptomycin pattern" were described by Demerec in 1948. Cavalli and Macaccaro (1952) have shown the chloramphenicol resistance in E.coli K12 follows a pattern similar to that followed by penicillin resistance in S. aureus. The "streptomycin pattern" on the other hand obviously involves genes of variable potency in both E. coli and the closely related S. typhimurium.

The idea of genes of variable potency being involved in antibiotic resistance suggests the possibility of different resistance mechanisms operating. There are two known mechanisms of streptomycin resistance in E.coli. The first, and by far the most widely studied, is the high level resistance due to mutation at the StrA

locus (Newcombe and Nyholm 1950). This resistance mechanism involves an alteration in the ribosomes which permits these cell organelles to function normally in the presence of streptomycin (Davis 1964).

The second resistance mechanism active against streptomycin involves enzyme inactivation of the drug. All the evidence for this resistance mechanism comes from studies of strains of E.coli which are resistant to streptomycin by virtue of carrying a streptomycin resistant R-factor.

R-factors are extrachromosomal genetic elements which carry the genetic information for resistance to one or more antibiotics. They are also capable of transferring from one species of the Enterobacteriaceae to another. Okamoto and Suzuki (1965) have described the extraction of streptomycin- and chloramphenicol-inactivating enzymes from R-factor carrying bacterial strains. These authors did not specify the type of enzyme which inactivated streptomycin but more recently Harwood and Smith (1969) have identified in an R-factor carrying strain, an adenylating enzyme which inactivates streptomycin.

The evidence that at least one other type of streptomycin resistance mechanism is operating is virtually non-existent and comes only from studies which involve interaction between R-factor mediated streptomycin resistance and chromosomal streptomycin resistance which is not due to mutation at the StrA locus.

Gundersen (1963) and Ginoza and Painter (1964) have described situations where the presence of an R-factor seems to increase the frequency of mutation to a higher level of streptomycin resistance. In both these instances the R-factors carried streptomycin resistance determinants. A similar situation has been described by Pearce and Meynell (1968) but they interpret the results somewhat differently.

Rather than the R-factor actually increasing mutation to streptomycin resistance they suggest that the presence of R-factor streptomycin resistance allows the inoculum to multiply to a density where one of the mutations to low level streptomycin resistance which occur comparatively often arises.

This explanation does not account for the fact that the resulting streptomycin resistant organisms have a very high resistance level. The combination of two different resistance mechanisms may be much more efficient than a double dose of the same resistance mechanism. Gundersen (1963) showed that the chromosomal mutation was not at the StrA locus and although the mutations observed by Pearce and Meynell (1968) have not been mapped it seems very unlikely that they were due to mutation in the StrA locus because of the frequency with which they occur.

The mutations to low level streptomycin resistance seem unlikely to be due to enzyme inactivation although there is some similarity between the resistance levels due to R-factor streptomycin resistance and low level chromosomal mutants. A fully functional enzyme could be produced as the result of a single step mutation only if that mutation were in a regulator gene and there is no evidence that regulator genes mutate any more frequently than structural genes. Thus the chromosomal mutations described by Gundersen (1963); Ginoza and Painter (1964) and Pearce and Meynell (1968) must involve some other resistance mechanism or mechanisms.

Watanabe and Watanabe (1959b) succeeded in showing that slow growing intermediate streptomycin resistant mutants in S. typhimurium could be transduced simultaneously to streptomycin

sensitivity and normal growth rate. These two effects are obviously due to the same or very closely linked loci. A similar connection between antibiotics and growth rate of bacteria has been described by Sasarman et al (1968) in E.coli K12. Mutants apparently resistant to low concentrations of Neomycin and streptomycin (5-40 μg/ml) are slow growing and are capable of producing only small colonies. They have been shown to lack catalase and certain cytochromes.

In 1961 Hancock described the relationship between streptomycin uptake and sensitivity to the drug. The more sensitive was the organism the more antibiotic was absorbed. Under anaerobic conditions the organism was more resistant. This finding suggests some correlation between resistance to and uptake of, streptomycin and aerobic respiration.

Permeability of cells to various antibiotics is well known e.g. R-factor mediated Tetracycline resistance is due to an inducible system which prevents Tetracycline from entering the cell (Franklin 1967). Reeve (1966) concludes that the single step chloramphenicol resistant mutants which he isolated in E. coli K12 modified the cell membrane.

It therefore seems possible that the mutations to low level streptomycin resistance which occur at a relatively high frequency may in some way be connected with uptake of the drug. This is not necessarily the only resistance mechanism which may operate because as Dubin et al (1963) have shown streptomycin causes several effects when streptomycin-sensitive bacteria are incubated in the presence of the drug.

Indeed, if low level streptomycin resistance can be due to the mutation of several genes as hypothesised by Demerec (1948) there is every reason why more than one resistance mechanism may operate. Therefore some low level streptomycin resistance mutants of *E.coli* K have been studied genetically and the possible resistance mechanism or mechanisms operating have been examined.

2. MATERIALS AND METHODS

(i) Bacterial strains, R-factors and Bacteriophages

All bacterial strains used are derivatives of *Escherichia coli* K12 and were obtained from Dr E.C.R.Reeve. They are listed in Table Ia.

Table I(a)

Bacterial strains

Stock Number	Relevant Characters
RE1	*proA trp his lac strA* F^-
RE12	*metB* F^+
RE26	*proA trp his lac* F^-
RE68	*metB* F^- (R57)
RE72	*metB* F^- (R46)
RE91	HfrH *thi*
RE91a	HfrH *thi proB*
RE174	HfrP4X *argH metB*
RE176	HfrP4X *argH metB*
RE226	HfrH *thi proC*
RE235	*thr leu thi lac* F^- (R1*drd*19)

The figures in brackets denote R-factors and these are listed in Table I(b).

Table I(b)

R-factors

R-factor	Resistance determinants	Source
R1*drd* 19(fi^+)	K C A S Su	Dr E. Meynell
R46 (fi^-)	A S T Su	Dr N. Datta
R57 (fi^-)	T Su	Dr N. Datta

Abbreviations:- A - Ampicillin; C- Chloramphenicol; K - Kanamycin; S - Streptomycin; Su - Sulphonamides; T - Tetracycline. fi^+ represents fertility inhibition exerted by the R-factor on F-factors. fi^- means there is no fertility inhibition. R1drd 19 means this is derepressed mutant number 19 of R1 which transfers the R-factor with a high frequency.

P1kc transducing phage was used for all co-transduction experiments. Lysates were stored over chloroform at 4°C.

Wild type T6 phage was used for some interrupted mating experiments.

Both types of phage were obtained from Dr E.C.R.Reeve.

(ii) Media

Nutrient broth	- 5gm NaCl and 5gm nutrient broth (~~Oxoid~~ Difco) per litre of distilled water.
Nutrient agar	- as above solidified with 1.5% Difco-Bacto agar.
L-broth + Thymine	- 10gm Difco Bacto Tryptone: 5gm Difco Yeast Extract; 5gm NaCl; 1gm glucose and 0.2gm Thymine per litre of distilled water.
L-broth Agar	- As above solidified with 1.5% Difco Bacto agar.
P1 Bottom Agar	- 10gm Difco Bacto Tryptone; 5gm Difco Yeast Extract; 10gm NaCl; 1gm Glucose and 12gm Difco Bacto Agar per litre of distilled water. 2mls 0.1M $CaCl_2$ added to each 100ml agar before pouring plates.
P1 Top Agar	- 10gm Oxoid Tryptone; 5gm NaCl and 7gm Difco Special Noble Agar per litre of distilled water. 0.1M $CaCl_2$ added at same concentration as above.
DST agar	- 40gm. Oxoid DST agar base per litre of distilled water.
EMB Lactose agar	- 3.75gm oxoed EMB agar base per 100ml of distilled water.
Water agar	- 15.0gm Difco Bacto agar per litre of distilled water.
M9 salts (10 X final concentration)	- 60.0gm Na_2HPO4 (anhydrous); 30gm KH_2PO_4; 5gm NaCl and 10gm NH_4Cl per litre of distilled water.
M9 minimal medium	- 1ml 0.01M $CaCl_2$; 1ml 0.1M M_9SO_4; 1ml 20% sugar and 10ml M9 salts (10 x's final conc^n.) were added to 87ml sterile distilled water.

$CaCl_2$; M_9SO_4; sugar solutions and M9 salts solution are sterilized separately at 15lbs for 15 minutes.

M9 agar - Requirements for 100ml of M9 minimal medium were added to 90ml of melted water agar.

VB minimal medium (50 x final concentration) - 10gm $MgSO_4$; 100gm citric acid, 500gm KH_2PO_4; 175 gm $NaNH_4HPO_4$ $4H_2O$ dissolved in 670 ml of distilled water. 2ml chloroform added per 100ml of VB medium to act as preservative. 2mlVB minimal medium (x50) added to 100ml melted water agar. (Vogel and Bonner 1956).

Amino acids - These were added at a final concentration of 50μg/ml as required.

Vitamins - Thiamine (Vitamin B_1) was also added at a final concentration of 50μg/ml when required.

Sugars - Prepared as 20% solutions and added to the medium to give a final concentration of 0.2%

Saline (0.85%) - 8.5gm NaCl dissolved in 1 litre of distilled water.

Buffer - 3gm KH_2PO_4; 7gm Na_2HPO_4 (anhydrous) 4gm NaCl and 0.2gm $MgSo_4 \cdot 7H_2O$ per litre of distilled water.

Sodium Citrate (50%) 50gm Tri-sodium citrate per 100ml of distilled water.

Antibiotic solutions Antibiotics were dissolved in distilled water and sterilised by filtration, immediately before use.

All media, except VB minimal medium (x 50) and antibiotic solutions, are sterilised at 15lbs for 15 minutes.

In the earlier experiments M9 minimal medium was used but I then changed to VB minimal medium because the high citrate concentration makes it particularly useful in transduction experiments. Phage P1 is unable to attack recipient cells after plating on VB medium because of the high citrate content. Comparative tests have shown that colony appearance and growth rate were very similar on the two media.

(iii) Selection of mutants resistant to low levels of streptomycin

0.1ml aliquots of a fully grown culture in L-broth were plated on nutrient agar plus 5μg/ml streptomycin. Resistant colonies were picked after overnight incubation at 37°C and purified on nutrient agar. This eliminated any streptomycin dependant mutations which may have arisen among the resistant mutants.

(iv) Resistance level determination

Two methods of measurement were used:-

(a) Streak testing after the method of Reeve (1966). A fully grown broth culture of the strain to be tested is diluted 10^{-2} into 0.85% saline. A single loopful of this dilution was streaked across appropriate test plates. The minimal inhibitory plate concentrations(MIPC) is the level of antibiotic which just permits confluent growth of the streak. After overnight incubation the streaks are classified as ++ (thick streaks); + (thin confluent growth) or - (no growth or a few isolated colonies).

This method gives good discrimination between sensitive and resistant strains but the MIPC tends to be rather higher than the concentration of antibiotic which prevents single colony formation in the strain.

(b) Viable count method. 0.1ml aliquots of a suitable dilution of the fully grown broth culture to be tested are plated on nutrient agar containing various concentrations of antibiotic and the viable counts are compared with the viable count on nutrient agar plates without antibiotic. The results are expressed graphically as the percentage survival on various antibiotic concentrations as

compared with 100% survival on plates with no antibiotic.

This method has also been used to determine the survival of various strains on crystal violet L-broth agar.

(vi) R-factor transfer.

R-factors were transferred in overnight matings, recipients being selected for transfer of one resistance determinant. After purification the presence of other resistance determinants was checked with Oxoid Multodisks, which were placed on Oxoid DST Agar plates previously spread with approximately 10^8 cells of the strain being tested. The plates were scored after overnight incubation at 37°C.

Only R-factor resistances can be measured by the Multodisk technique. Strains which have low level chromosomal streptomycin resistance appear as streptomycin sensitive when tested with Multodisks.

(vii) Interrupted Mating Experiments

Log phase donor cells (10^8) were added to 2 x 10^8 stationary phase recipient cells in warm broth. Incubation at 37°C, with no shaking was continued for 5 minutes to allow pair formation. The mixture was then diluted 0.1ml into 20ml fresh warmed broth. This was taken as time zero and incubation continued, aliquots were withdrawn at specific times for interruption by one of the following methods:-

(a) Interruption by T6 phage. 1ml of the mating mixture was diluted into 5ml of broth containing T6 phage at approximately 10^6 p.f.u/ml. The mixture was incubated at 37°C, without

shaking, for 15 minutes, and was then filtered to remove unadsorbed phage. The bacteria were washed from the filter into 5ml buffer and 0.1ml aliquots from this buffer suspension were plated onto appropriate selective plates.

(b) Mechanical Interruption. 1ml of the mating mixture was added to 7ml buffer in a 25ml bottle (square section) and the suspension was treated with an MSE Microhomogeniser at $^{3}/_{4}$ speed for 1 minute. 2ml of broth was added to help recombinant formation and 0.1ml aliquots were then plated onto selective plates.

After purification recombinant colonies were tested for unselected markers.

(viii) Transduction Experiments

The strain to be transduced was grown to an $O.D_{550}$ 0.4 in L-broth + 0.4% glucose. Then 0.4ml of 0.1M $CaCl_2$ was added to 20ml of culture to help phage adsorption and incubation was continued for 20 minutes. The culture was cooled in ice and divided into 2 x 10ml aliquots. One aliquot was added to 10^8 phage particles in a fresh flask (this gives a multiplicity of infection of 1.0) and was incubated at 37°C for 20 minutes, without shaking. The untreated culture was used to determine pre-treatment viable counts and mutant frequency.

After incubation the phage treated culture was centrifuged at 7,000 r.p.m for 10 minutes. The supernatant was removed and the pellet was resuspended in 10ml buffer which contains 0.5% sodium citrate. Suitable dilutions from the buffer suspension were plated out for transductants. When a number of transduct-

ions were being done the experimental procedure was altered slightly. The strains to be transduced were grown up in 1ml tubes overnight. Next morning they were diluted 1/10 into fresh broth +0.002 M $CaCl_2$ in 1ml tubes, and incubated for 2 hours. Donor phage at MOI 1.0 was added and incubation continued for 20 minutes. The cultures were filtered and the bacteria were resuspended in 1ml buffer + 0.5% sodium citrate. 0.1ml samples from the buffer suspension were plated onto selective plates.

(If M9 minimal medium was used sodium citrate was added to all plates at a final concentration of 0.5%. This was not necessary with VB minimal medium which contains a high proportion of citrate.)

(ix) Preparation of P1 donor phage

The donor strain was fully grown in L-broth and 0.2ml of this culture was added to tubes containing 2.5ml molten P1 top agar and 10^7 phage particles at 45°C. The tubes were mixed gently by rotation (to prevent bubble formation) and poured onto P1 bottom agar. (For the preparation of donor phage bottom agar plates were poured at the rate of 3/100ml of agar, since the deeper agar gives a better yield of phage.) After overnight incubation the phage were harvested in the usual way and the lysate stored over chloroform at 4°C.

The titre was assayed in the usual way on a suitable indicator strain of E.coli K12.

(x) Effect of Tetracycline on growth rate in L-broth

(a) L-broth culture of the strain to be tested, in log phase at $O.D._{550}$ 0.1, was cooled in ice and divided into four 25ml samples in 100ml shaker flasks in ice. The samples were challenged with tetracycline as follows:-

1) No tetracycline was added

2) Tetracycline was added at a final concentration of 1.0 or 2.0 μg/ml at time 0.

3) Tetracycline was added at a final concentration of 20.0; 40.0 or 80.0 μg/ml at time (0+15)minutes.

4) Tetracycline was added as described in 2) followed by a second addition of the antibiotic as described in 3).

The flasks were incubated from time 0 with shaking in a water bath at 37°C and at 20 minute intervals 2ml samples were withdrawn and cooled in ice for optical density measurements in the Beckmann DB spectrophotometer at 550mμs.

(b) An L-broth log phase culture at $O.D_{550}$ 0.1 was cooled in ice. In order to test for the effect of low concentrations of Tetracycline on the growth rate the culture was divided into 3 portions, each of 25ml, in 100ml shaker flasks. The samples were treated as follows:

1) No tetracycline was added.

2) Tetracycline was added at a final concentration of 0.25μg/ml at time 0.

3) Tetracycline was added at a final concentration of 0.5 μg/ml at time 0.

2ml samples were withdrawn and cooled in ice at intervals of 20 mimutes during incubation with shaking at 37°C. The $O.D_{550}$ of these samples was read in the Beckman DB spectrophotometer.

(xi) Mutagenesis with N-methyl-N' nitro-N-nitroguanidine

See Appendix.

(xii) Ethylene diaminetetraacetate (E.D.T.A.) treatment

Log phase cells (4×10^8 per ml) were diluted 10^{-2} into 1mMEDTA in distilled water and incubated at 37°C. Over a period of 2 hours samples were withdrawn at 30 minute intervals and diluted in 0.85% saline to give 500-800 colonies per ml. 0.1ml samples from these dilutions were plated onto Nutrient agar (3 plates per strain) for viable count determination.

(xiii) Test for Production of Catalase

The enzyme catalase is responsible for the release of oxygen from hydrogen peroxide according to the equation below.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2\uparrow$$

The presence of the enzyme is detected by putting 1ml of 10 vol. hydrogen peroxide on a 24hr culture, on an agar slope, of the strain to be tested. Almost immediately bubbles of oxygen appear on the surface of the culture if the strain is catalase positive.

(xiv) Detection of possible nucleotide excretion

An overnight culture in minimal medium of the strain to be tested was diluted $^{1}/_{20}$ into 40ml fresh minimal medium. After ninety minutes incubation when $O.D._{550}$ was 0.05 the culture was divided into 2ml portions in 100ml shaker flasks. Streptomycin was added to one sample at a final concentration of 60μg/ml and the other acted as a control. The samples were incubated at 37°C with shaking and every 30 minutes the optical densities were read at 260mμ and 550mμ and a 5ml sample was centrifuged at 7000rpm for 10 minutes and $O.D_{260}$ of the supernatant was determined.

(xv) Growth in certain basic dyes

This generally followed the procedure given by Nagal de Zwaig et al (1967) except that the cultures were not shaken during the 4 hours incubation.

The dyes tested were Acriflavine, Acridine, Orange, Methylene Blue and Eosin-Y. 2mlL-broth samples, in tubes, of the dyes at appropriate concentrations were inoculated with a 10^{-2} dilution of an overnight broth culture. The tubes were scored after 4 hours incubation at 37°C as turbid or clear.

3. RESULTS

(i) First selection of low level streptomycin resistant mutants in RE26 and RE91

Mutants resistant to a low level of streptomycin (denoted by str-r in the text) were selected in RE26 and RE91 by plating 0.1ml samples of a fully grown L-broth culture of each strain on nutrient agar containing 5μg/ml streptomycin. Apparently resistant colonies were visible after overnight incubation at 37°C with a frequency of approximately 1×10^{-6}.

Twenty colonies were picked from the selective plates of each strain and purified by streaking on nutrient agar. One mutant of RE26 and two from RE91 failed to grow on the purification plates and were assumed to be streptomycin dependent.

After purification the resistant mutants were tested for MIPC by streaking a 10^{-2} dilution of a fully grown L-broth culture across antibiotic agar plates. The MIPC for the various mutants and the sensitive parental strains are given in Table II.

Demerec (1948) stated that mutants with a resistance to at least 4.0μg/ml streptomycin could be regarded as being more resistant to the antibiotic than the parental strains. Since the sample of each strain used for selection of the mutants came from a single culture it cannot be assumed that the individual mutants are independent. Therefore one mutant from each strain with an MIPC 5.0μg/ml was picked and used in further investigations.

Figure I(a) and (b) illustrate the percentage survival of each mutant and its parent strain on low concentrations of

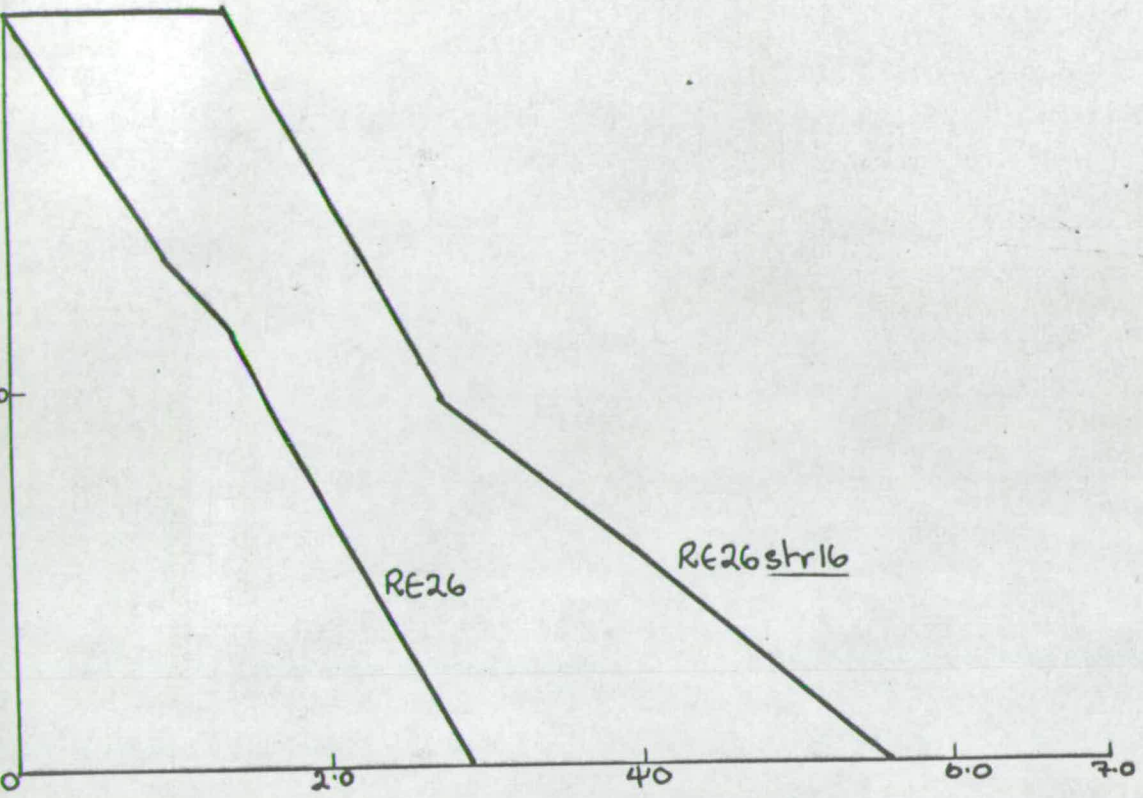
TABLE II

MIPC of RE26, RE91 and low level streptomycin resistant mutants of both strains.

Strain	MIPC (μg/ml)	No. of mutants
RE26 str-s	1.4	-
RE26 str-r	2.8	5
	5.0	14
RE91 str-s	1.0	-
RE91 str-r	2.8	2
	4.0	6
	5.0	10

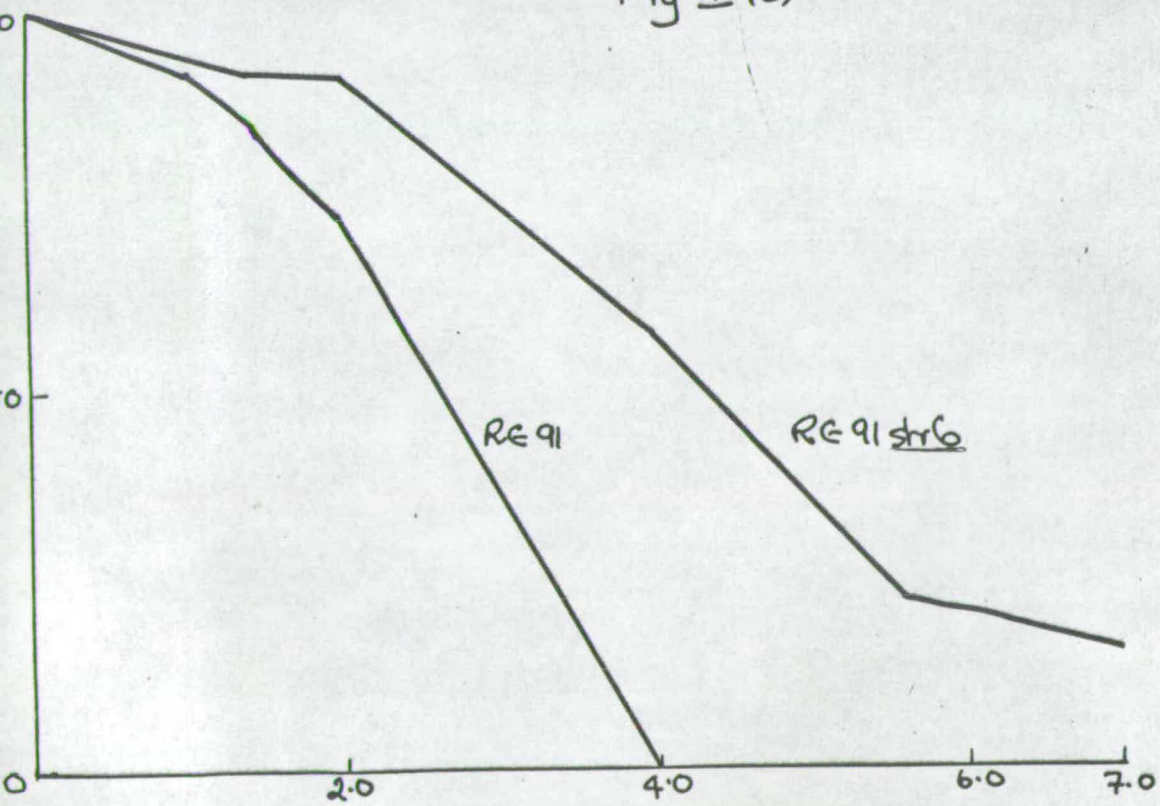
MIPC was obtained by streaking loopfuls of a 10^{-2} dilution of a fully grown broth culture across antibiotic nutrient agar plates. The concentrations of streptomycin listed in column 2 are those which permit thin confluent growth of the streak after overnight incubation.

Fig I (a)



μg/ml Streptomycin

Fig I (b)



μg/ml Streptomycin.

streptomycin as determined by the viable count method. Comparison of MIPC given in Table II with the resistance pattern of each mutant as shown in Figure I illustrates the difference in resistance levels obtained by each method. A reduction in percentage survival to 10% of the viable count will still allow thin confluent growth when the mutant is streak tested.

All MIPC determinations were done on nutrient agar containing streptomycin. It is a known fact that higher resistance levels are obtained with streptomycin resistant mutants if the strain is tested on supplemented minimal medium plus streptomycin rather than antibiotic nutrient agar. In later tests e.g. with transductants, where supplemented minimal medium was used the sensitive strain was tested and shown not to grow on the concentration of streptomycin used.

It is interesting that none of the mutants picked in either strain was resistant to more than 5.0μg/ml streptomycin. The occurrence of mutants with MIPC lower than the concentration of antibiotic used in their selection is also unexpected. A possible explanation of this phenomenon is a variation in the effective plate concentration of streptomycin. This could have been caused by the uptake of antibiotic from the medium by the large number of sensitive cells plated in the selection experiments.

(ii) Mapping Experiments on RE26str16 and RE91str6

The mutant RE26 str16, which is resistant to 5μg/ml of streptomycin, was used as the recipient strain in a series of interrupted mating experiments. The donor strain was HfrH which

transfers genetic material in a clockwise direction (see Figure II) and after15 minutes of mating has transferred the wild type pro and lac alleles into the recipient strain. Since RE26 has mutations in both these loci interruption of mating after 15 minutes with selection for Pro^+ and Lac^+ recombinants allows one to test whether this region of the chromosome carries the sensitive allele for the streptomycin resistance mutation in RE26str16.

For mating experiments donor and recipient strains were combined in the ratio 1:2 as described in Methods. Mating was interrupted with T6 phage after 15 minutes and Pro^+ and Lac^+ recombinants were selected. The recombinants were purified and tested for streptomycin resistance by streaking across supplemented minimal agar plates containing 4μg/ml streptomycin. Over a number of experiments the results consistently showed that pro^+ and lac^+ recombinants were also streptomycin sensitive (Table III).

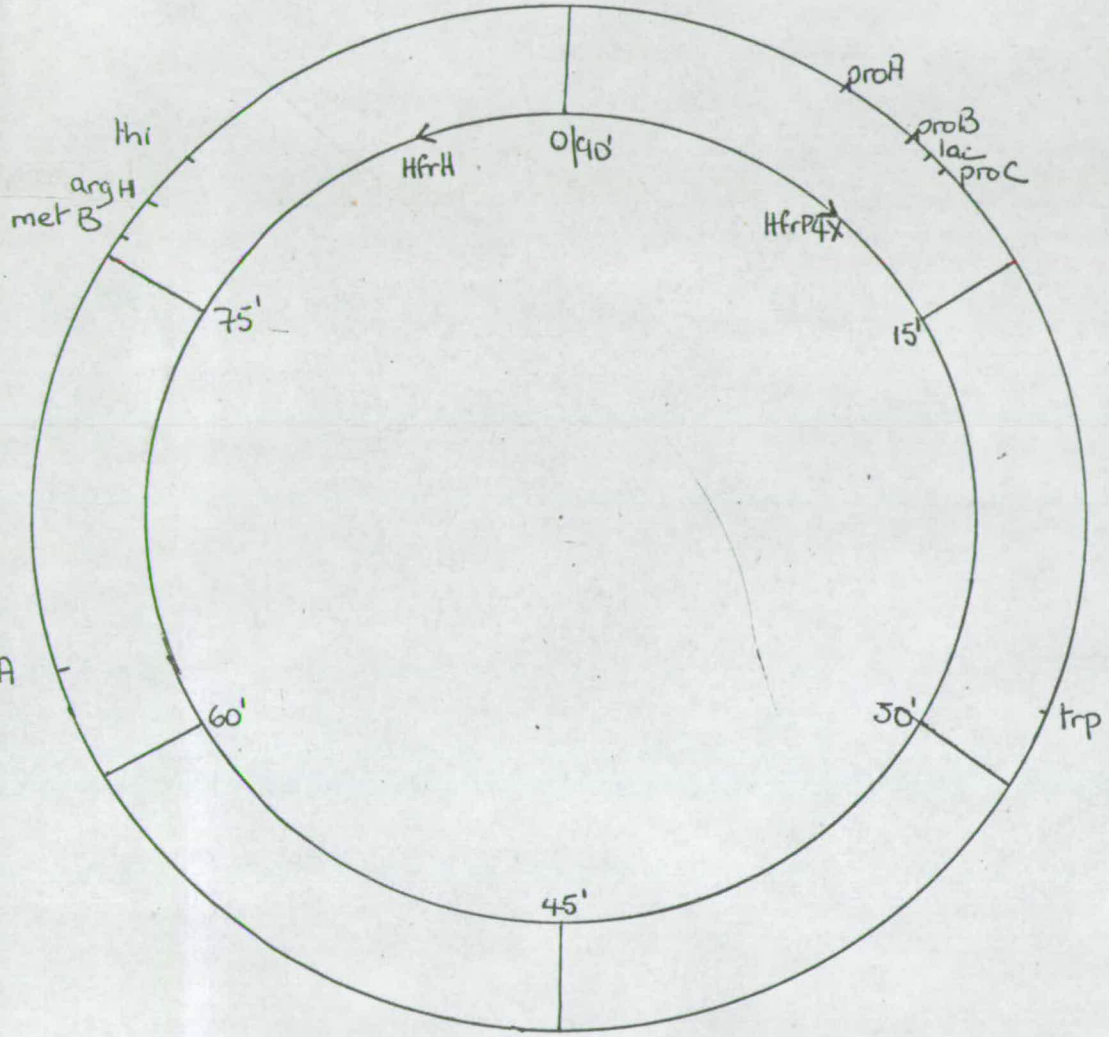
These results suggest that str16 is located close to the pro and lac loci on the E.coli linkage map (Taylor and Trotter 1967). Further mating experiments have confirmed this hypothesis. P4X type Hfr strains have their origin between proA and proB and transfer genetic material in an anticlockwise direction (Low 1965).

Two HfrP4X strains were mated to RE26str16 and after 5 minutes the mating was interrupted mechanically and Pro^+ recombinants were selected. Streptomycin resistance of the purified recombinants was tested on supplemented minimal antibiotic agar (Table IV).

The selection of a number of Pro^+ recombinants after 5 minutes of mating indicates that the mutation for proline requirement

Figure 2

-inkage map of Escherichia coli showing relevant genes and origins of Hfr strains. (after Taylor and Trotter 1967)



The map is not drawn to scale but shows the relative positions of the relevant genes.

TABLE III

Hfr H *thi* str-s mated to RE26 *pro try his str16*

Time of Interruption	Marker selected	Number of recombinants tested	% streptomycin sensitive
15'	Pro^+	727	96.6%
15'	Lac^+	2000	100.0%

After 15 minutes the mating was interrupted with T6 phage. Pro^+ and Lac^+ recombinants were selected separately and tested for streptomycin resistance by streak-testing on selective plates containing 4.0 μg/ml streptomycin. No growth or a few isolated colonies on the streak meant the recombinant was streptomycin sensitive.

TABLE IV

P4X Hfr str-s mated to RE26 *proA try his str16*.

Strain	Marker selected	Number tested	% streptomycin sensitive
RE174	$proA^+$	279	22.0%
RE176	$proA^+$	242	26.5%

The matings were interrupted mechanically after 5 minutes. *ProA*$^+$ recombinants were purified and tested for streptomycin sensitivity as described for previous mating experiments.

in RE26 is at the proA locus. If the mutation had been in the proB or proC cistrons there would have been no recombinants with P4X Hfr strains because both these alleles are transferred as terminal markers by these strains.

22.0% and 26.5% of the proA$^+$ recombinants were found to be streptomycin sensitive and this indicates two possible locations of str16. If this gene is located distal to proA with respect to the origin of the donor strain a few streptomycin sensitive recombinants would have been detected. The second possible location of the mutant gene is between proA and the origin of the P4X Hfr strains. Theoretically if the gene were located there one would expect all proA$^+$ recombinants to be streptomycin sensitive. However, Low (1965) has shown that for markers close to the origin of a donor strain the frequency with which they appear as unselected markers is very much lower than expected. This phenomenon is due to the necessity of one cross-over event occurring between the origin and the male marker before it can be expressed in a recombinant. If this region is very short (the case in point above) there is a reduced chance that a cross-over event will occur and this consequently reduces the frequency with which the male marker will appear in recombinants.

Thus as a result of mating experiments with two different donor strains it is possible to say that str16 is located near proA. It is not possible to state which side of proA the gene is located.

Str16 can be located more precisely by attempting to co-transduce RE26 str16 to proA$^+$ and streptomycin sensitivity with P1Kc transducing phage. Two loci are co-transducible if they are

separated by not more than two minutes on the E coli linkage map. Taylor and Trotter (1967) have correlated co-transduction frequencies and map distance in minutes for the genome of E.coli.

Donor phage was prepared on a Pro^+ str-s strain and used to transduce RE26str16 to proline independence (For procedure see Methods). After selection and purification of the Pro^+ transductants they were tested for streptomycin sensitivity. The results of four separate experiments are given in Table V. The co-transduction frequency between proA and str16 is consistent with an interlocus distance of approximately 0.5minutes (Taylor and Trotter, 1967).

It has therefore been established that str16 is located approximately half a minute from proA. If the second of the alternative locations of str16 is correct, i.e. if it is clockwise of proA it may be possible to co-transduce proB and str16. (See Fig. II). None of the bacterial strains available carried a proB mutation, since strains assumed previously to be proB or proC were found to carry a proA mutation. A proB mutation of RE91 was therefore obtained as described in the Appendix.

In the experiments to determine co-transduction between proB and str16 donor phage was prepared on RE26str16 and used to transduce RE91a to Pro^+. The Pro^+ transductants were selected and after purification were tested for lactose fermenting ability and streptomycin resistance as unselected markers. The experimental results and co-transduction frequencies are given in Table VI.

In both experiments where the transductants were tested for lactose fermenting ability 22.0% and 28.0% had received the

TABLE V

Transduction of RE26 proA str16 to Pro$^+$ to determine co-transduction between proA and str16 (Four tests)

Experiment	No. of Pro$^+$ transductants	No. streptomycin sensitive	Co-transduction frequency %
1	146	64	44.0%
2	448	166	37.0%
3	247	79	32.0%
4	26	12	46.0%
Σ 1-4	867	321	37.0%

=Average co-transducti frequency

Pro$^+$ transductants were purified and tested for streptomycin senstitivity by streak-testing on supplemented minimal antibiotic agar.

TABLE VI

Transduction of RE91 *proB* Lac^+ to Pro^+ with P1 (Pro^+ Lac^- *str16*) (4 tests)

Experiment	No. of Pro^+ transductants	Unselected markers		Co-transduction frequency	
		Lac^-	*str16*	*proB*-Lac	*proB*-*str16*
1	198	56	16	28.0%	8.0%
2	198	50	12	26.0%	6.0%
3	276	-	14	-	5.0%
4	198	-	14	-	7.0%
Σ 1-4	850	-	56	-	6.6%
Σ 1-2	396	106		26.7%	

Averag co- trans- ductio frequenc

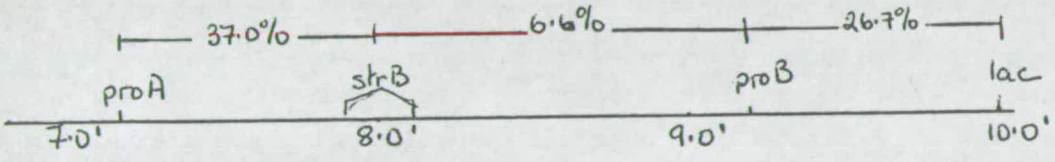
Pro^+ transductants were purified and tested for loss of the ability to ferment lactose and streptomycin sensitivity

lac$^-$ gene from the donor phage. Markovitz (1963) has reported a co-transduction frequency between *proB* and *lac* of 23.0%. 5.0-8.0% co-transduction between *proB* and *str16* is consistent with a map separation between the two loci of about one minute. No co-transduction between *lac* and *str16* was obtained.

Figure 3 shows the approximate map location of *str16*, deduced from the transduction data. The map positions of *proA*, *proB* and *lac* are based on Taylor and Trotter (1967) and *str16* is placed at about 8.0 minutes on their map. This locus will now be designated *strB* to distinguish it from the major streptomycin resistance locus *strA* (Newcombe and Nyholm, 1950).

The time taken for a gene to express its phenotype after incorporation into the cell genome is termed "phenotypic lag". Some genes, e.g. *proA*$^+$, *lac* are expressed immediately after twenty minutes phage treatment whereas others require post-treatment incubation in broth which allows the cells to divide, before they become fully functional and expressed in transductants. Several experiments were made to determine whether *strB* had any appreciable phenotypic lag before the expression of its resistance was complete. The technique involved using donor phage grown on a Pro$^+$ *strB* line, which had been obtained in a transduction experiment, to transduce RE26 to *proA*$^+$ and *proA*$^+$ *strB* simultaneously. In all experiments it was found that the number of colonies which appeared on Pro$^+$ streptomycin selective plates was approximately 40% of the number of colonies which grew on plates selecting only for Pro$^+$ transductants.

Figure 3.
Enlarged detail of *E. coli* linkage map showing approximate location of *strB*.



The map has been compiled using co-transduction frequencies obtained from the experiments described in the text. The positions of *proA*; *proB*; and *lac* were obtained from Taylor and Trotter (1967).

Thus one may conclude that strB has no appreciable phenotypic lag because the number of double transductants compared with the number of Pro^+ transductants was approximately equal to the co-transduction frequency which had previously been obtained between the two loci.

A second str-r strain, RE91str6 was tested by transduction to determine if str6 had a similar map location to strB. Donor phage was prepared on RE91str6 and used to transduce RE26 to $proA^+$. Purified Pro^+ transductants were tested for streptomycin resistance as an unselected marker but as illustrated in Table VII none of the Pro^+ transductants was streptomycin resistant.

A similar experiment was carried out using the donor phage prepared on RE91str6 to transduce RE91proB to $proB^+$. A number of purified Pro^+ transductants were tested for streptomycin resistance but none was found to be streptomycin resistant (Table VII). This shows that mutations causing low level streptomycin resistance can occur in at least two different loci.

(iii) Tests on further mutants in RE26

Twenty independent str-r mutants were selected in RE26 by plating several 0.1ml samples from each of twenty independent overnight L-broth cultures on nutrient agar containing 5μg/ml streptomycin. As in the first selection experiment resistant colonies appeared after overnight incubation with a frequency of approximately 10^{-6}.

After purification MIPC of the various mutants was determined by the streak-testing method (See Table VIII). As noted in the

TABLE VII

Co-transduction between *str6*, *proA* and *proB*

Donor characteristics	Recipient characteristics	No. of Pro^+ transductants	No. str-r	Co-transduction frequency %
proA$^+$ *str6*	*proA str-s*	160	0	0
proB$^+$ *str6*	*proB str-s*	225	0	0

In both co-transduction experiments, streptomycin resistance was tested by streaking Pro^+ transductants across supplemented minimal agar which contained 2μg/ml and 4μg/ml streptomycin. The lower concentration was tested when no growth had been obtained on 4μg/ml. The sensitive strain does not grow on 2μg/ml streptomycin.

TABLE VIII

MIPC of Independent Streptomycin Resistant Mutants of RE26

MIPC (μg/ml)	No. of mutants
2.8	8
4.0	10
5.0	2
<1.4	Parent

MIPC was obtained by streaking loopful of 10^{-2} dilution of a fully grown broth culture across antibiotic nutrient agar plates. MIPC concentrations of streptomycin are those which permit thin confluent growth of the streak after overnight incubation.

previous selection experiments none of the mutants was resistant to more than 5.0μg/ml streptomycin.

Each of the independent mutants was transduced to Pro^+ with donor phage which had been grown on a Pro^+ Str-s strain of E.coli. The transductions were done in small tubes containing 1ml broth according to the procedure described in Methods. Two independent transduction experiments were carried out on each mutant in which a maximum of forty Pro^+ transductants were tested for streptomycin sensitivity as an unselected marker.

The results of the preliminary transduction tests suggested the mutants could be divided into four distinct groups according to the measure of co-transduction between $proA^+$ and str-r. Two mutants were therefore selected from each group and subjected to more extensive transduction analysis. In the third transduction experiment the results were in good agreement with those obtained in the preliminary tests. However the fourth test proved to be unreliable when the results were examined in the light of the previous experiments. The reason could be connected with some error in the antibiotic concentration of the test plates because in each instance the percentage of streptomycin sensitive transductants had increased.

Table IX shows the results of the four separate transduction tests and gives the co-transduction frequency between $proA^+$ and str-s for each independent mutant based on the results of the first three experiments. In Table X the mutants have been grouped according to these co-transduction frequencies.

TABLE IX

Co-transduction between proA and str-r (4 tests)

Mutation No.	Number str-s No. of Pro^+ tranductants	% Co-transduction I - III	IV
str21	$^{3}/_{20}$; $^{1}/_{12}$	12.5%	-
str22	$^{12}/_{18}$; $^{5}/_{18}$; $^{7}/_{50}$; $^{32}/_{50}$	31.7%	74.0%
str23	$^{3}/_{20}$; 0/18.	7.9%	-
str24	$^{9}/_{19}$; $^{0}/_{16}$.	0	-
str25	$^{19}/_{20}$; $^{9}/_{20}$; $^{5}/_{40}$; $^{16}/_{47}$	41.0%	34.0%
str26	$^{6}/_{20}$; $^{0}/_{20}$; $^{3}/_{54}$; $^{45}/_{50}$	9.6%	90.0%
str27	$^{4}/_{7}$; $^{10}/_{20}$; $^{7}/_{32}$; $^{7}/_{49}$	35.5%	14.0%
str28	$^{2}/_{18}$; $^{0}/_{16}$.	5.9%	-
str29	$^{4}/_{18}$; $^{1}/_{12}$	16.8%	-
str30	$^{0}/_{19}$; $^{0}/_{12}$	0	-
str31	$^{18}/_{20}$; $^{8}/_{20}$; $^{9}/_{16}$; $^{50}/_{50}$.	62.5%	100%
str32	$^{0}/_{20}$; $^{1}/_{19}$.	2.5%	-
str33	$^{8}/_{20}$; $^{0}/_{20}$; $^{10}/_{36}$; $^{7}/_{44}$	23.0%	15.8%
str34	$^{0}/_{3}$; $^{0}/_{20}$	0	-
str35	$^{0}/_{20}$; $^{0}/_{19}$; $^{3}/_{17}$; $^{28}/_{49}$	5.3%	57.0%
str36	$^{0}/_{19}$; $^{0}/_{16}$	0	-
str37	$^{1}/_{19}$, $^{0}/_{20}$	2.5%	-
str38	$^{0}/_{20}$; $^{1}/_{17}$	2.7%	-
str40	$^{0}/_{19}$; $^{0}/_{20}$, $^{0}/_{14}$, $^{13}/_{50}$	0	26.0%

The second column gives the number of streptomycin sensitive Pro^+ transductants. The mutants have been numbered 21-40 to avoid confusion with previously selected mutants.

TABLE X

Low-level str-r mutants grouped according to co-transduction between proA and str-r.

Co-transduction between proA and str-r	Number of Mutants	Mutant Designation
0	5	str24; str30; str34; str36; str40.
0 - 20%	10	str21; str23; str26; str28; str29; str32; str35; str37; str38; str39.
21 - 40%	3	str22; str27; str33.
41 - 60%	2	str25; str31.

This classification table is based on co-transduction frequencies calculated from the results of the first three co-transduction experiments.

This grouping is rather arbitrary, but the range of co-transduction frequencies suggests that str-r mutations occur at more than one locus close to proA and at least one locus not co-transducible with proA. Co-transduction tests with proB should distinguish these groups more definitely, but time has not been available to carry these out.

(iv) Catalase Content of Mutants

None of the streptomycin resistant mutants isolated on 5μg/ml streptomycin was slow-growing or characterised by small colony size. However Săsărman and Horodniceanu (1967) have isolated slow growing dwarf colony antibiotic-resistant mutants of E.coli on 5 - 40μg/ml of streptomycin or Neomycin. These mutants (Ncf^-) fall into two groups: one fails to produce catalase (the enzyme which catalyses the release of oxygen from hydrogen peroxide) and the other is catalase positive (like normal E.coli) but fails to synthesize d-Aminolaevulonic acid which is necessary for the first step in the synthesis of haem.

The catalase negative mutants were located in the lac region of the E.coli genome Ncf and lac were linked by interrupted mating experiments and were co-transducible by P1. The co-transduction frequency between Ncf and lac was claimed to be 95-100%, but this may have been boosted artificially because the selected marker is always Ncf^+ Mutants in the second group which form normal sized colonies on d-Aminolaevulonic acid-supplemented medium, were due to mutation of a gene located in the purB - trp region of the genome.

All the mutants selected on 5μg/ml streptomycin had normal sized colonies on media which did not contain d-Aminolaevulonic

acid and consequently those which gave no linkage with proA are unlikely to be synonymous with Ncf⁻ mutants located in the purB - trp region of the genome. However all mutants were tested for the production of catalase and found to be catalse positive, thus excluding the possibility that some of the mutants which were co-transducible with proA may have been identical with the Ncf⁻ mutants described by Sasarman & Horodniceanu (1967) and located near lac.

(v) Interaction of Mutants with R-factors

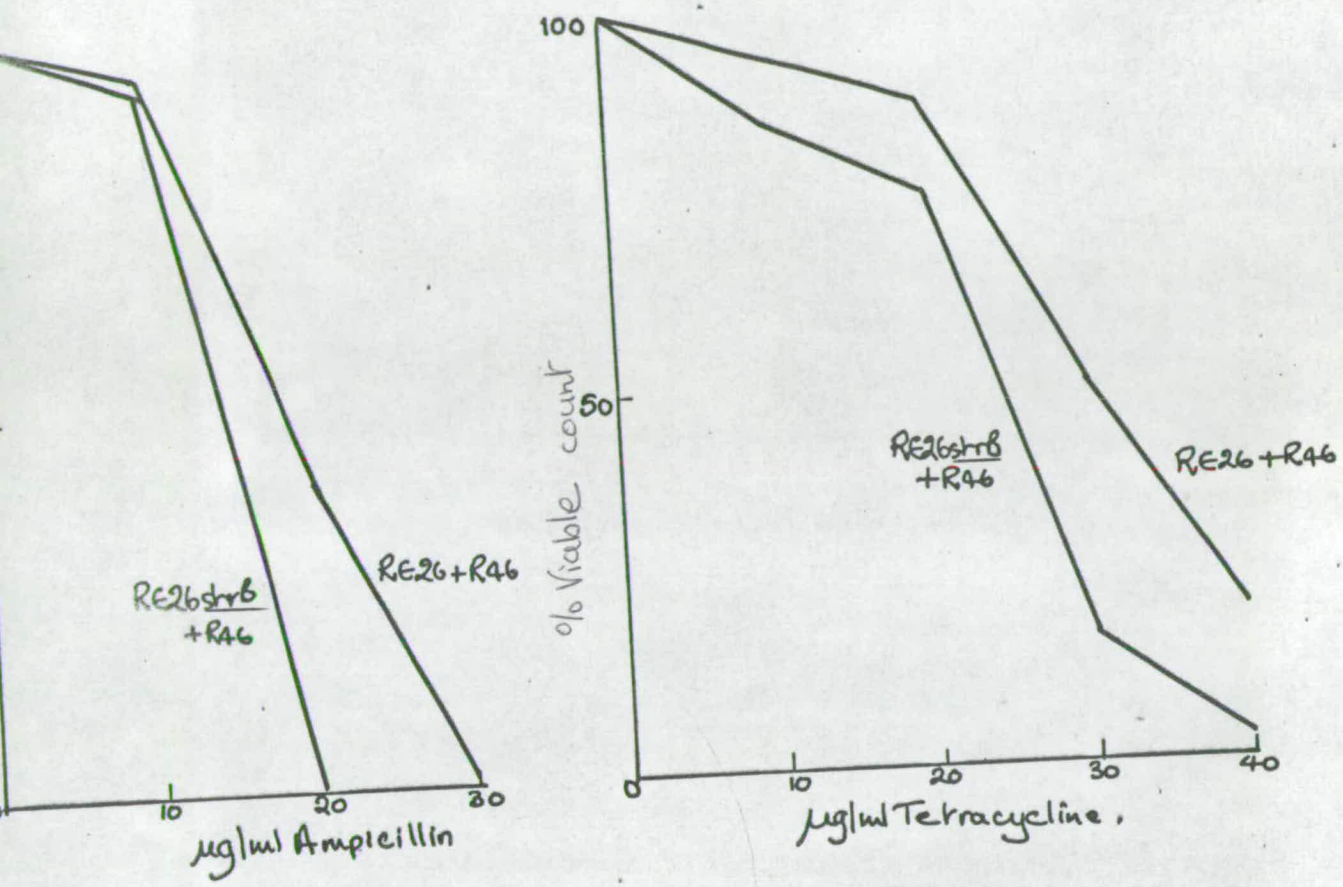
The degree of streptomycin resistance obtained as a result of the mutations studied here is comparable with the resistance levels obtained with some streptomycin resistant R-factors. Reeve (1966) when studying low level chloramphenical resistance in E.coli K12 demonstrated interaction between chromosomal and extrachromosomal resistance genes for this antibiotic.

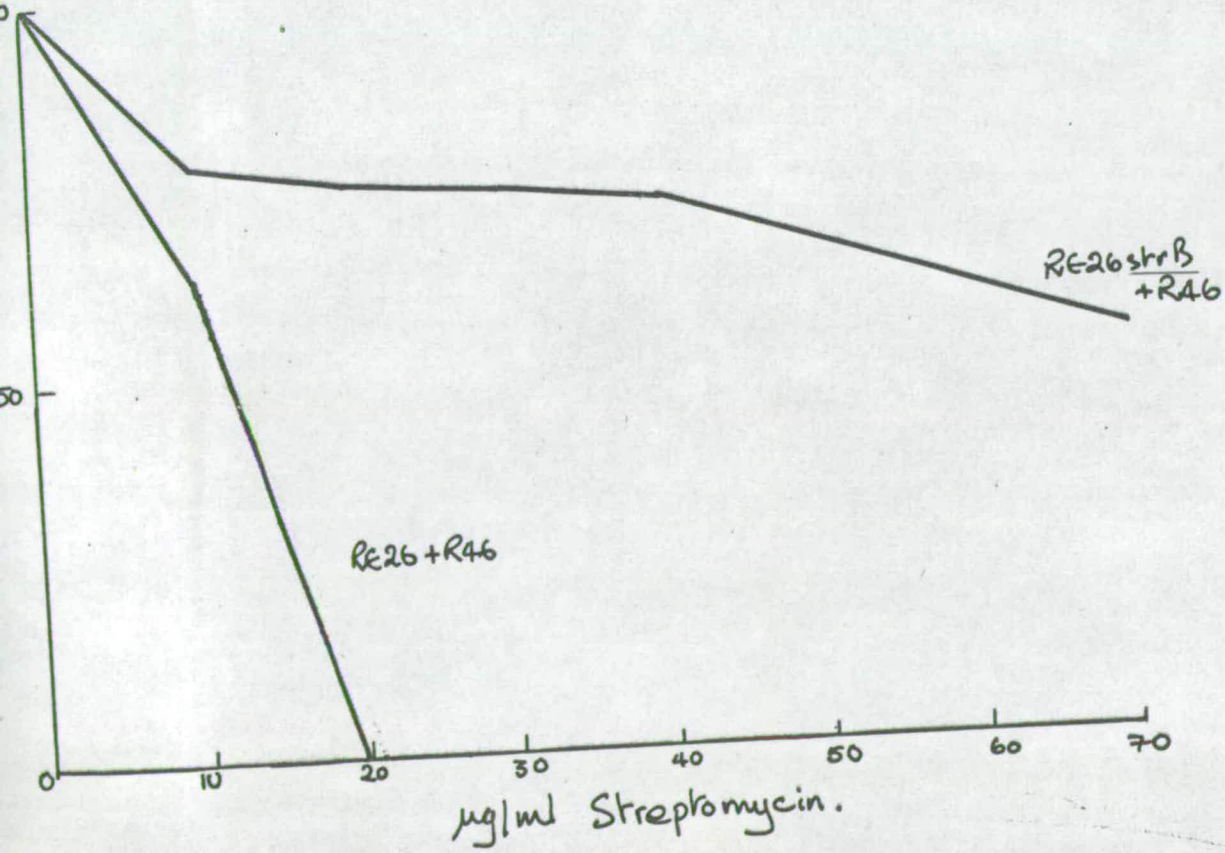
A similar interaction between R-factor streptomycin resistance and strB has been demonstrated. R-factor R46 which, in addition to streptomycin resistance, also carries resistance determinants for Ampicillin, Tetracycline and Sulphonamides, was introduced into RE26strB. Streptomycin resistance in the presence of both resistance determinants is increased severalfold compared with each individual resistance determinant. Pearce and Meynell (1968) have described very high streptomycin resistance levels when R-factor and chromosomal resistances are combined.

The survival curves of RE26(R46)and RE26strB(R46) with respect to Ampicillin, Streptomycin and Tetracycline are illustrated in Figure 4. From Figure 4 it is obvious that the interaction

Figure 4

Effect of strB on the expression of the resistance determinants of R46





between chromosomal and R-factor resistance genes is not restricted to resistance determinants for the same antibiotic. Both Ampicillin and Tetracycline resistances are reduced in the presence of strB.

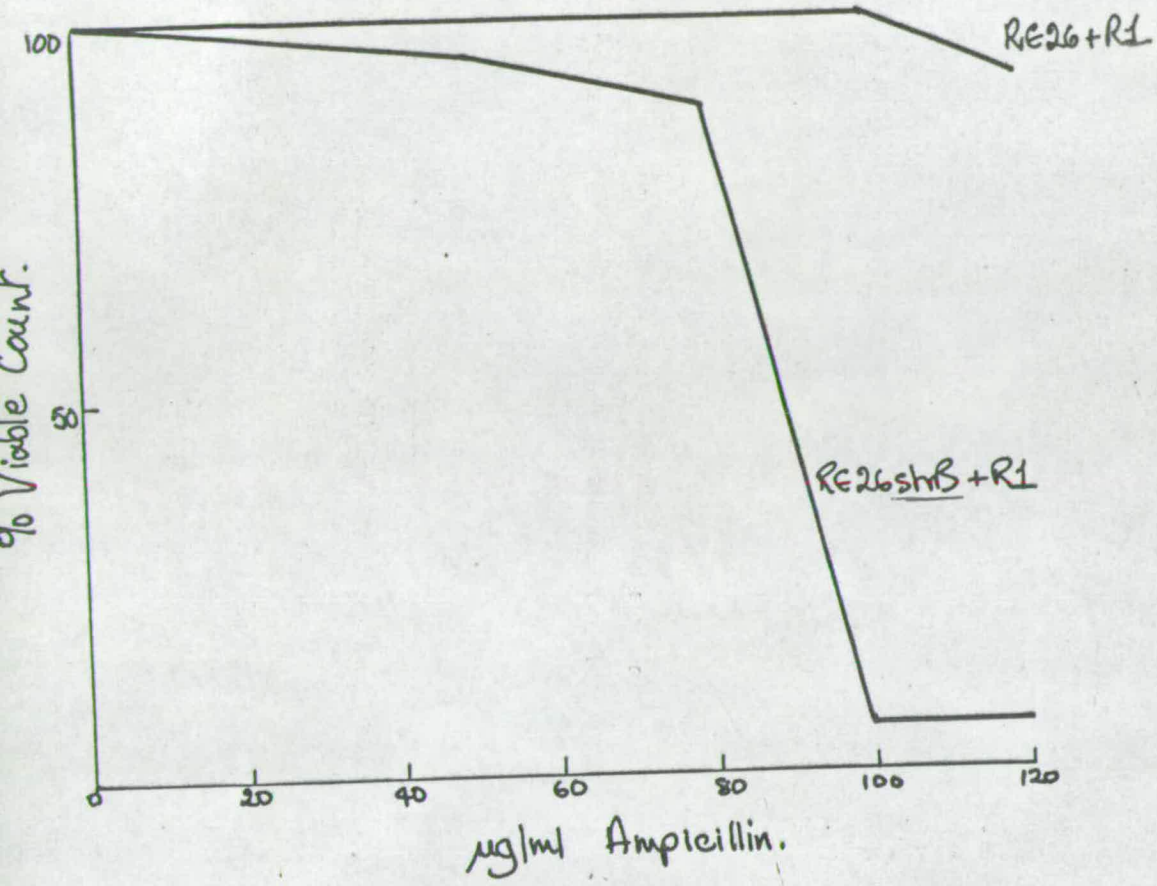
Figure 5 illustrates the effect of strB.on the expression of the resistance determinants of R1(drd19). As with R46 the streptomycin resistance is increased and Ampicillin resistance is reduced. StrB also interferes with R-factor mediated Chloramphenicol resistance. R1(drd19) also carries a Kanamycin resistance determinant and when Kanamycin resistance was tested as far as 2000μg/ml strB had no apparent effect on the expression of the R-factor resistance. If one had to speculate I think it likely that strB would increase resistance to Kanamycin in view of the similarity between the antibiotics Kanamycin and streptomycin. Another point in favour of this theory is that strB per se increases Kanamycin resistance of the strain (See Table XII).

Both the R-factors described so far have carried streptomycin resistance and the effects observed on the other resistance determinants may have been caused by the interaction of chromosomal and extrachromosomal streptomycin resistance determinants. To test this hypothesis R57, which carries only Tetracycline resistance, was introduced into RE26strB. Theeffect of strB on the expression of tetracycline resistance in R57 is illustrated in Figure 6 with RE26 (R57) for comparison purposes. Quite clearly the effect on Tetracycline resistance is due to the presence of strB alone.

Four of the independent str-r mutants of RE26 were infected with R46. The survival curves of these four mutants on Ampicillin and Tetracycline, with RE26 (R46) for comparison are illustrated in

Figure 5

Effect of strB on the expression of some resistance determinants of R1 (drd 19)



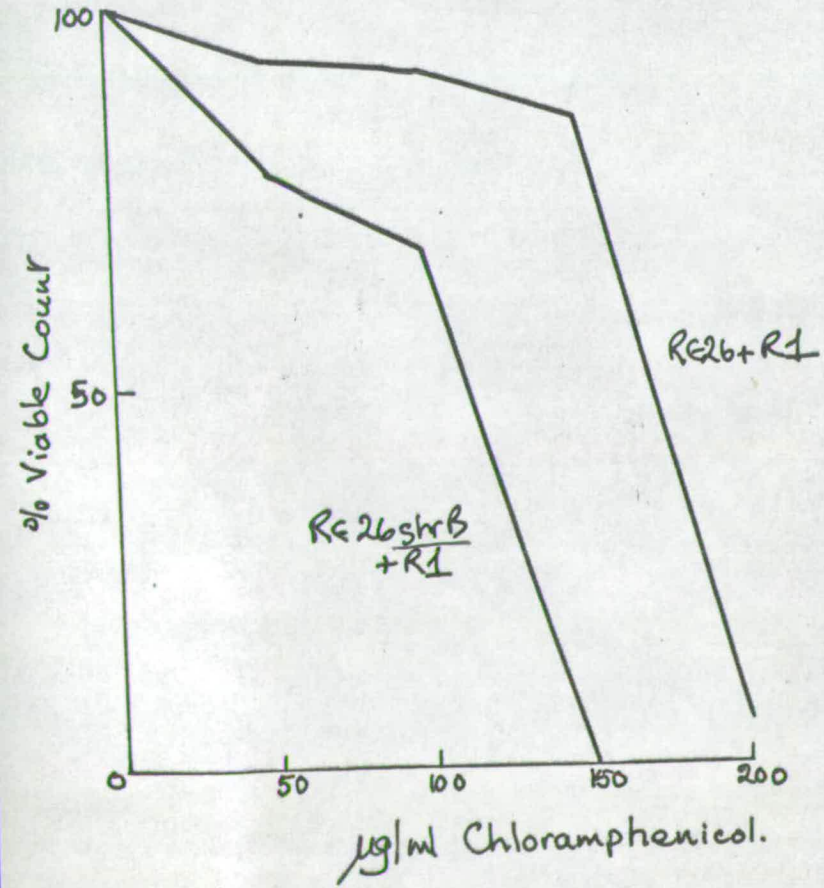


Figure 5 (contd.)

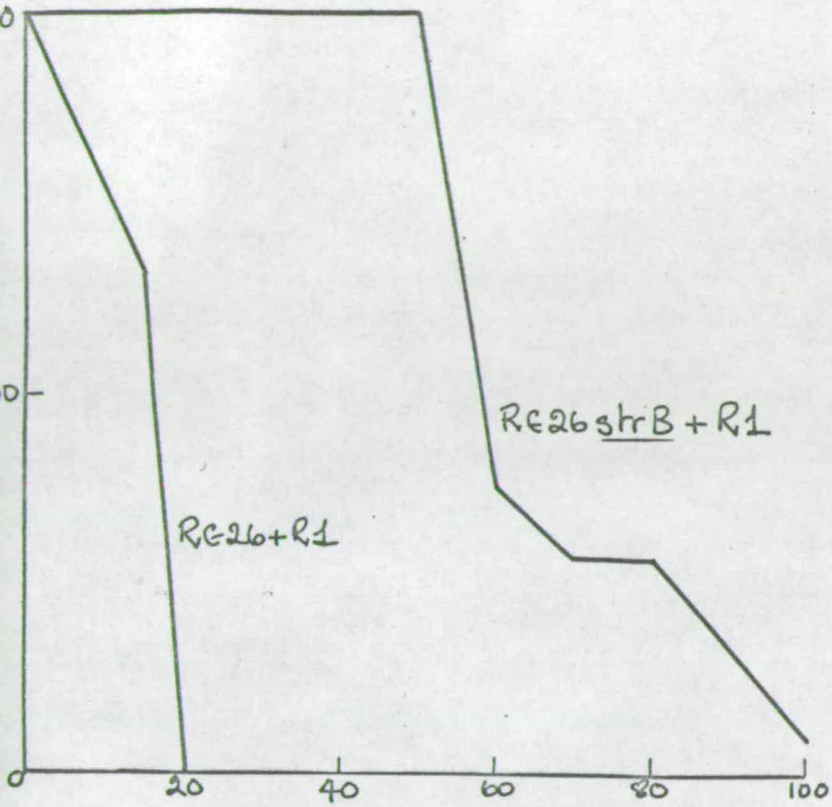


Figure 6

Effect of strB on the expression of R57 Tetracycline resistance

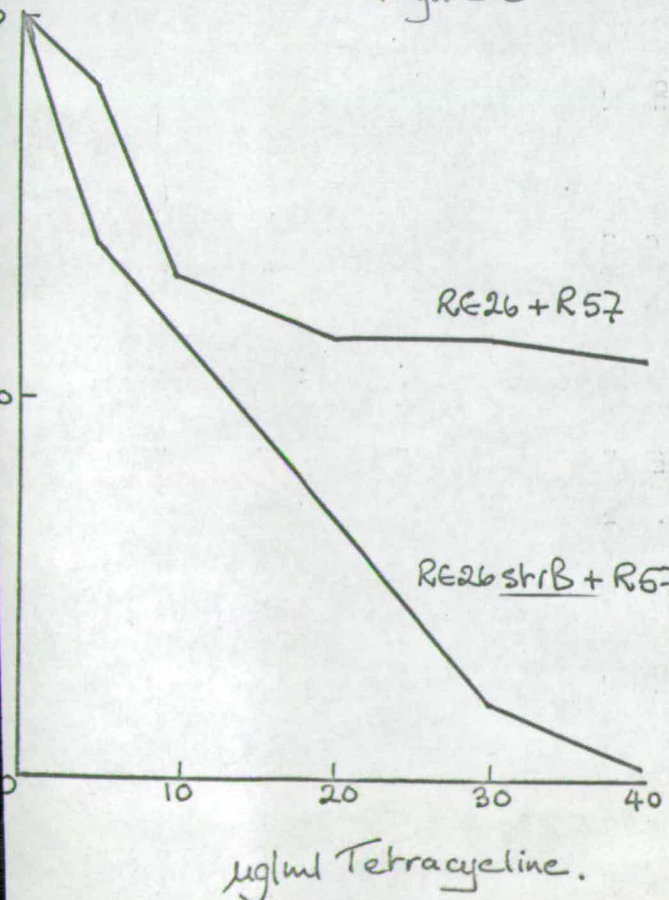


Figure 7. Ampicillin resistance is reduced in the presence of each mutant and in only one mutant, str33 is the Tetracycline resistance unaltered. The streptomycin resistance was not measured.

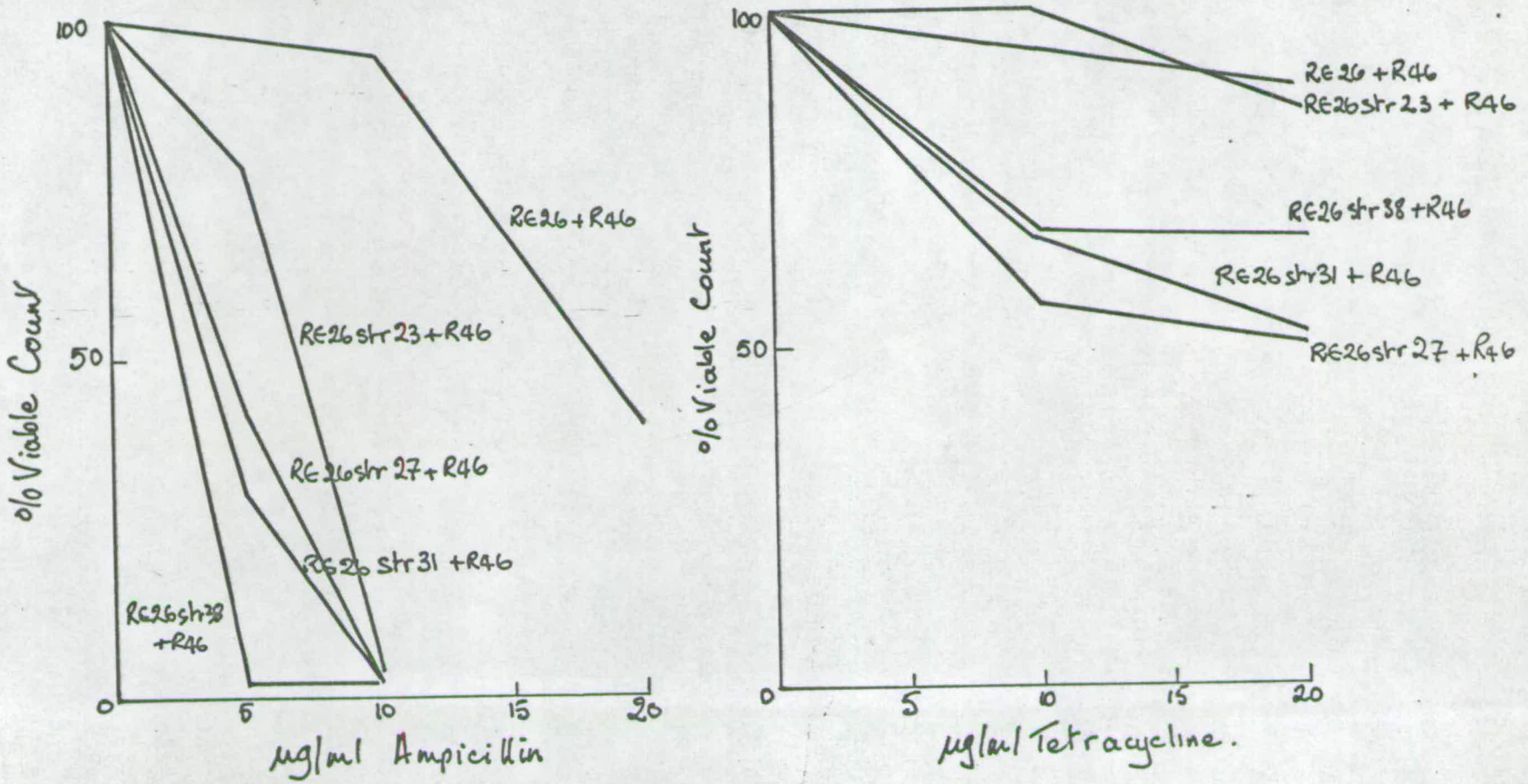
Each of the str-r mutants examined in conjunction with R46 is co-transducible with proA. RE91str6, which is not located in the same region of the chromosome, has similar effects on the resistance determinants of R46.(Figure 8). Thus the effect of str-r on R-factor Ampicillin and Tetracycline resistance is a general property of str-r mutants regardless of their location on the chromosome.

The interaction observed between the str-r mutants and R-factor Tetracycline resistance can be investigated more fully on account of the inducible nature of R-factor Tetracycline resistance. Franklin (1967) has shown that R-factor strains pre-incubated in low, sub-inhibitory concentrations of Tetracycline are more resistant to the drug than those strains which have not undergone pre-incubation. The increased resistance observed is associated with a decrease in the uptake of Tetracycline as measured by the amount of radioactivity associated with the cells which had been incubated in radioactive Tetracycline. Izaki et al (1966) noted a similar reduction in the absorption of Tetracycline after pre-incubation in low concentrations of the antibiotic.

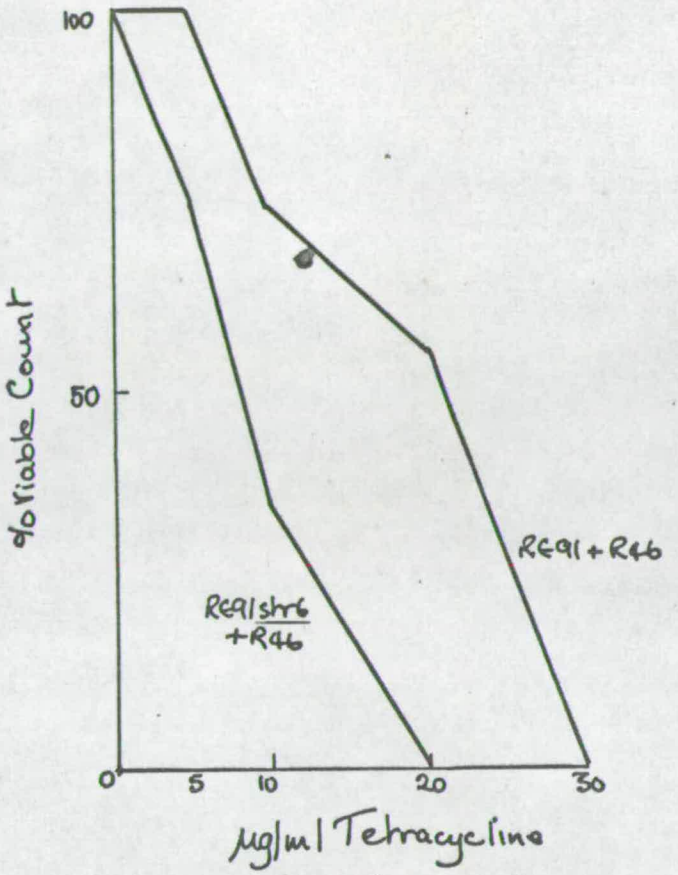
Reeve (unpublished) has developed a technique based on the growth rate of cells in L-broth when challenged by high and low concentrations of Tetracycline which also illustrates the inducible nature of R-factor Tetracycline resistance. When the R-factor

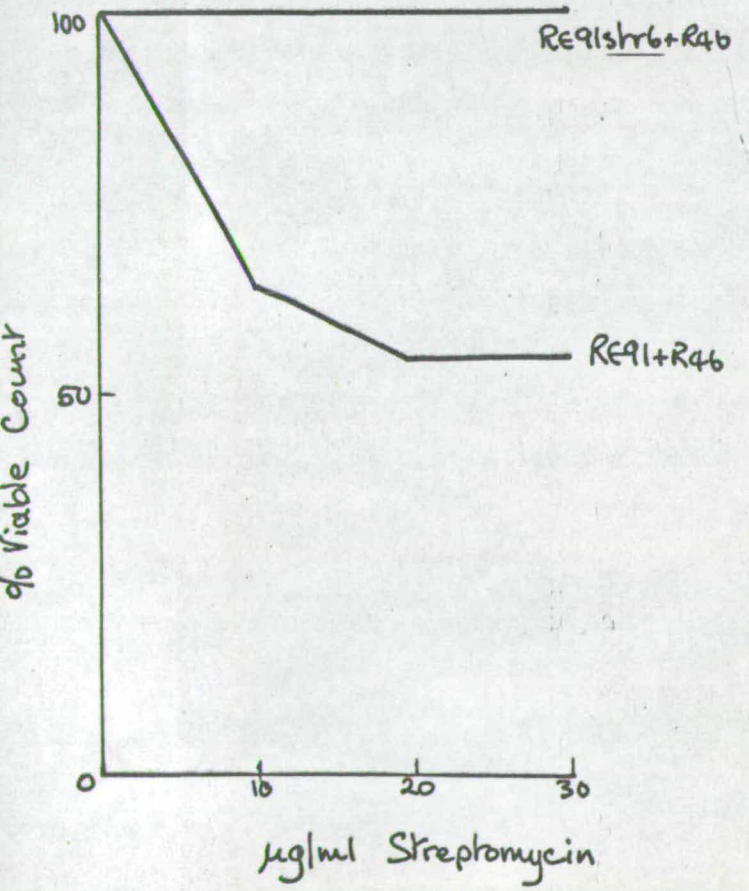
Figure 7.

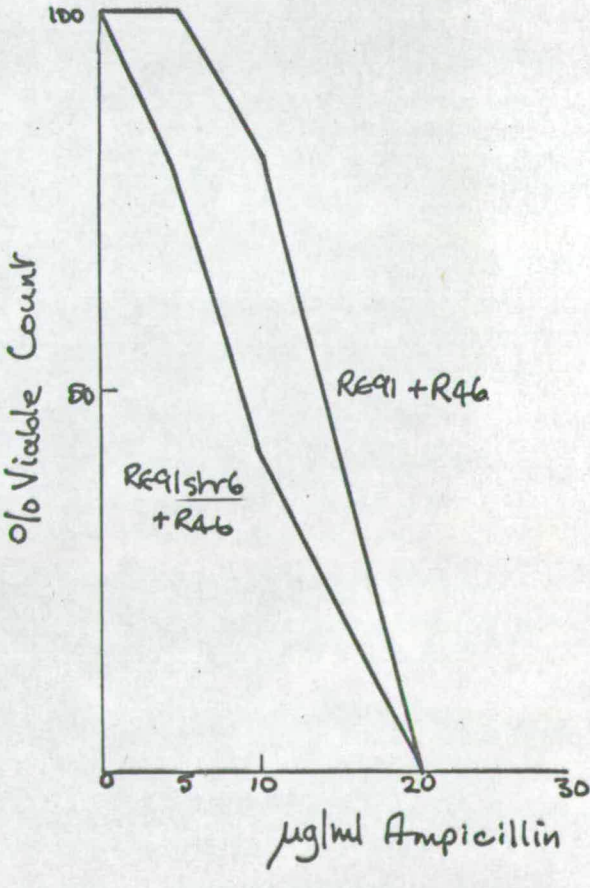
Effect of four str-r mutants on the expression of Ampicillin and Tetracycline resistance of R46.



Effect of strb on the expression of the resistance determinants of R46.







strain is incubated in a high concentration of Tetracycline the growth rate falls to virtually zero for several hours. This bacteriostatic effect is abolished by pre-incubation for fifteen minutes in a low concentration of Tetracycline before adding the higher dose of antibiotic.

The experimental procedure for investigating the effect of Tetracycline on the growth rate of R-factor strains has been described in Methods (X)(a). The graphs were obtained by plotting a logarithmic function of OD_{550} against the time in minutes.

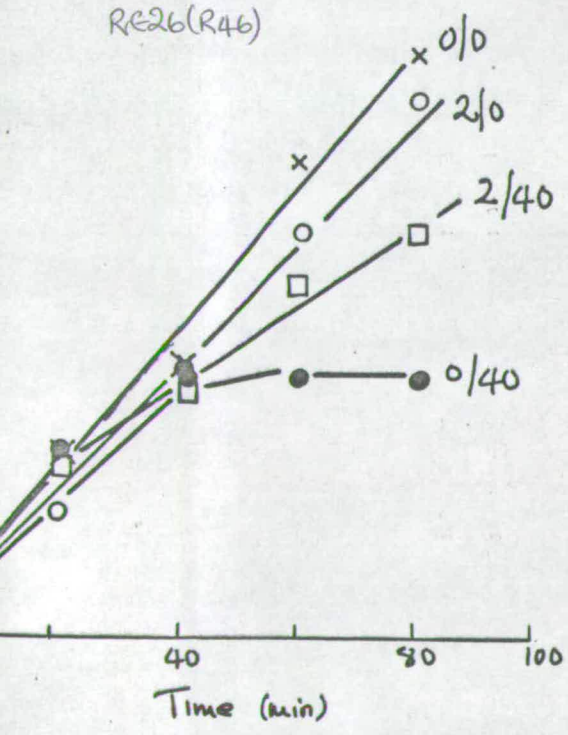
The first graph in Figure 9 illustrates the effect of the different Tetracycline treatments on the growth rate of RE26 (R46). The 0/0 curve is the normal growth rate of RE26 (R46) in L-broth. Addition of 2μg/ml Tetracycline at time 0 slows the growth rate slightly (compare 0/0 line with 2/0 line). When 40μg/ml Tetracycline is added fifteen minutes after incubation has started the growth rate falls to zero after approximately one generation. The 0/40 curve illustrates this point. The bacteriostatic effect of the high concentration of Tetracycline can be removed by the addition of 2μg/ml of Tetracycline fifteen minutes before the addition of 40μg/ml of the antibiotic (2/40 curve). As a result of the pre-incubation with 2μg/ml Tetracycline the growth rate of RE26 (R46) in 40μg/ml of the antibiotic is slower in comparison with the 2/0 curve.

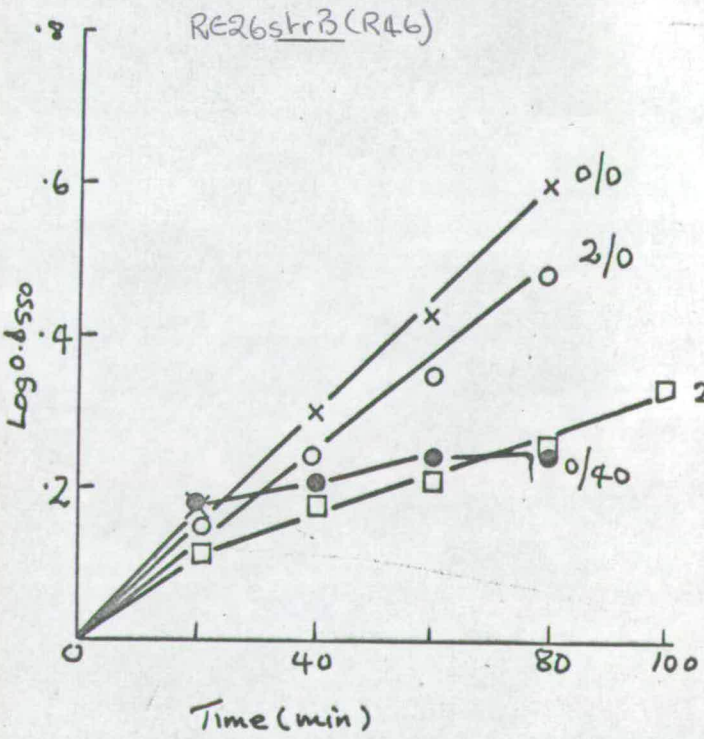
When the same treatments with Tetracycline are done on RE26<u>strB</u> (R46) the pattern of results obtained is somewhat different. Referring to the second graph in Figure 9 addition of 2μg/ml Tetracycline again slows down the growth rate (cf 0/0 and 2/0 curves)

Figure 9.

Growth of RE26(R46) and RE26strB(R46) when challenged with Tetracycline.

Symbol	Label	Tetracycline added (μg/ml) at time 0	Tetracycline added (μg/ml) at time 0+15
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40





The 0/40 curve deviates from the pattern of the 0/40 curve in RE26 (R46): the bacteriostatic effect of Tetracycline becomes apparent almost immediately. Pre-incubation with 2μg/ml Tetracycline (2/40 curve) again abolishes the bacteriostatic effect but the growth rate remains very much slower than the 2/0 curve. Comparison of the two graphs in Figure 9 makes the effect of strB on the expression of Tetracycline resistance of R46 very apparent.

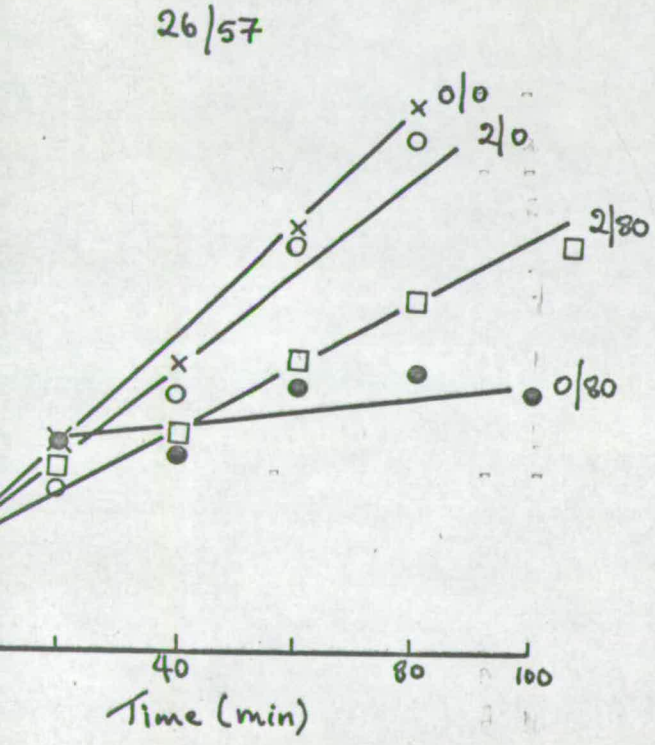
The effect of strB on RE26(R57) has been examined in the same way and the results are illustrated in Figure 10. R57 gives a much higher Tetracycline resistance and the challenge dose of Tetracycline was increased to 80μg/ml. RE26(R57) responds in much the same way as RE26(R46) to the various treatments of Tetracycline with the exception that bacteriostasis occurs almost immediately after the addition of 80μg/ml Tetracycline (0/80 curve). Again the presence of strB has the general effect of slowing down the growth rate of the treated cultures as evidenced by the 2/0 and 0/80 curves of RE26strB(R57). The failure to obtain induction to high level resistance in RE26strB(R57) (curve 2/80) was a repeatable result.

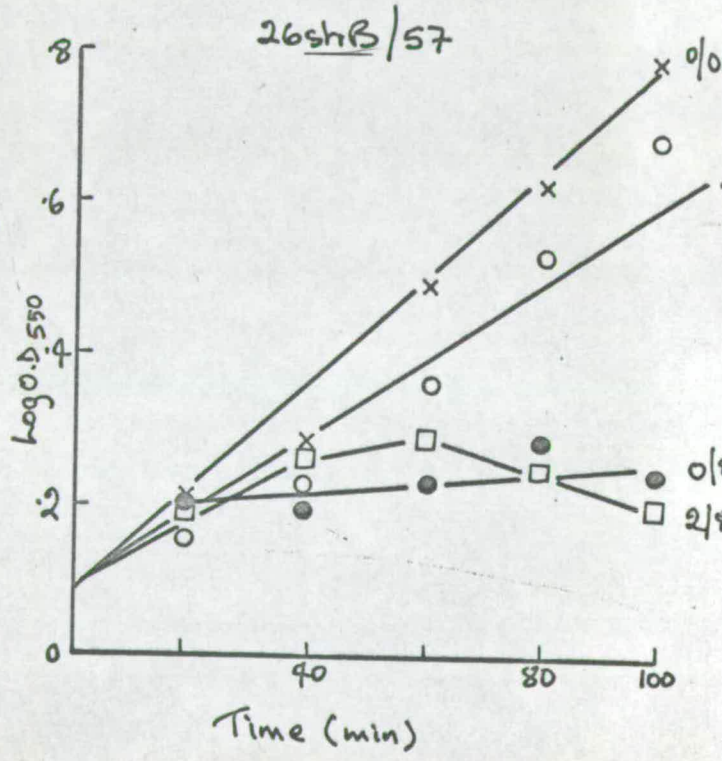
Three independent str-r mutants of RE26 were examined in the same way and the graphs in Figure 11 illustrate the results obtained. When compared with RE26(R57) the most striking effect of the mutants is shown by the 0/80 curves. In the presence of an str-r mutation the self-induction obtained with RE26(R57) is reduced (str23(R57)) or abolished (str31(R57) and str38(R57)). None of the mutants has any effect on the 2/0 or 2/80 curves.

Figure 10

Growth of RE26(R57) and RE26strB (R57) when challenged with Tetracycline.

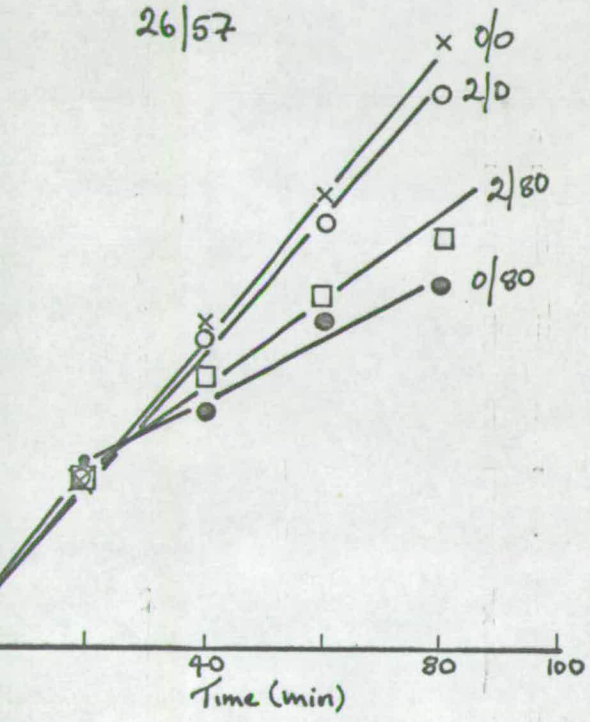
Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time (10 + 15)
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/80	—	80
□——□	2/80	2	80

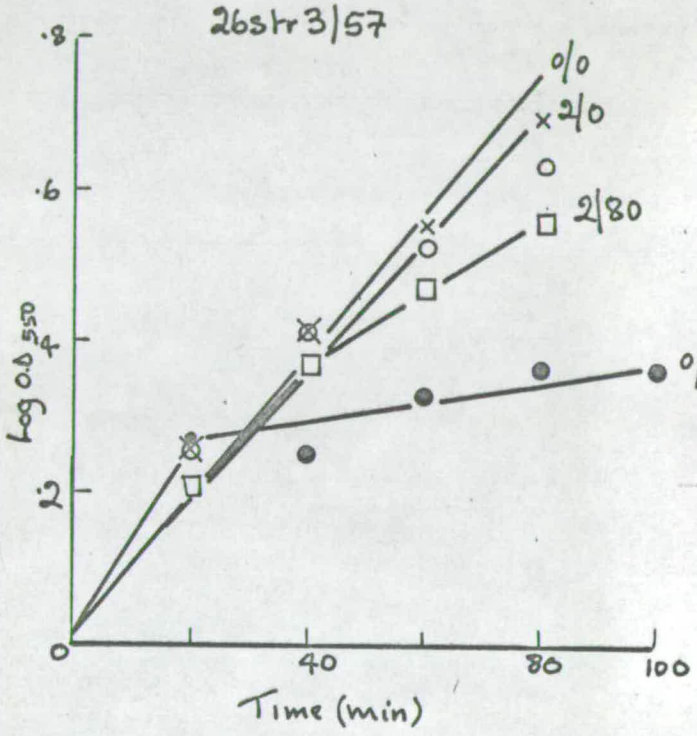


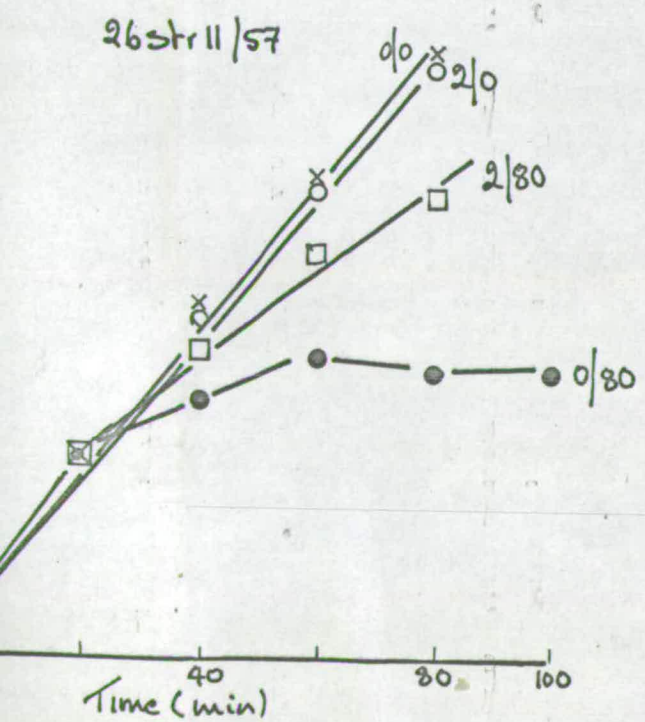


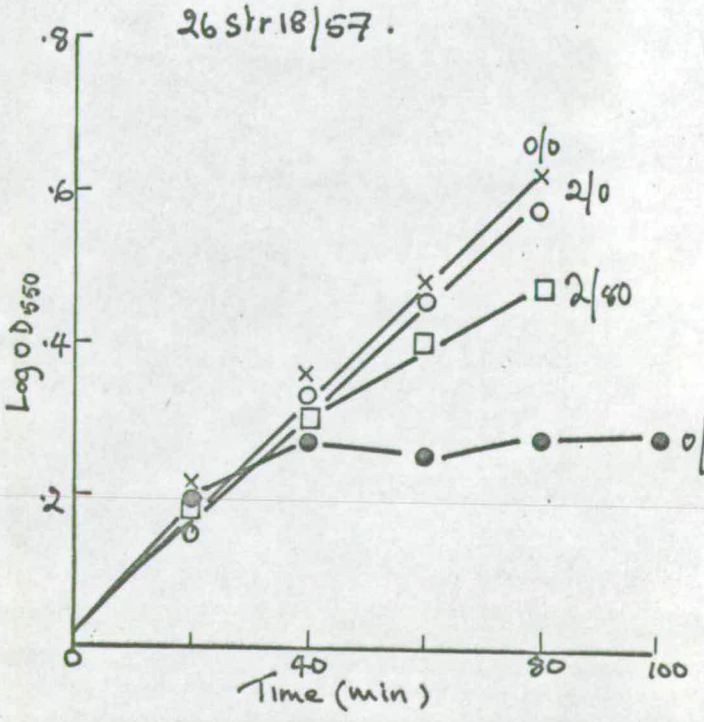
Effect of three independent str-r mutants on tetracycline resistance of

Symbol	Label	Tetracycline added (ug/ml) at Time 0	Tetracycline added (ug/ml) at Time 0+15
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/80	—	80
□——□	2/80	2	80









Each of the mutants examined so far has been co-transducible with proA to some extent and more evidence that the interaction between these mutants and R-factor Tetracycline resistance is a general property of str-r mutants regardless of map location is shown by the effect of str6 on R-factors R46 and R57. The two graphs in Figure 12 illustrate the effect of str6 on R46 Tetracycline resistance. The challenge dose of Tetracycline was reduced to 20μg/ml because RE91 is more sensitive to this antibiotic than RE26. In spite of the slower growth rate of RE91str6(R46) it is very obvious that str6 interferes with the expression of Tetracycline resistance in much the same way as strB as shown by curves 2/0; 0/20 and 2/20 in the graphs.

The combination of str6 and R57 was examined in the same way and the results are illustrated in Figure 13. The effect of str6 is less apparent in the presence of R57.

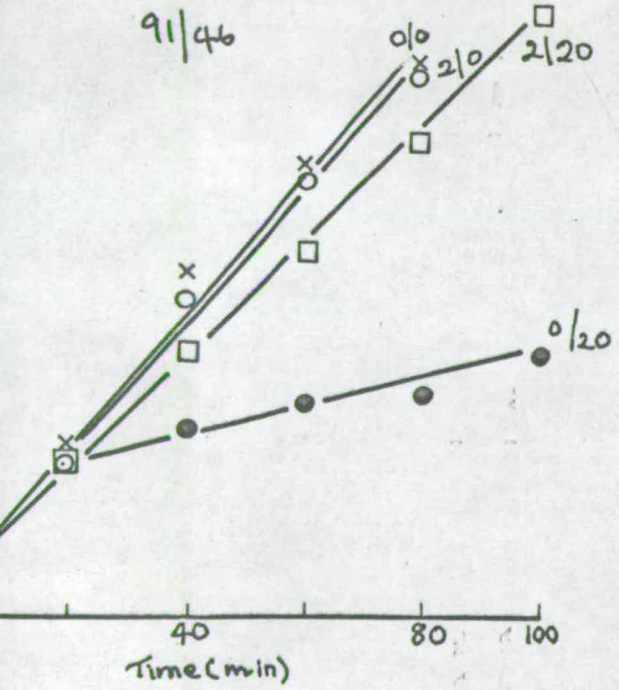
When a number of growth curves of RE26strB(R46) was examined it was observed that the growth rate decreased after the culture had been challenged with 40μg/ml Tetracycline. A typical example is illustrated in the 0/40 curve of RE26strB(R46) in Figure 14. The 0/40 curve of RE26(R46) represents bacteriostasis due to the challenge of 40μg/ml Tetracycline. The decrease in growth rate of RE26strB(R46) continues during longer incubation. Viable counts were determined at time zero, zero+60 minutes and at zero+100 minutes for the four cultures of each strain. The results are given in Table XI.

The viable counts of 0/0 and 2/0 cultures in each strain increase as expected. However the bacteriostatic effect observed

Figure 12

Effect of strb mutation on RE91(R46) when challenged with Tetracycline.

Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time 0+15
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/20	—	20
□——□	2/20	2	20



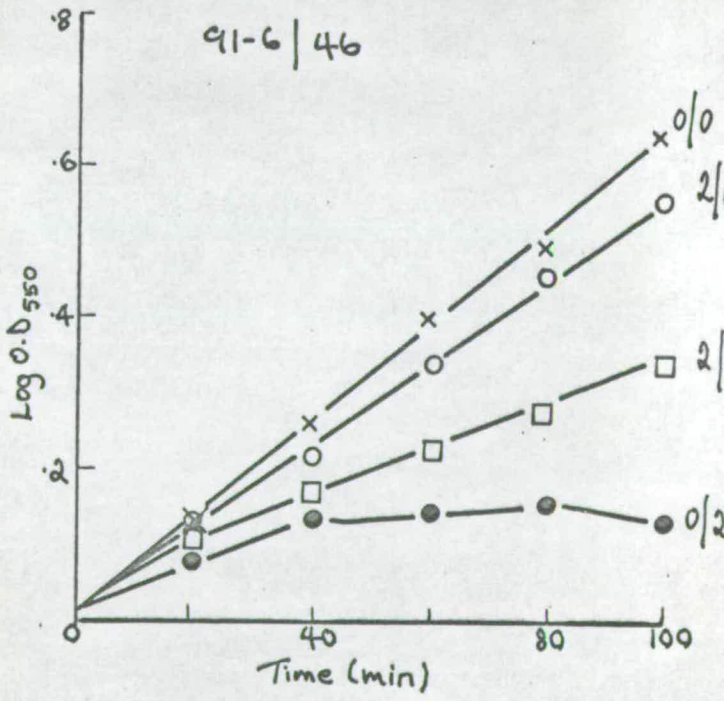
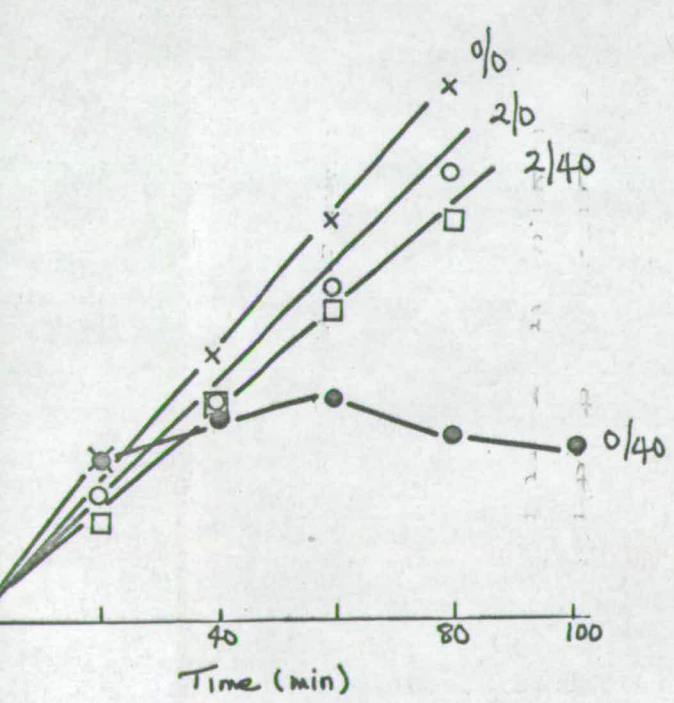


Figure 13

Effect of strb mutation on RE91 (R57) when challenged with Tetracycline.

Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time 0+15
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40



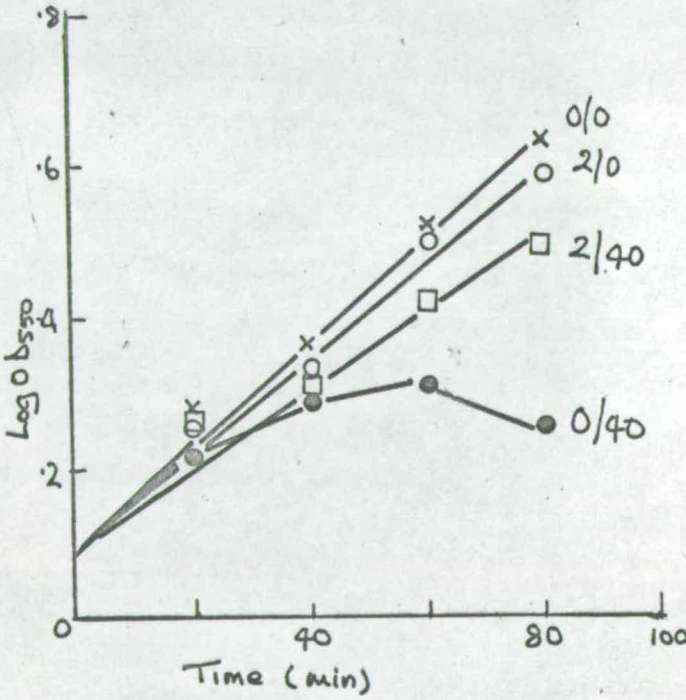
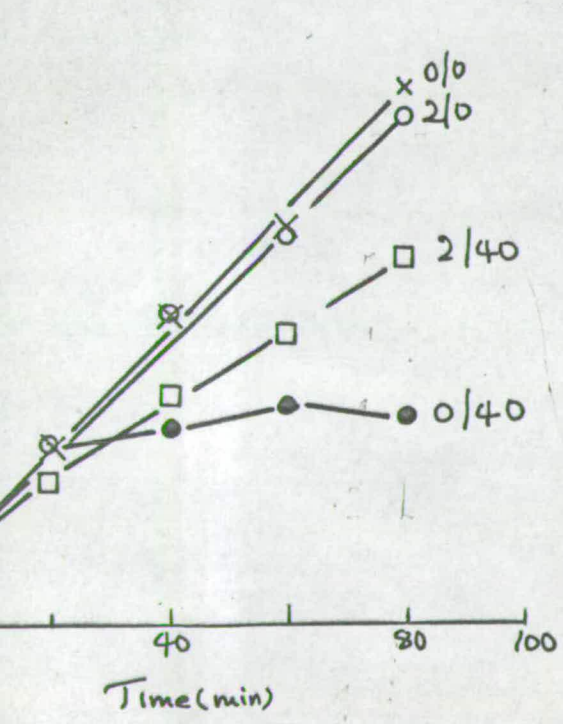


Figure 14

Effect of strB on RF26(R46) when challenged with ● Tetracycline.

Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time 0 + 15′
x——x	0/0	—	—
o——o	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40



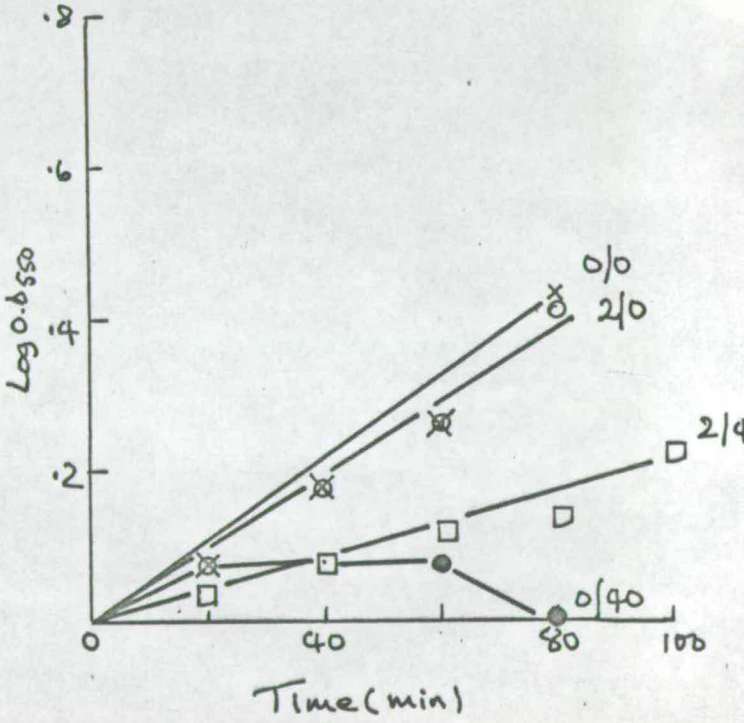


TABLE XI

Effect of Tetracycline on the Viable Count of RE26(R46) and RE26strB(R46).

Culture	Viable Counts (cells/ml)					
	RE26(R46)			RE26strB(R46)		
	0	0+60	0+120	0	0+60	1+120
0/0	10^8	4.2×10^8	$6.4 \times 10^{8*}$	5×10^7	1.2×10^8	2.9×10^8
2/0	10^8	2.9×10^8	$5.8 \times 10^{8*}$	5×10^7	1.3×10^8	2.9×10^8
0/40	10^8	2×10^7	$<10^7$	5×10^7	5×10^7	$<10^7$
2/40	10^8	10^8	2.5×10^8	5×10^7	10^7	4×10^7

The viable counts were obtained from the average of three plates.

*These figures were obtained after (0 + 100) minutes and correspond to $O.D_{550}$ 1.8 as measured on the Beckmann DB spectrophotometer.

in the 0/40 culture of RE26(R46) is not apparent in the viable count determinations; the number of viable cells per ml. in this culture decreases. The viable counts of the 2/40 curve in RE26(R46) do not follow the steady increase in cell number which is illustrated by the graph.

In RE26strB(R46) the viable counts of the 0/40 curve also represent a decrease in the number of viable cells. The steady increase in the cell density of the culture shown by the 2/40 curve is not due to a similar increase in the number of viable cells. There is an initial decrease in cell number before the increase which is observed during the latter part of the incubation. Therefore the increase in $O.D_{550}$ which is observed for the 2/40 curves does not represent only an increase in cell number but may also reflect an uptake of Tetracycline by the cells. In 1963 Arima and Izaki observed that uptake of Tetracycline by sensitive cells caused the absorption of cells at 550 mμs to increase. The situation in the 0/40 culture is rather more complex: from the viable count determinations one would expect a steady decrease in $O.D._{550}$. The 0/40 curve obtained may therefore represent an uptake of Tetracycline.

(vi) The Effect of Tetracycline on Mutants

The interaction which occurs between the str-r mutants and R-factor mediated Tetracycline resistance could be caused in one of two ways. Either such mutations could produce "collateral sensitivity" to Tetracycline or they could interfere specifically with the R-factor Tetracycline resistance mechanism.

Collateral sensitivity was the term used by Szybalski and Bryson (1952) to describe the effect of mutation to resistance to one antibiotic causing sensitivity to another.

In the first instance str-r strains will be more sensitive to Tetracycline than the parent; in the second instance the interaction between the two resistances will be apparent only in Tetracycline resistant R-factor strains.

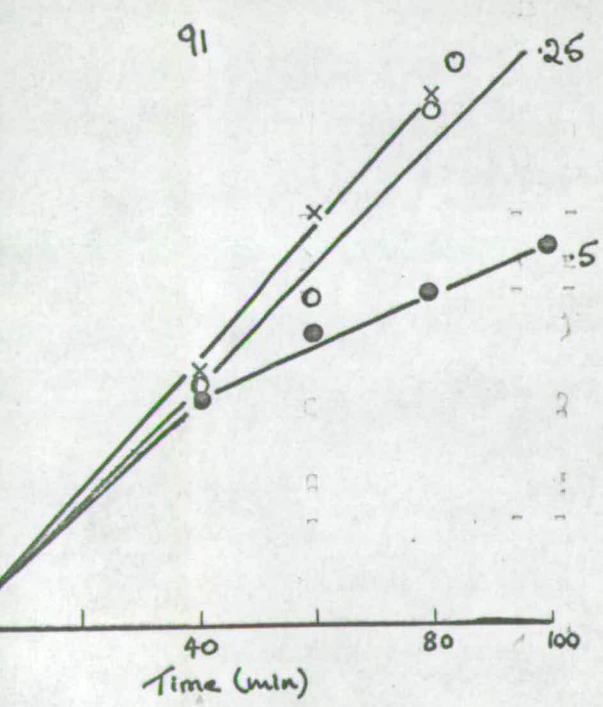
These hypotheses were tested by measuring the growth rate of str-r mutants and the parent strains in L-broth containing low concentrations of Tetracycline. Log phase L-broth cultures of str-r mutants were treated with 0.25 and 0.5μg/ml Tetracycline as described in Methods (X)(b). The results of the experiments are expressed graphically by plotting a logarithmic function of $O.D_{550}$ against time in minutes.

Figure 15 illustrates the effect of Tetracycline on the growth rates of RE91 and RE91str6. The presence of str6 clearly makes the cell more susceptible to Tetracycline. A similar test on str-r mutants of RE26 produced the results shown in Figure 16. The effect of Tetracycline on RE26 is a general decrease in growth rate and this response occurs in several str-r mutants to varying degrees. The results of these experiments support the hypothesis that str-r mutants have acquired collateral sensitivity to Tetracycline. In two of the mutants, str21 and str24 there is no growth in the presence of Tetracycline. Thus the increased sensitivity to Tetracycline is expressed to varying degrees but it is not possible to correlate this with any difference in the location of the mutants. The two mutants most seriously affected by Tetracycline do not have the same map location according to Table X:

Figure 15

Response of RE91 and RE91 str6 to low concentrations of Tetracycline.

Symbol	Label	Tetracycline added (µg/ml) at time 0
x——x	0	—
o——o	·25	0·25
●——●	·5	0·5



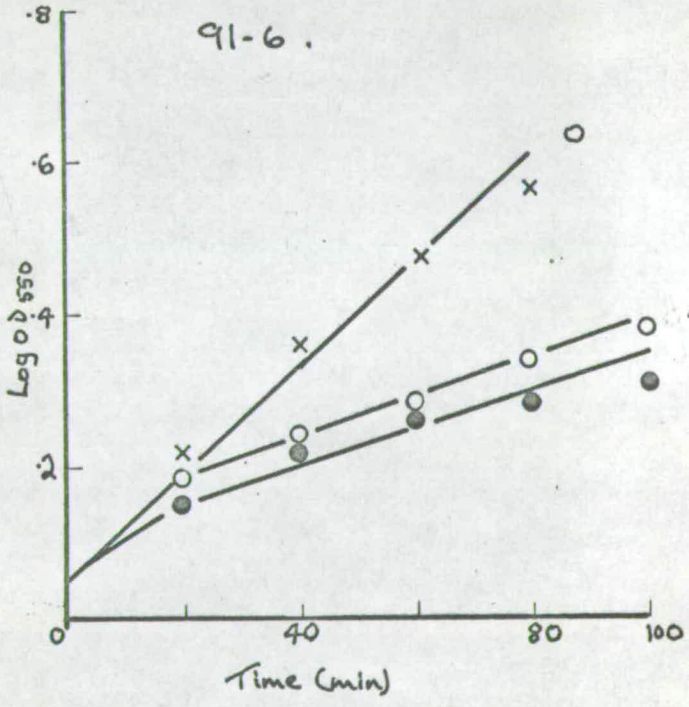
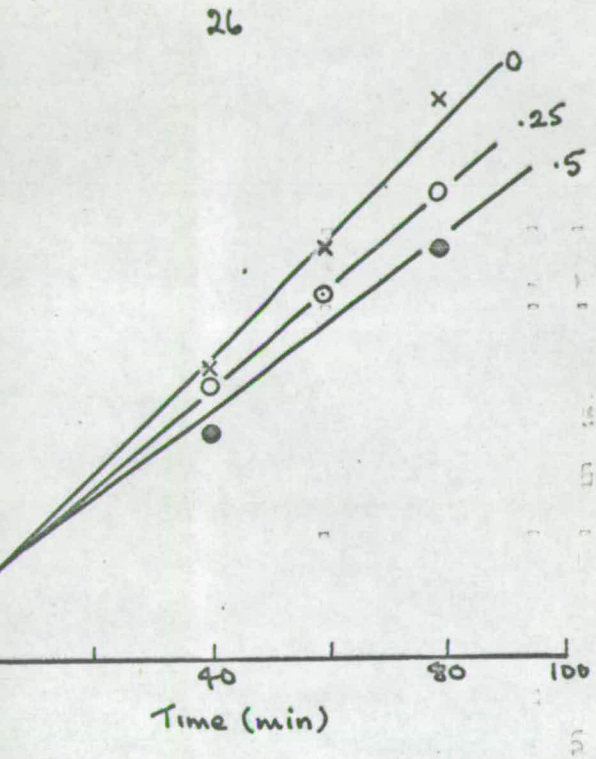
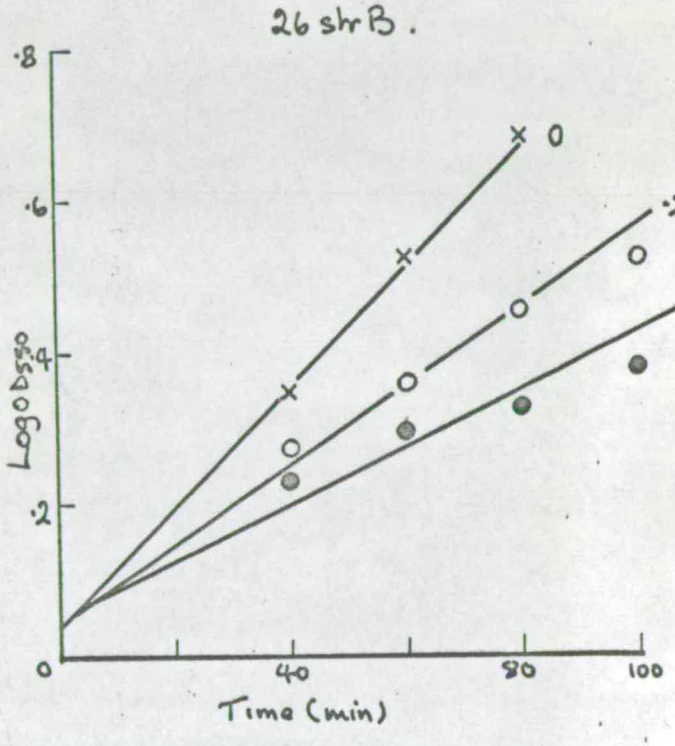


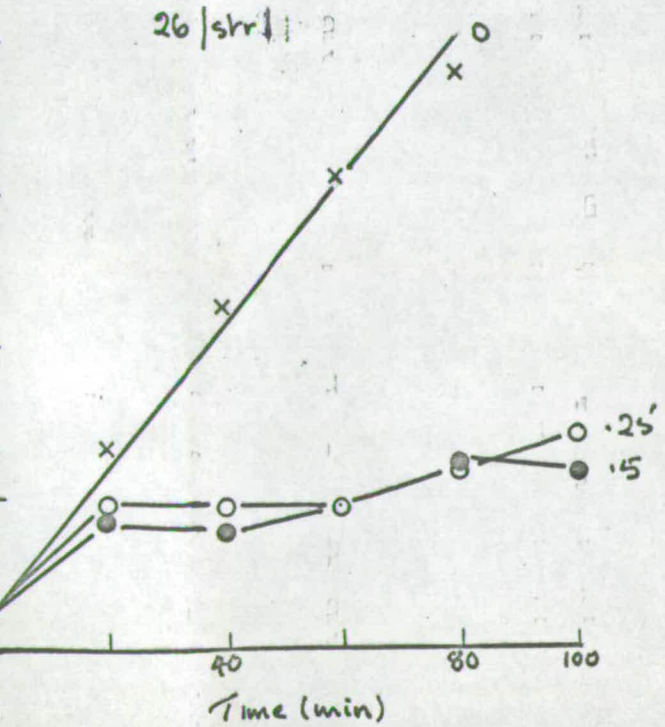
Figure 16

Response of RE26, RE26 strB, and other str-r mutants of RE26 to low concentrations of Tetracycline.

Symbol	Label	Tetracycline added (μg/ml) at time 0
x——x	0	—
o——o	.25	0.25
●——●	.5	0.5







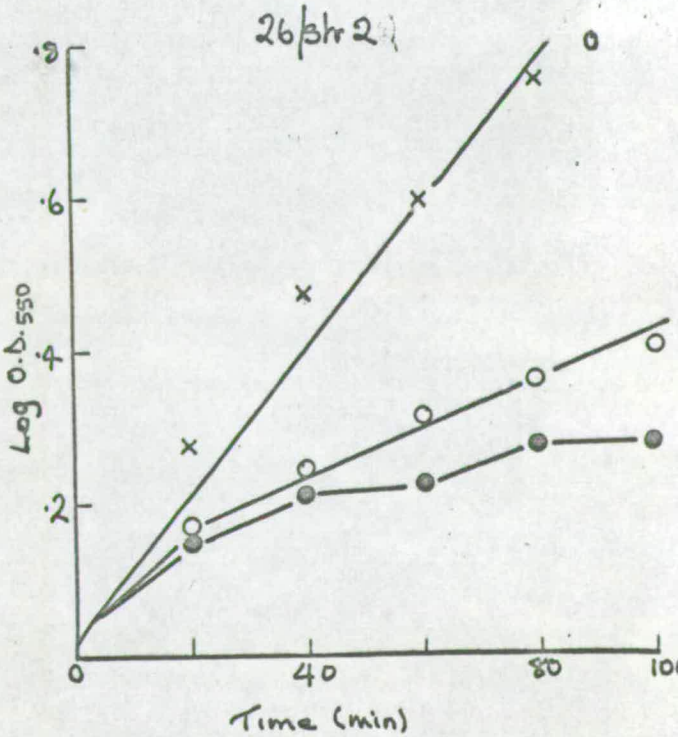
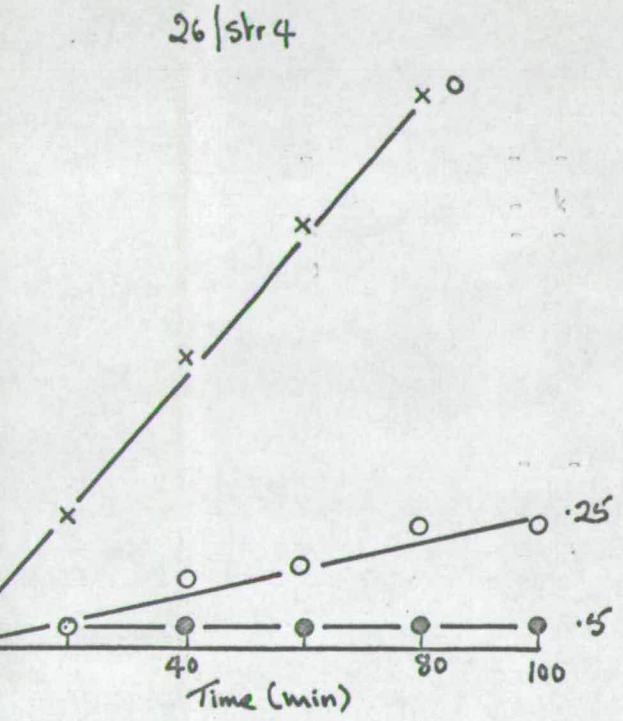
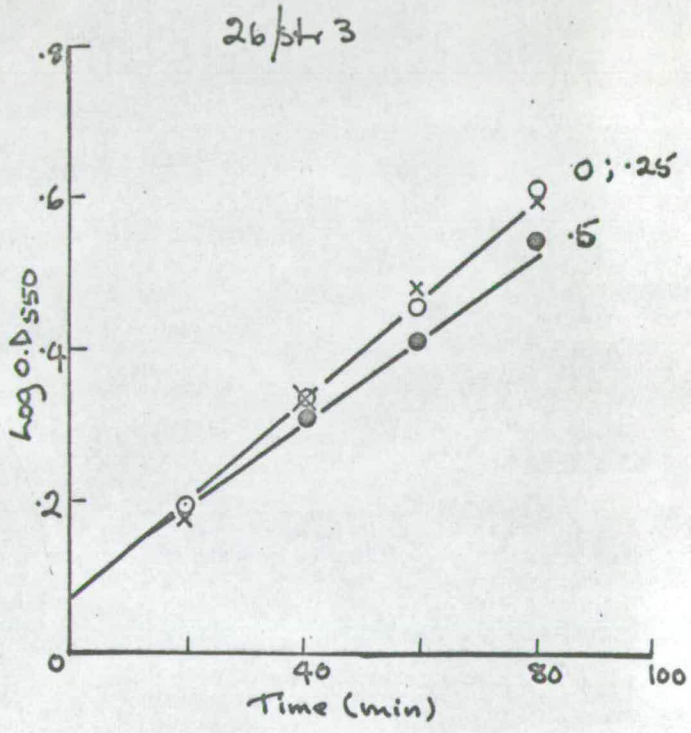
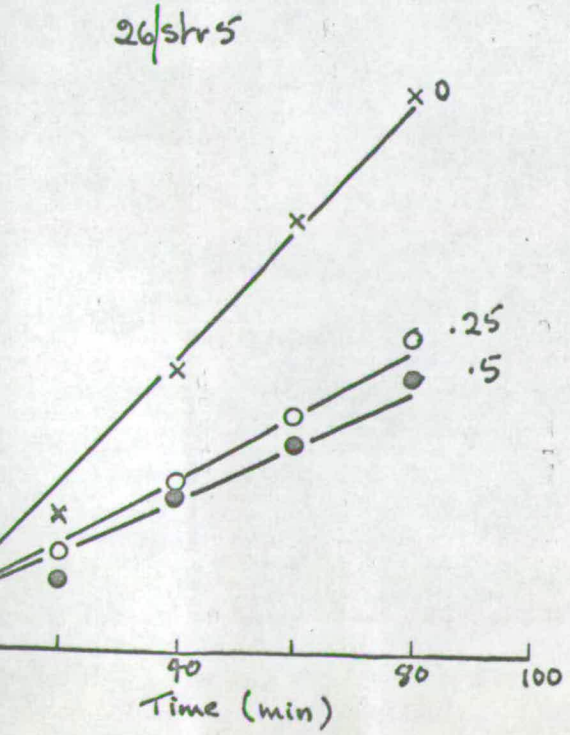


Figure 1b (contd)

Symbol	Label	Tetracycline added (μg/ml) at time 0
×——×	0	—
○——○	·25	0·25
●——●	·5	0·5







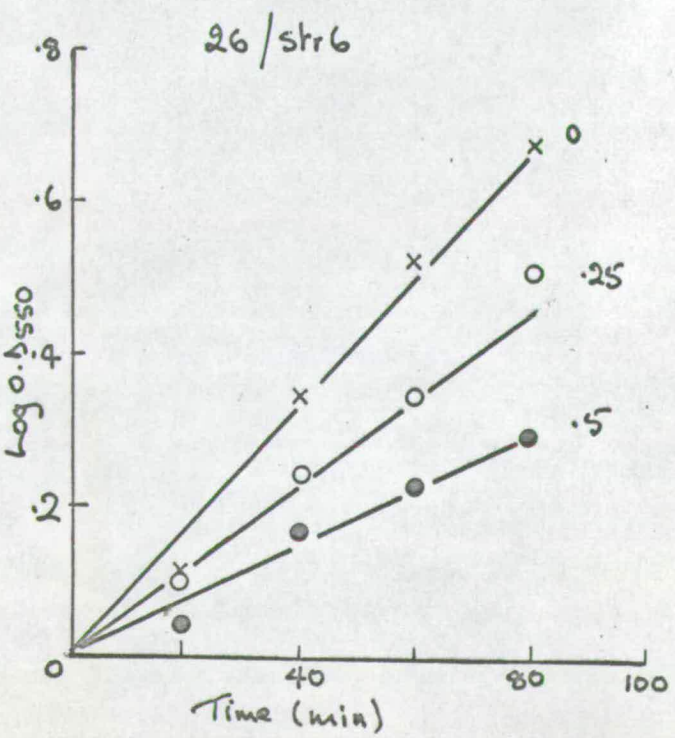
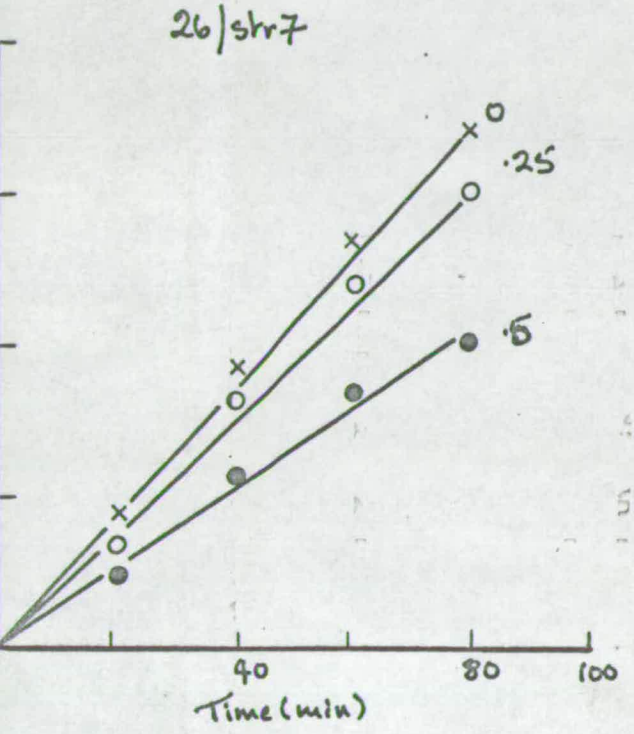
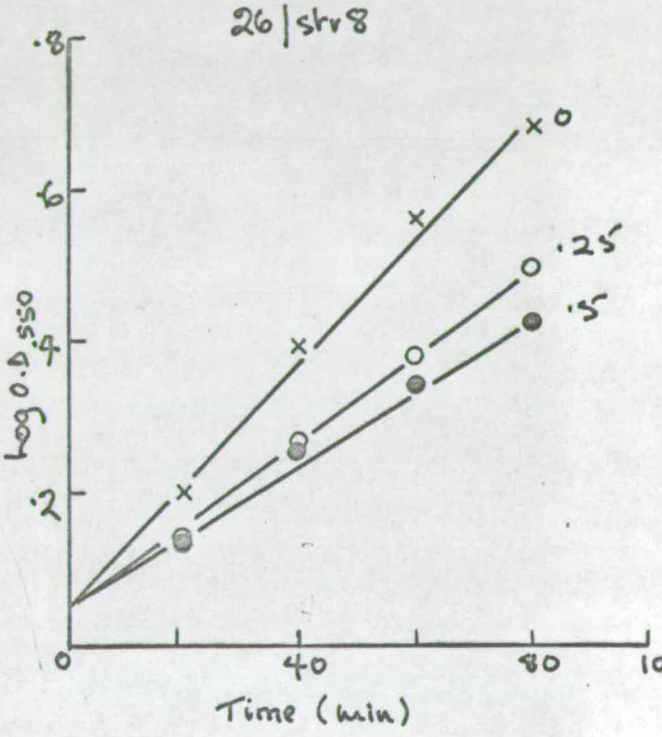
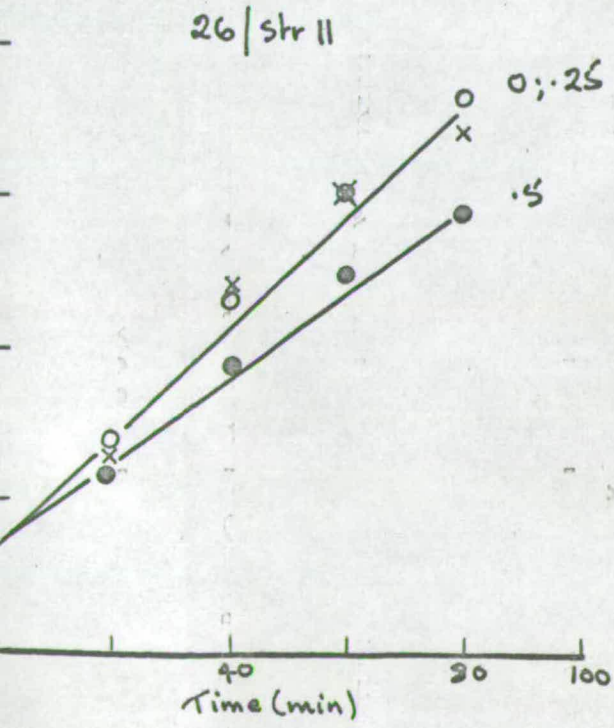


Figure 16 (contd.)

Symbol	Label	Tetracycline added (µg/ml) at time 0
×——×	0	—
○——○	·25	0·25
●——●	·5	0·5







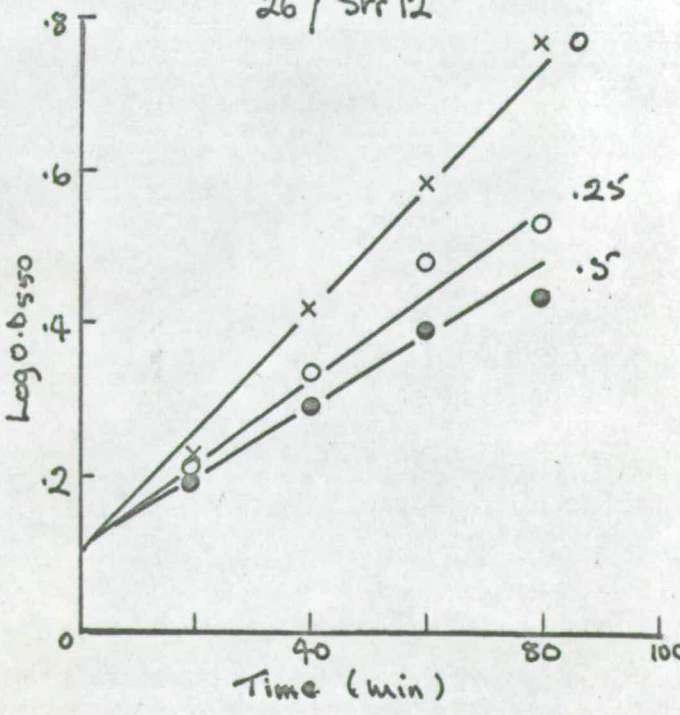
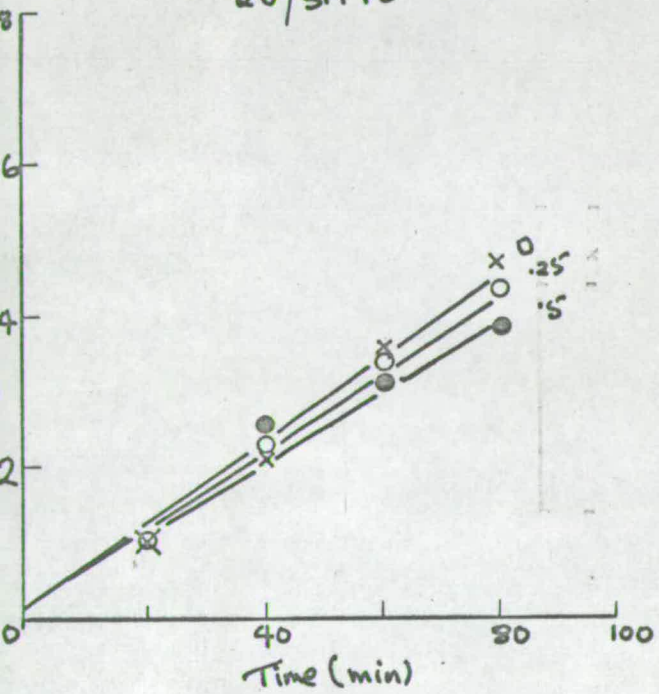


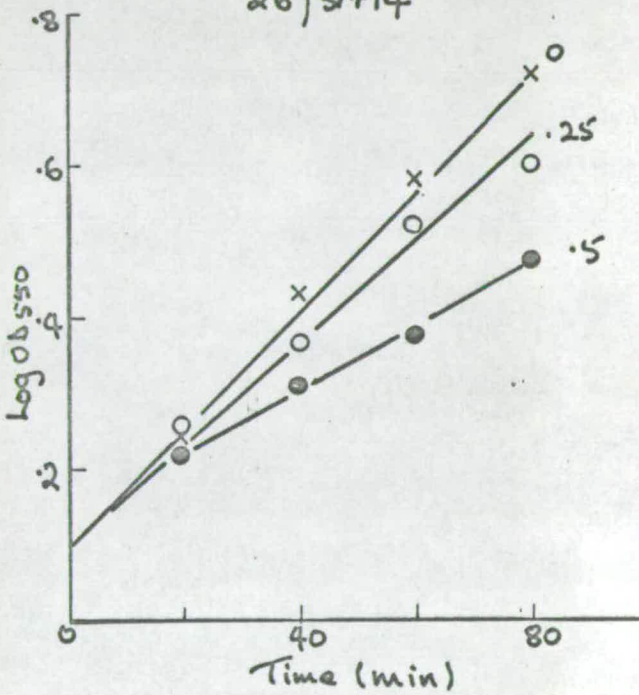
Figure 16 (contd)

Symbol	Label	Tetracycline added (µg/ml) at time 0
×——×	0	—
○——○	·25	0·25
●——●	·5	0·5

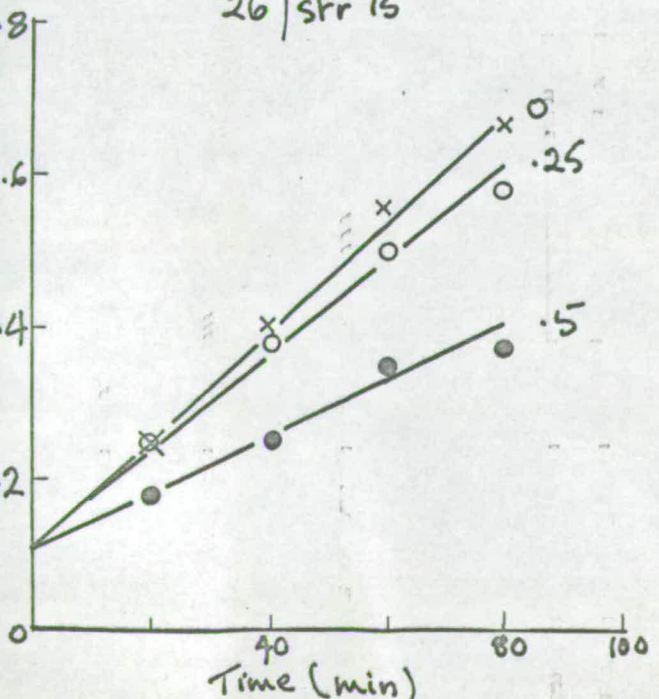
26/str 13



26/str 14



26/str 15



26/str 16

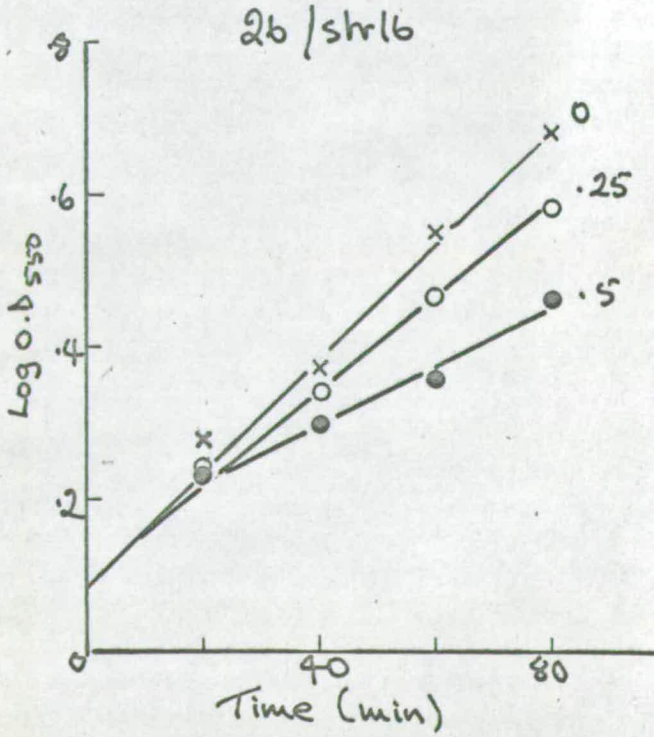
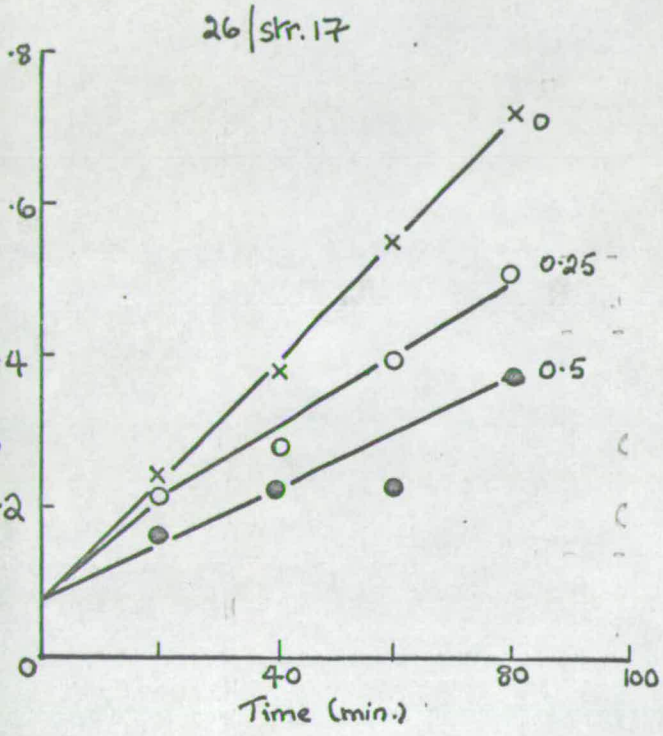
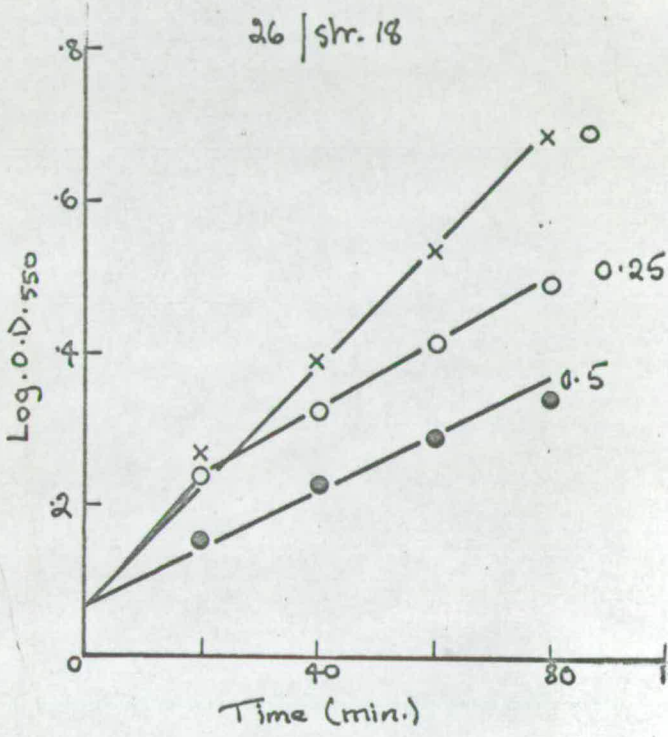
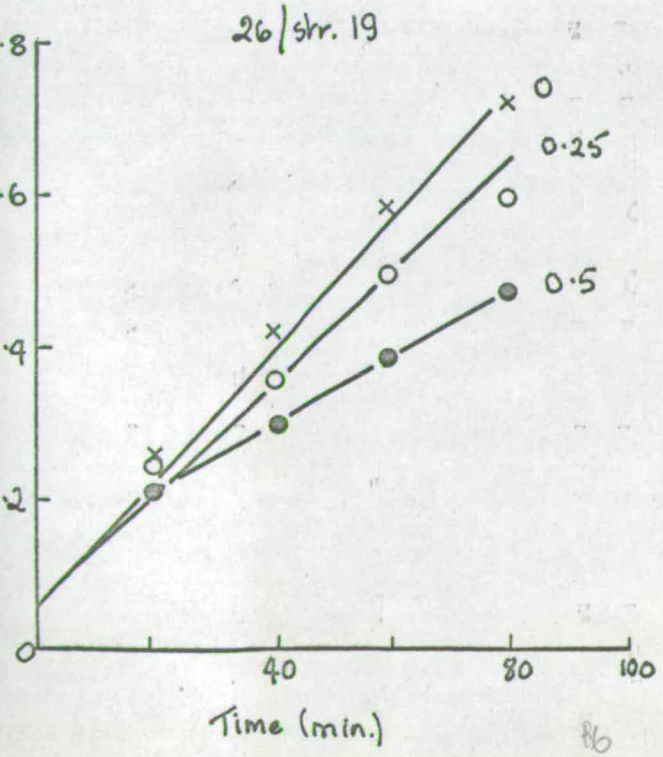


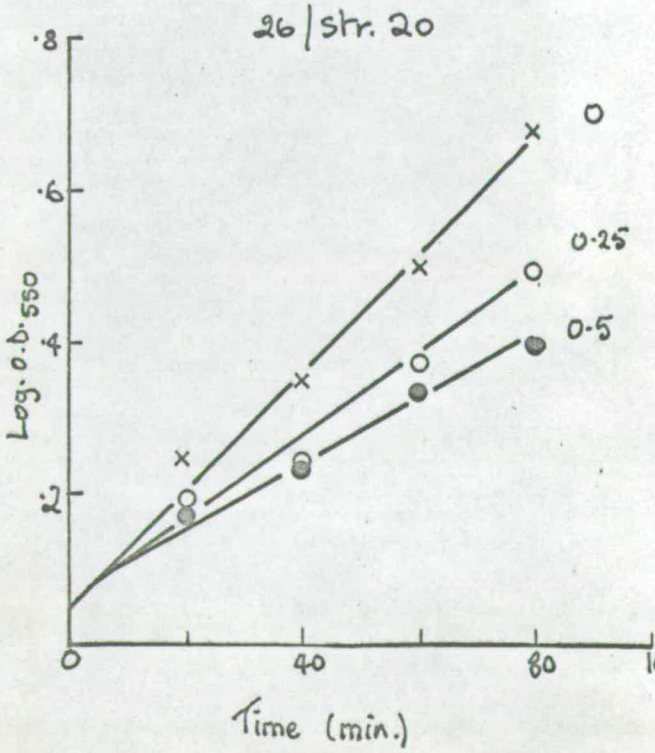
Figure 16 (contd.)

Symbol	Label	Tetracycline added (μg/ml.) at time 0
×——×	0	—
○——○	0·25	0·25
●——●	0·5	0·5









str21 is 12.5% co-transducible with *proA* but str24 is not co-transducible with that locus.

Although not strictly connected with Tetracycline resistance it seems logical at this point to mention cross-resistance of these mutants to some other antibiotics. All mutants were tested with Novobiocin sensitivity disks (30μg/ml) for their reaction to this antibiotic. RE26 *strB* alone among the str-r mutants and the parent strains was sensitive to Novobiocin. The zone of inhibition around the sensitivity disc on the lawn of RE26*strB* was 11.0mm in diameter. Brock (cited in Gottlieb and Shaw 1967) has shown that Novobiocin causes permeability defects in *E.coli* which are associated with Mg^{2+} deficiency.

In the other tests for cross resistance only RE26 and RE26*strB* were examined. Each of the strains was tested forresistance to Kanamycin and Paromomycin (antibiotics related structurally to streptomycin) and D-cycloserine. The percentage survival of each strain on the three antibiotics is illustrated in Table XII. It seems doubtful whether the presence of *strB* alters the resistance of *RE26* to D-cycloserine. This antibiotic inhibits two enzymes specifically involved in cell wall mucopeptide synthesis (Strominger et al 1960).

For the two antibiotics related to streptomycin one might expect the presence of *strB* to cause increased resistance. Certainly RE26*strB* is more resistant to Kanamycin than RE26 but the effect is less pronounced with respect to Paromomycin.

TABLE XII

Cross resistance of RE26strB to other antibiotics.

Strain	Kanamycin (μg/ml)				Paromomycin (μg/ml)				D-cycloserine (μg/ml)		
	1.0	1.4	2.0	4.0	1.0	1.4	2.0	4.0	5.0	10.0	15.0
RE26	65%	59%	-	-	71%	36%	-	-	84%	73%	47%*
RE26strB	100%	75%	61%	-	87%	42%	12%*	-	88%	82%	64%

*These colonies were very small.

In the presence of D-cycloserine it is very noticeable that the colony size decreases with increased concentration of the antibiotic.

The figures quoted are the percentage survivors on the antibiotic agar plates as compared with the viable count on nutrient agar.

(vii) Selection of Streptomycin resistant Mutants in R-Factor strains.

The interaction between low level streptomycin resistance and Tetracycline resistance has been investigated by selecting for mutants in RE26 (R57). (R57 carries only Tetracycline resistance.) The selection procedure was identical to that used for the independent mutants of RE26. Overnight incubation of the selection plates at 37°C again produced apparently streptomycin resistant mutants at a frequency of 10^{-6}. The mutant frequency which is identical with that for RE26 and RE91 is evidence that the presence of an R-factor does not alter mutation rate.

Ten independent mutants were picked from the selection plates and MIPC determined after purification. As with the mutants selected previously some were obtained which only grew on a lower concentration of streptomycin than that used for selection purposes.

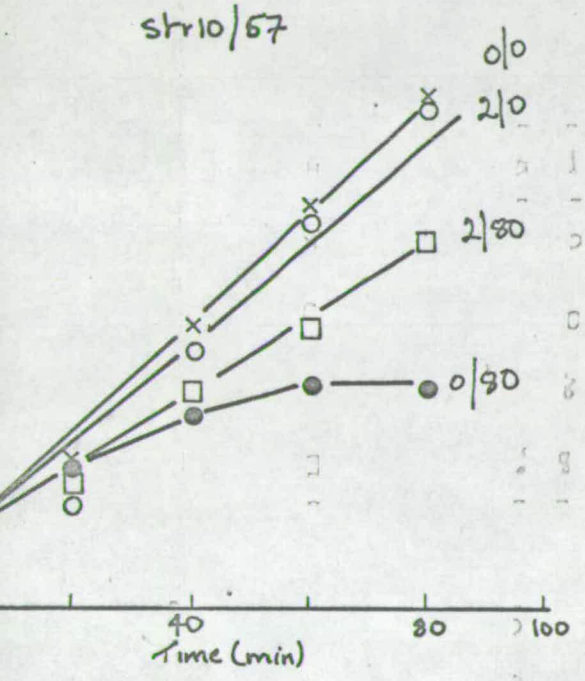
Three of the str-r mutants of RE26 (R57) str 1/57; str 8/57 and str 10/57 were tested for the effect of the streptomycin resistance on the expression of the Tetracycline resistance. The procedure used has been described in Methods (X)(a), and again the results are presented graphically. Figure 17 illustrates the growth rates of the three mutant strains of RE26 (R57) when challenged with the two concentrations of Tetracycline.

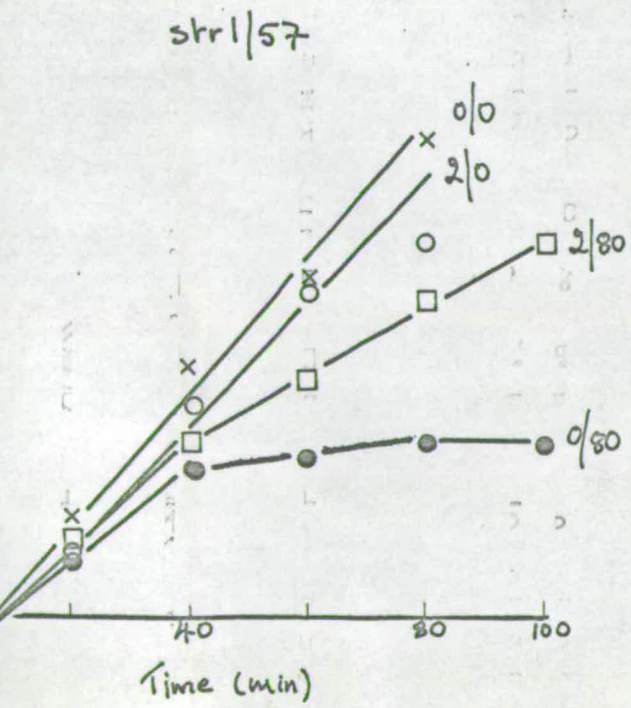
In the three mutants tested there is only partial induction when the culture is challenged with 80μg/ml Tetracycline. The 2/80 curves show that the presence of an str-r mutation reduces the growth rate when high level Tetracycline resistance is induced.

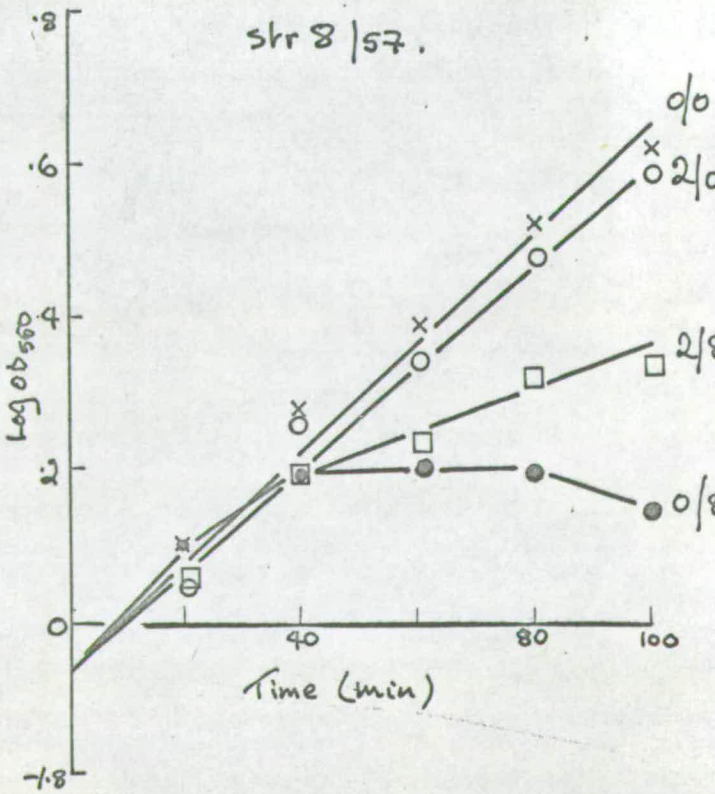
Thus, one again observes the interaction between str-r mutants and Tetracycline resistance. This interaction has been

Growth of three str-r mutants of RE26(R57) when challenged with Tetracycline.

Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time (0+15)
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/80	—	80
□——□	2/80	2	80







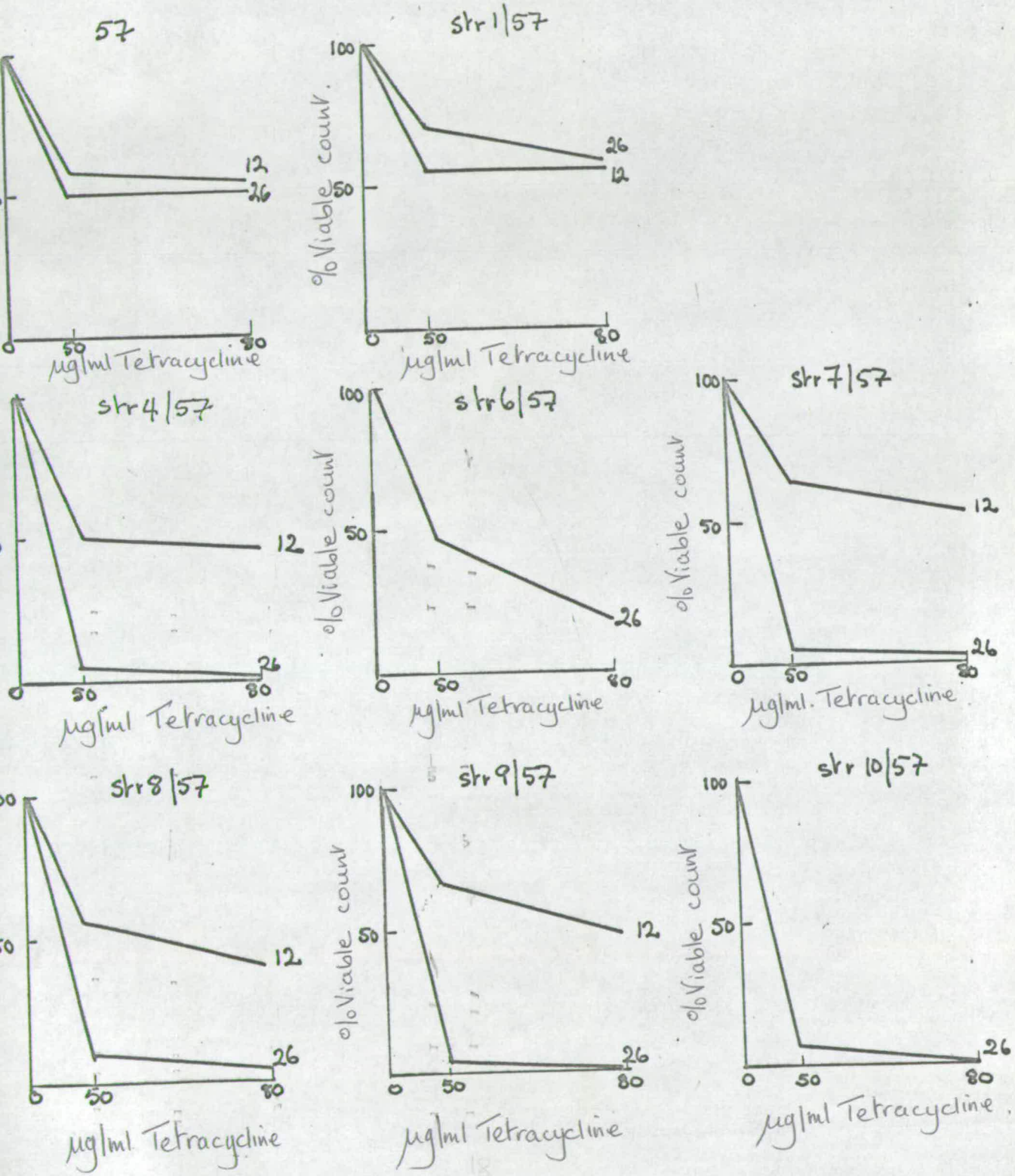
investigated more fully by transferring the R-factors from the mutant strains into RE12, a streptomycin sensitive strain of E.coli. The Tetracycline resistance levels of the str-r strains of RE26 (R57) and of RE12 (R57) obtained from them are given in Figure 18 which also includes the Tetracycline resistance level of RE26 (R57) and RE12 (R57) as a control.

With the exception of mutant str1/R57 all str-r mutants of RE26 (R57) have a decreased Tetracycline resistance in comparison with RE26 (R57). After transfer from the str-r strains of RE26 into RE12 the Tetracycline resistance returns to that of the control RE12 (R57). Thus the transfer experiments show that the str-r mutants obtained in RE26 (R57) have at least one property in common with the str-r mutants of RE26 - the effect on Tetracycline resistance.

The streptomycin resistance determinant of R46 in combination with an str-r mutation results in an organism which is resistant to a high level of streptomycin (see Figure 4; 5 and 8). Consequently selection of str-r mutations in RE26 (R46) has to be done indirectly, relying on the increased resistance due to the presence of the two resistance determinants. Ten independent mutants of RE26 (R46) were isolated on nutrient agar containing 50μg/ml streptomycin. Colonies appeared on the selective plates at a frequency of 10^{-6}: the same frequency with which str-r mutants appeared in other strains. Thus there is some evidence that the presence of an R-factor carrying a streptomycin resistance determinant does not alter the mutation rate of a strain to streptomycin resistance. Gundersen (1963) and Ginoza and Painter (1964)

Figure

Tetracycline resistance of RE26(R57) str-r mutants and the expression of R-factor resistance after transfer into RE12.



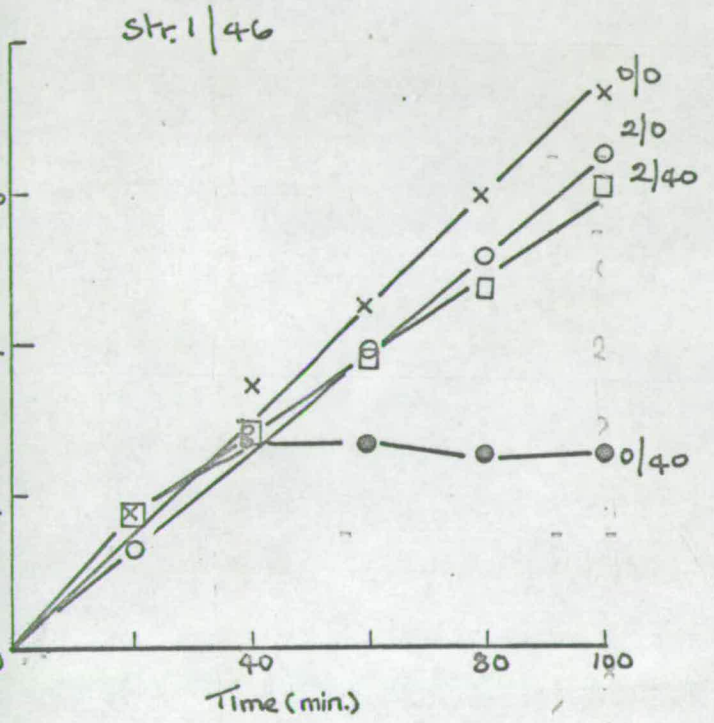
postulated that R-factor streptomycin resistance increased the frequency with which high level chromosomal streptomycin resistance occurred. Pearce and Meynell (1968) have suggested that the increase in the frequency with which highly resistant variants of streptomycin resistant R-factor strains appear is due to a synergistic effect of two resistance mechanisms. The streptomycin resistance of the R-factor allows the population to reach a sufficient density on a high concentration of streptomycin for one of the frequent mutations to low level streptomycin resistance to occur. The results of selecting for resistance to 50μg/ml streptomycin in RE26 (R46) supports this theory.

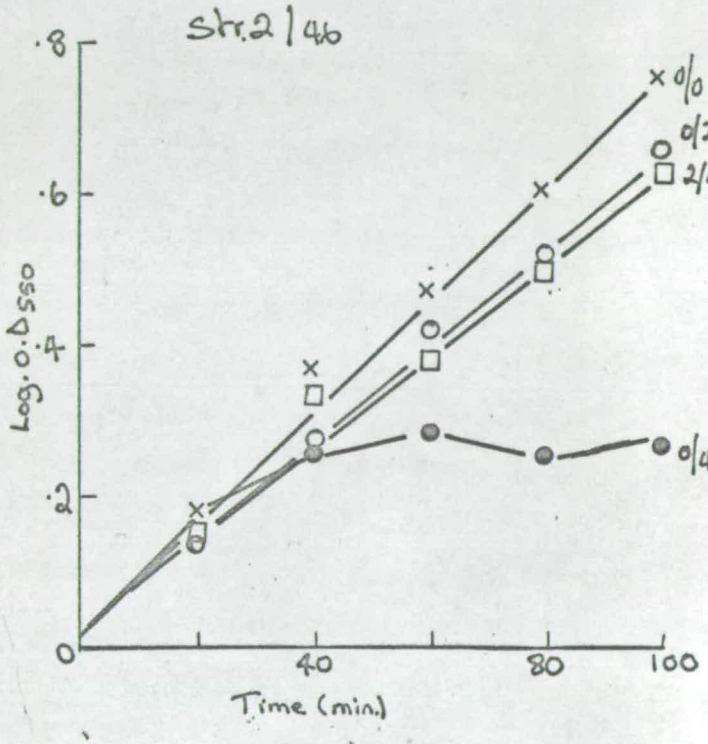
The similarity between the mutation frequencies obtained in selection experiments on RE26 and RE26 (R46) is not in itself sufficient evidence for the identity of the mutants. R46 carries resistance determinants for Tetracycline and Ampicillin in addition to Streptomycin and this provides another way of investigating the mutants of RE26 (R46). If the mutants are the result of a combination of R-factor resistance and str-r mutations it should be possible to detect an alteration in the Tetracycline resistance.

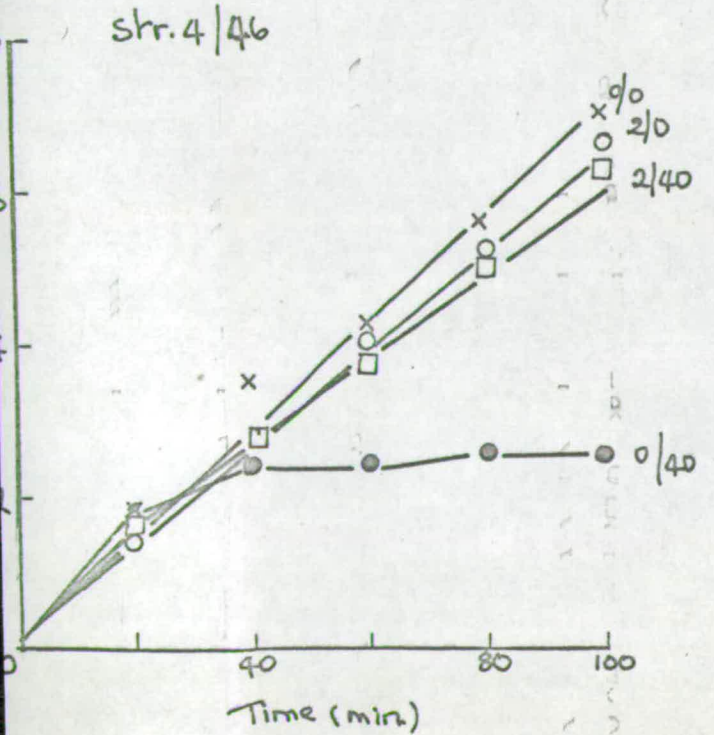
Each of the ten mutants was examined for the effects of adding Tetracycline to a log phase L-broth culture as described in Methods (X)(a). The results are illustrated by the graphs in Figure 19. All the mutants show an increase in sensitivity to Tetracycline in comparison with RE26 (R46). Two of the mutants, str6 (46) and str10 (46) have a decreased growth rate when challenged with 40μg/ml. Tetracycline fifteen minutes after the beginning of incubation (0/40 curves). In the other mutants the response to

Growth of RE26(R46) str-r mutants when challenged with Tetracycline

Symbol	Label	Tetracycline added (ug/ml)	
		at time 0	at time (0+15)
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40







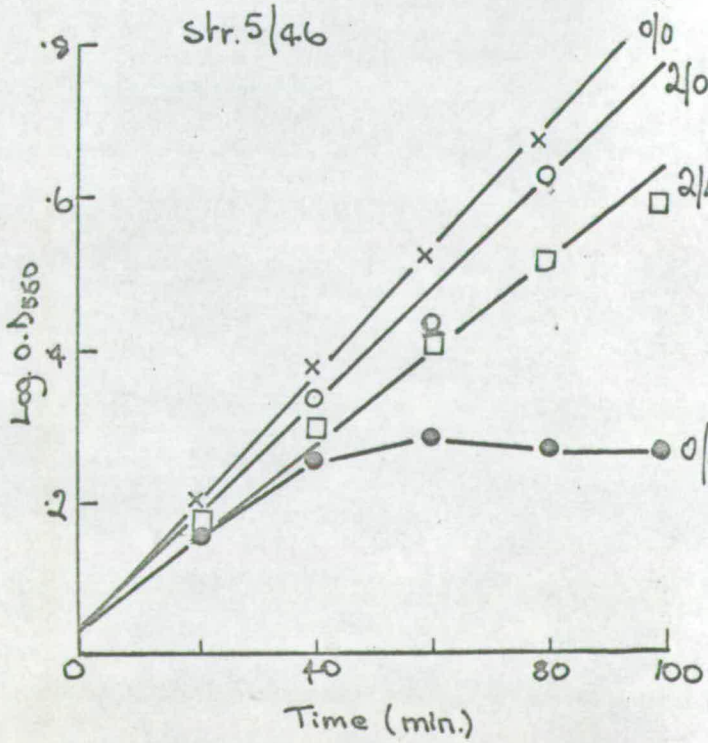
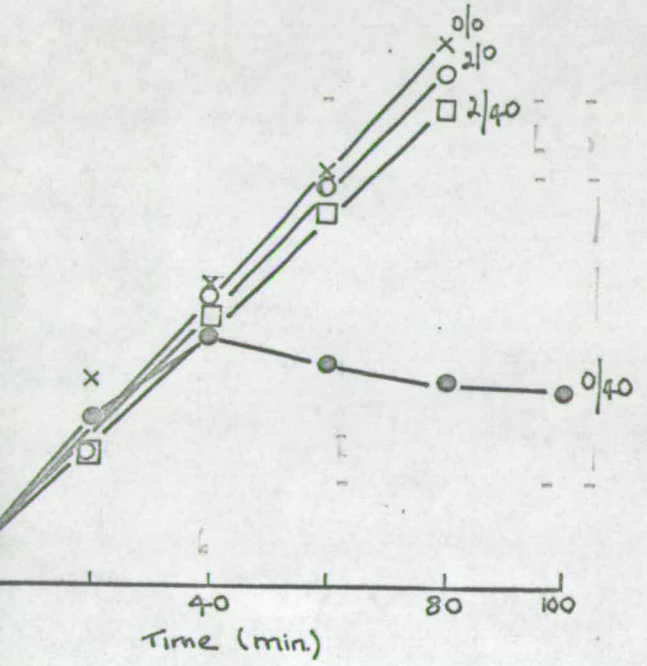


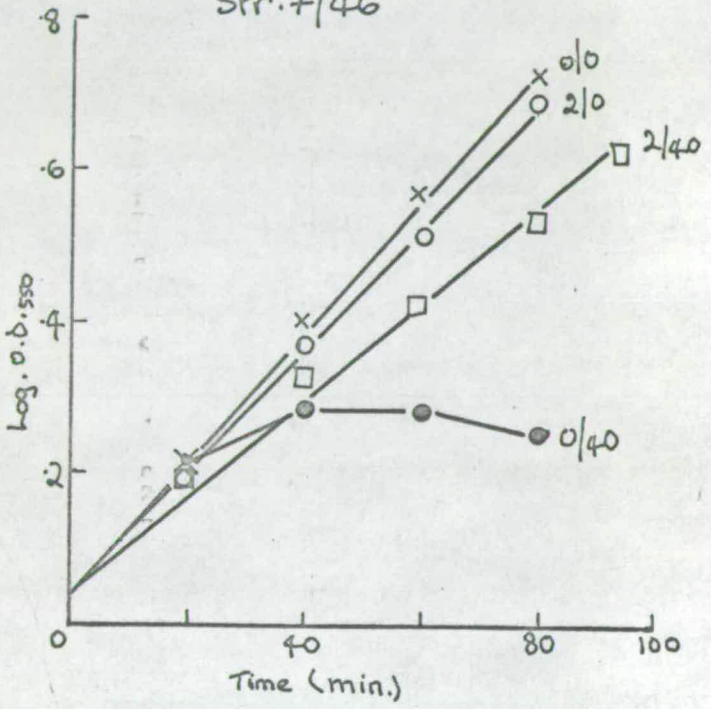
Figure 14 (cont'd.)

Symbol	Label	Tetracycline added (μg/ml)	
		at time 0	at time (0+15)
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40

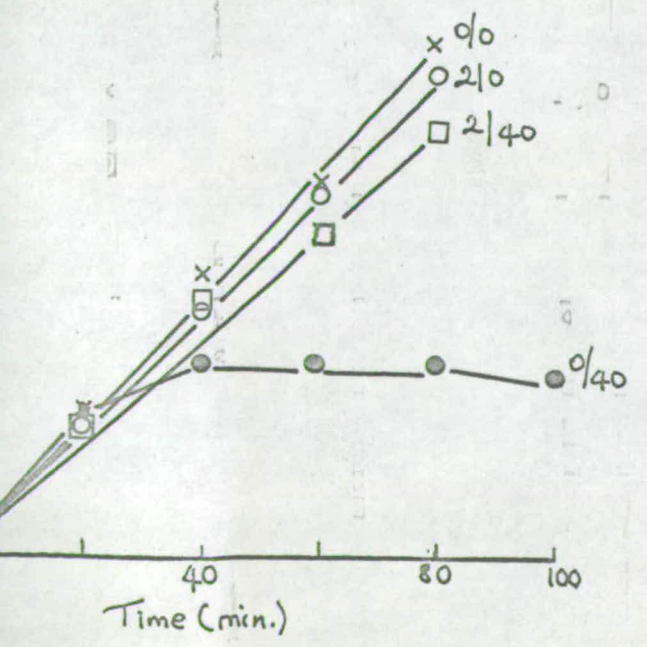
Str. 6/46



Str. 7/46



Str. 8/46



Str. 9/46

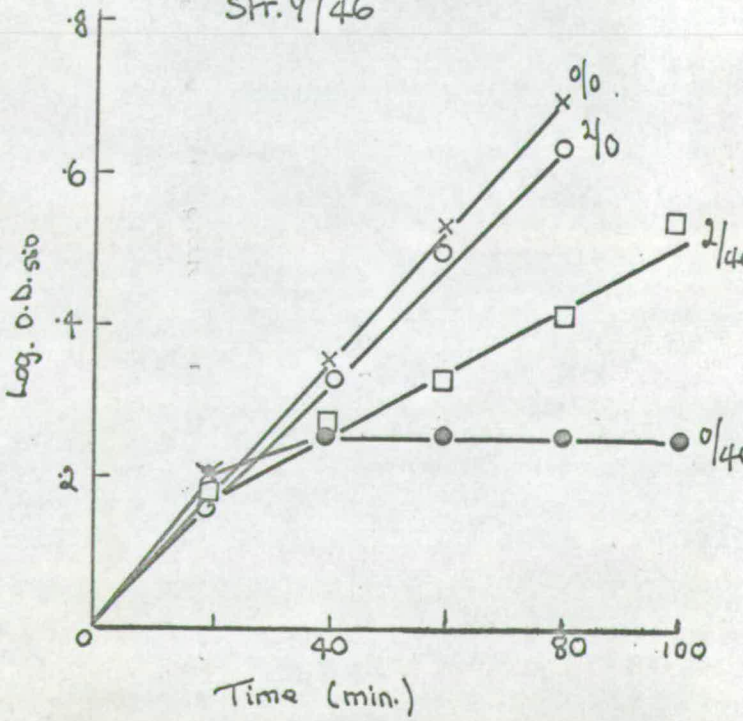
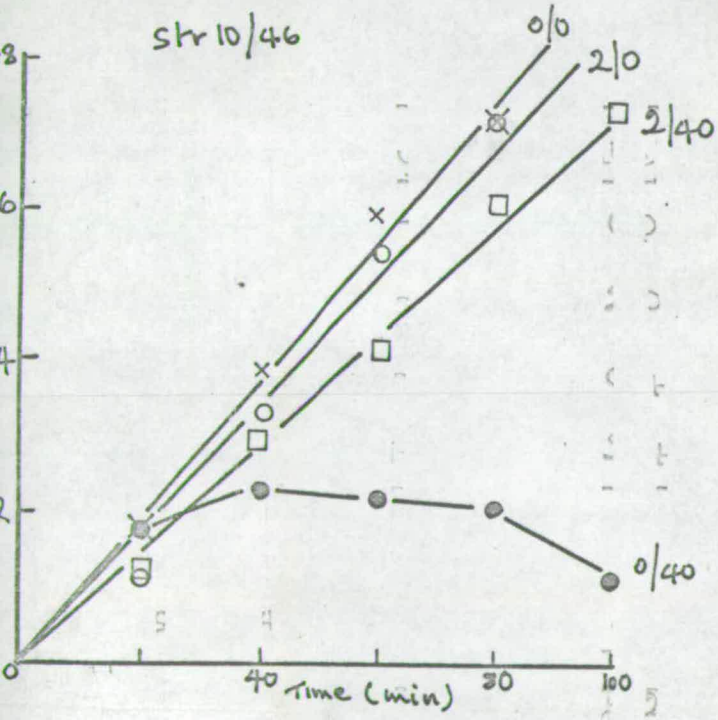
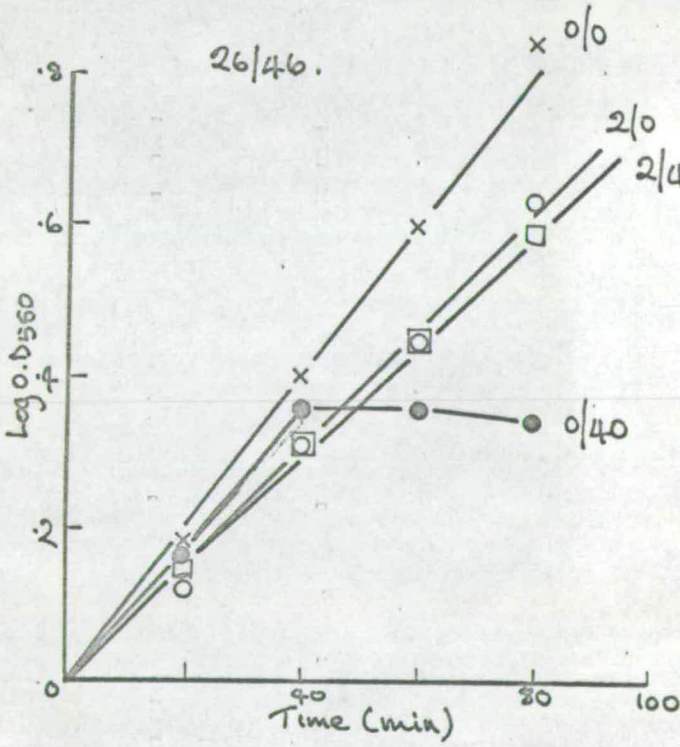


Figure 19 (contd.)

Symbol	Label	Tetracycline added (μg/ml) at time 0	at time (0+15)
×——×	0/0	—	—
○——○	2/0	2	—
●——●	0/40	—	40
□——□	2/40	2	40



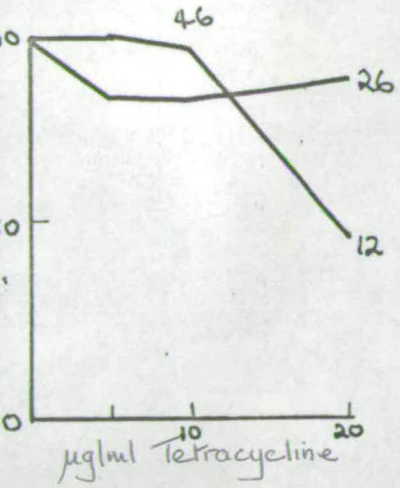


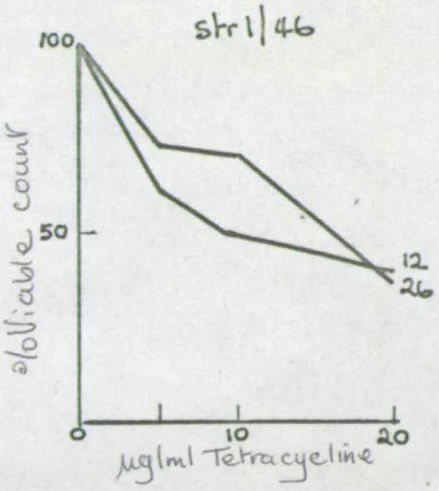
to 40μg/ml Tetracycline is more rapid than in RE26 (R46) (0/40 curves). The growth rate in Tetracycline after induction (2/40 curves) is variable from one mutant to another.

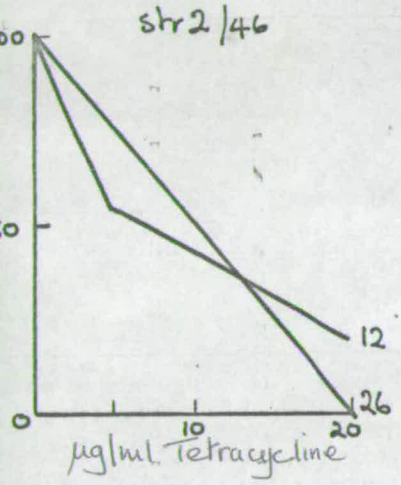
The presence of str-r mutants also affects the survival rate of Tetracycline resistant R-factor strains on nutrient agar containing Tetracycline (see Figures 4, 6 and 8). This same effect is observed with the ten mutants of RE26 (R46) as shown in Figure 20. The first graph in the series gives the survival curve on Tetracycline agar of RE26 (R46). It is easily seen from the other graphs that in the presence of increased streptomycin resistance the Tetracycline resistance is affected to a greater or lesser extent.

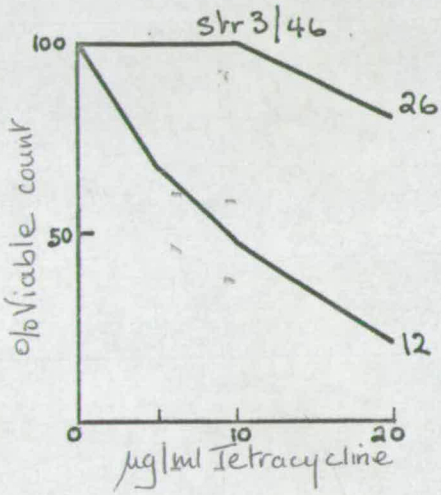
Evidence that the mutations causing increased streptomycin resistance in RE26 (R46) are located on the chromosome can be obtained by using the mutant strains as donors of R46. After transfer into RE12, a streptomycin sensitive strain of E.coli K12 the Tetracycline resistance of R46 were measured by the viable count method. The Tetracycline resistance levels of R46 in RE12 from the various mutants are also illustrated in Figure 20. The Tetracycline resistance levels of the RE12 (R46) strains obtained are a little lower than in the control RE12 (R46) which was obtained by transferring R46 from RE26. However it is quite clear that after transfer from the mutant RE26 (R46) strains the Tetracycline resistance of the R-factor is unaltered, thus suggesting a chromosomal location for the mutations being investigated in RE26 (R46).

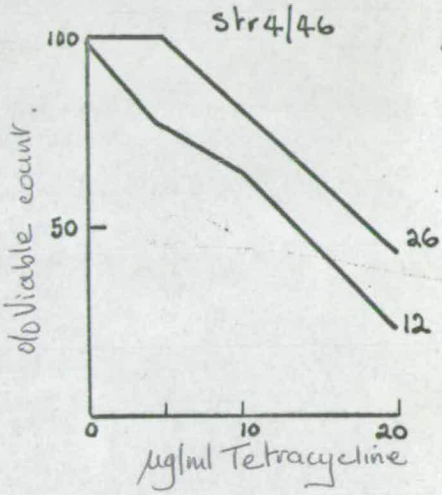
Ampicillin resistance is also affected by the presence of str-r mutations. The effect of increased streptomycin resistance

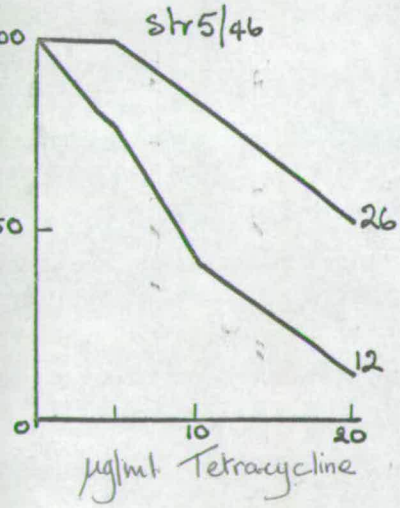

46
26
12
0
10
20
µg/ml Tetracycline

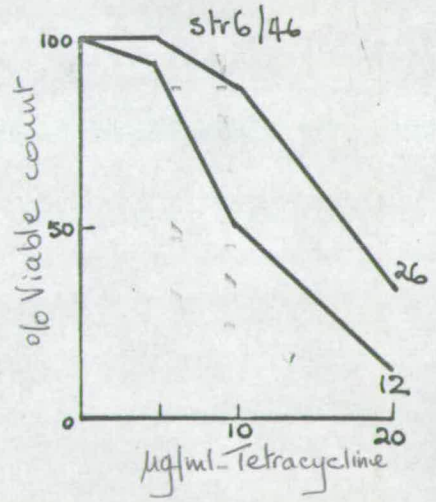

Str 1/46
100
50
0
% Viable count
12
26
0
10
20
µg/ml Tetracycline

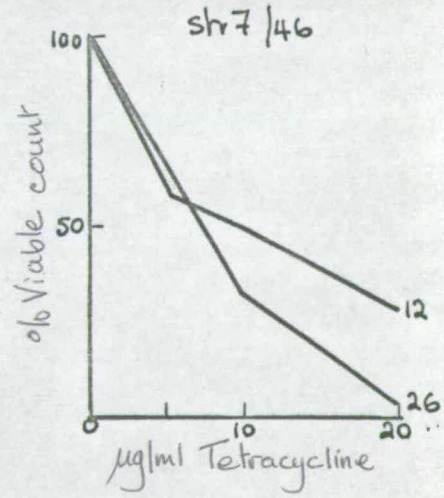

Str 2/46
12
26
0
10
20
µg/ml Tetracycline

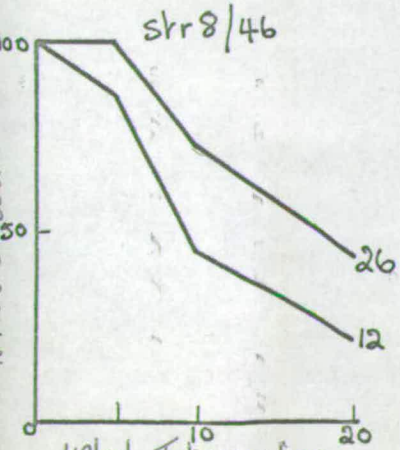

Str 3/46
100
50
0
% Viable count
26
12
0
10
20
µg/ml Tetracycline

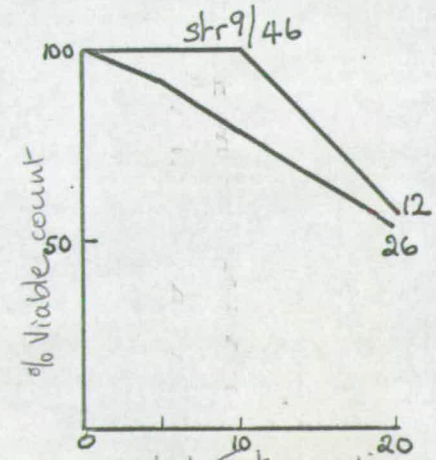

Str 4/46
100
50
0
% Viable count
26
12
0
10
20
µg/ml Tetracycline

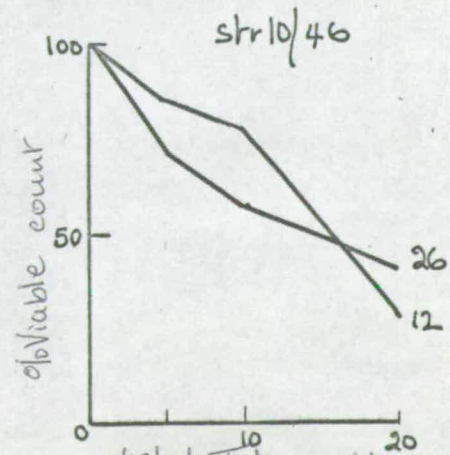

Str 5/46
26
12
0
10
20
µg/ml Tetracycline


Str 6/46
100
50
0
% Viable count
26
12
0
10
20
µg/ml Tetracycline


Str 7/46
100
50
0
% Viable count
12
26
0
10
20
µg/ml Tetracycline


Str 8/46
26
12
0
10
20
µg/ml Tetracycline


Str 9/46
100
50
0
% Viable count
12
26
0
10
20
µg/ml Tetracycline


Str 10/46
100
50
0
% Viable count
26
12
0
10
20
µg/ml Tetracycline

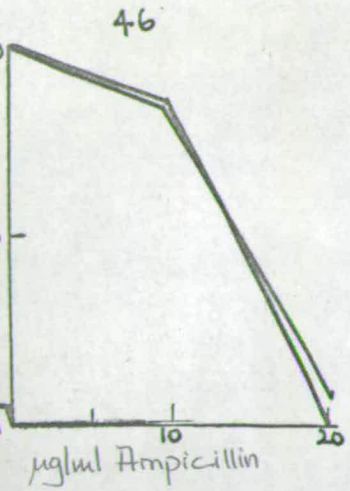
on the Ampicillin resistance of R46 is illustrated in Figure 21. The Ampicillin resistance of R46 in RE26 and RE12 is very similar (see the first graph in the series). Some of the mutants have a reduced percentage survival on the levels of Ampicillin tested but in some instances e.g. str1 (46) the Ampicillin resistance is obviously increased. A chromosomal str-r type mutation may thus have two affects on R-factor Ampicillin resistance; it may cause an increase or decrease in that resistance.

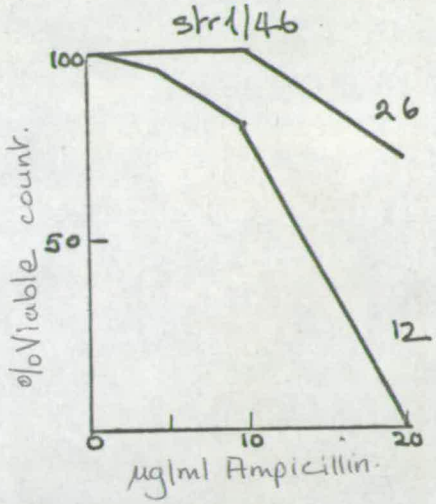
The changes in Ampicillin resistance in the mutants of RE26 (R46) are due to a chromosomal gene because after transfer of the R-factors into RE12 the Ampicillin resistance reverts to that of the control strain.

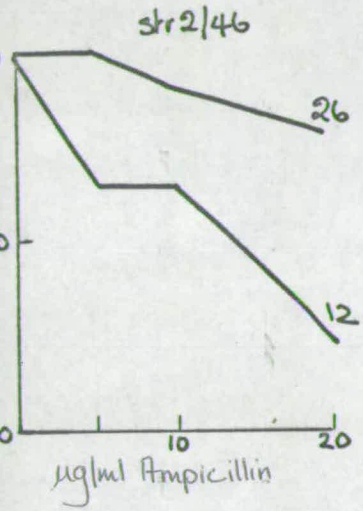
From the evidence of the mutation frequency, the effect on the Tetracycline resistance of R46 and the effects on Ampicillin resistance of R46 it is certain that the mutants of RE26 (R46) with increased streptomycin resistance are a result of combined R-factor streptomycin resistance and a chromosomal str-r type mutant. Additional evidence for this hypothesis comes from the decrease in streptomycin resistance when R46 is transferred into RE12. If the increased streptomycin resistance had been the result of an alteration in the R-factor streptomycin resistance in RE12 (R46) obtained from the mutants of RE26 (R46) would have had streptomycin resistance higher than the control RE26 (R46).

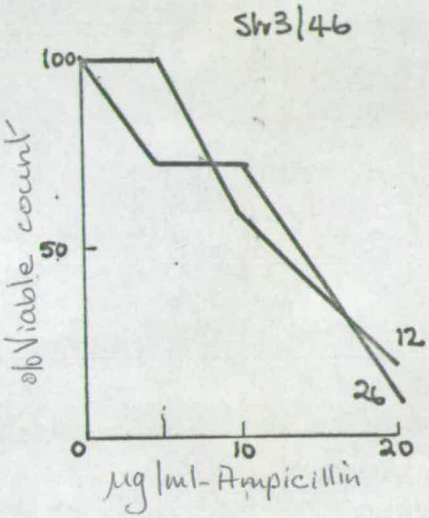
Table XIII summarises the effect of each of the ten independent mutants on the other R-factor resistance determinants. Three groups of mutants have emerged, the common feature of all is that Tetracycline resistance is decreased. Only three of the ten

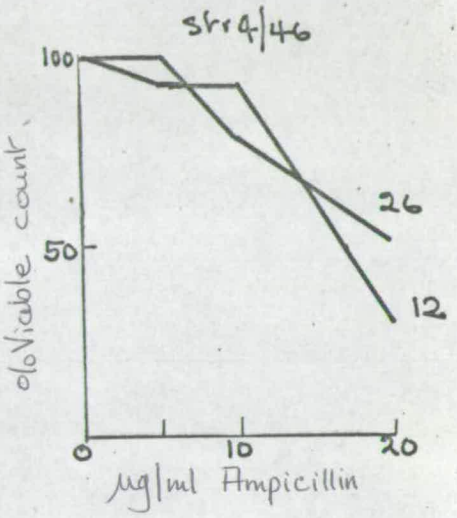
Figure 21

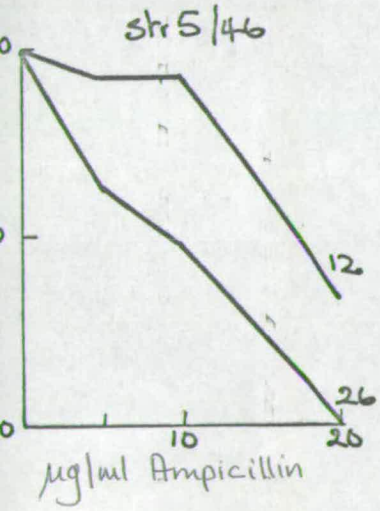

46
10
20
µg/ml Ampicillin

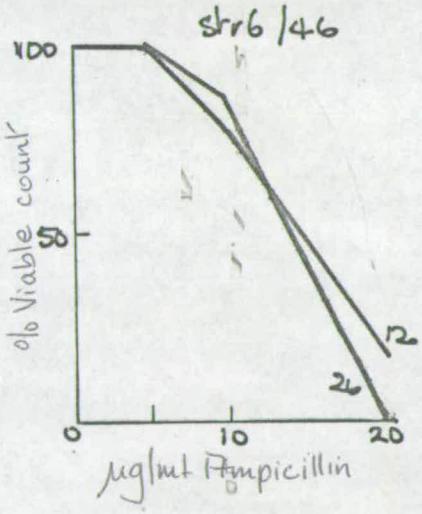

Str1/46
100
50
% Viable count
26
12
0
10
20
µg/ml Ampicillin

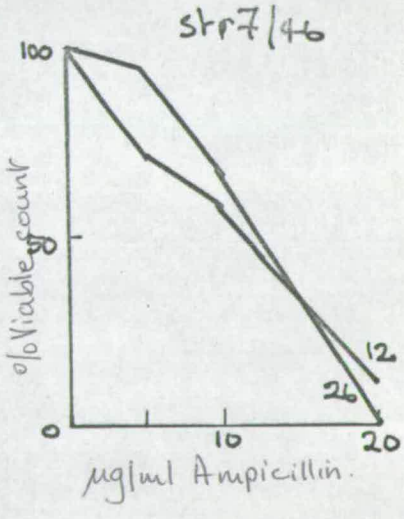

Str2/46
26
12
0
10
20
µg/ml Ampicillin

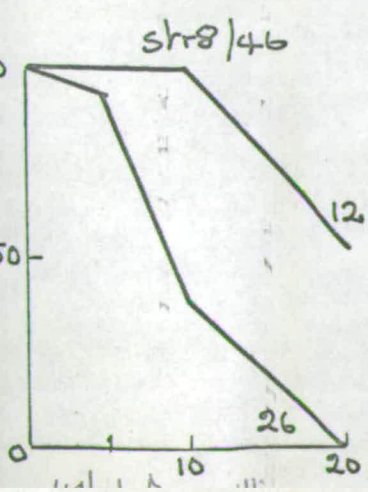

Str3/46
100
50
% Viable count
12
26
0
10
20
µg/ml Ampicillin

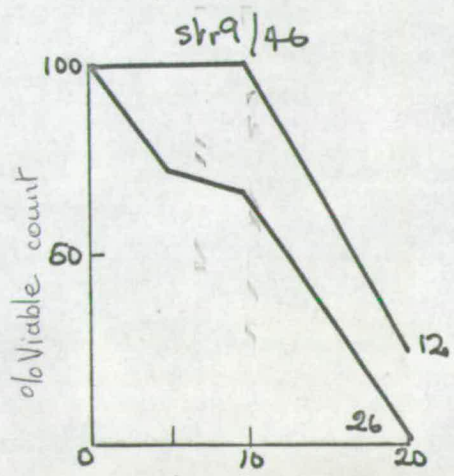

Str4/46
100
50
% Viable count
26
12
0
10
20
µg/ml Ampicillin


Str5/46
12
26
0
10
20
µg/ml Ampicillin


Str6/46
100
50
% Viable count
12
26
0
10
20
µg/ml Ampicillin


Str7/46
100
% Viable count
12
26
0
10
20
µg/ml Ampicillin


Str8/46
12
26
0
10
20


Str9/46
100
60
% Viable count
12
26
0
10
20

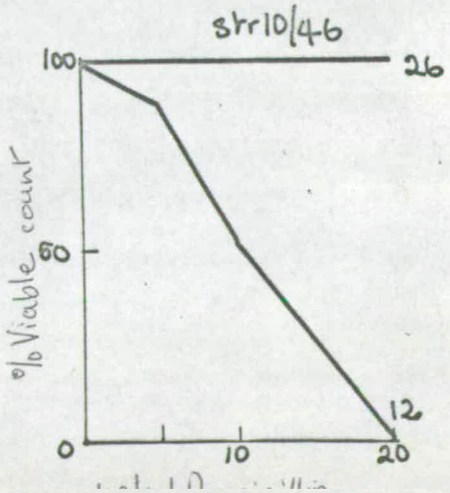

Str10/46
100
26
60
% Viable count
12
0
10
20

TABLE XIII

Summary of Mutants selected in RE26 (R46)

Mutant Phenotype	Number of Mutants	Mutant Designation
A and T resistances decreased	3	str5/46; str8/46; str9/46.
A and T resistances increased	0	-
A resistance increased and T resistance decreased (or slightly reduced)	5	str1/46; str2/46; str3/46; str4/46; str10/46.
A resistance decreased and T resistance increased	0	-
A resistance unchanged and T resistance decreased	2	str6/46; str7/46

Abbreviations:- A-Ampicillin; T-Tetracycline.

The mutants have been classified according to their percentage survival on antibiotic nutrient agar in comparison with RE26 (R46.)

mutants have the responses characteristic of strB and str6.

The existence of the three groups of mutants which differ in their effect on Ampicillin resistance is evidence of the variability of mutations which cause streptomycin resistance. Any mutations which have such a varied effect on other properties of the cell are likely to be due to a non-specific biological change e.g. an alteration in permeability.

The hypothesis that this is the resistance mechanism which is operating in these mutants and those selected in RE26 and RE91 will be tested in the next section.

(viii) Physiological Properties of the Mutants

The results of the previous section indicate that str-r mutations may involve a non-specific biological change such as an alteration in the permeability of the cell. The effect which the str-r mutations have on other antibiotic resistance determinants is obviously the result of an interaction between a non-specific biological function and other, specific, properties of the cell. Dubin et al (1963) have described a number of effects produced by incubating streptomycin sensitive E.coli in liquid culture media containing streptomycin. The first response is an uptake of streptomycin, followed by an increase in the loss of K^+ ions from the cell.

In view of these facts and the interaction between the mutations and other resistance determinants the mutants were screened for alterations in permeability. The group of substances known as the basic dyes can be used to determine any alterations in the permeability of cells.

When RE26 and RE26strB were incubated in the presence of basic dyes as described in Methods (XV) both strains behaved identically in Eosin-Y and Acridine Orange (see Table XIV). The concentrations of the dyes used were those given by Nagel de Zwaig and Luria (1967) when investigating the alteration in the permeability barrier of colicin tolerant mutants of E.coli. Tests with Acriflavine show that RE26strB is more sensitive to this dye than RE26, RE26strB did not grow in 25μg/ml of the dye.

Although both strains grew equally well in Methylene Blue after four hours incubation the tubes inoculated with RE26 had been decolourised but not those inoculated with RE26strB. The incubations were carried out in tubes 14.5cm x 1.3cm (int. diameter) without shaking. Consequently the incubations were done with the minimum of aeration. The decolourisation of methylene blue is caused by the presence of reducing substances and is reversed by atmospheric oxygen. Therefore the difference in RE26 and RE26 strB would not have been observed if the tubes had been shaken during incubation.

The lack of decolourisation in RE26strB cultures could have been caused by the production of less reducing substances as the result of a slower growth rate. This was shown to be true because both cultures had decolourised equally after overnight incubation.

Therefore strB has not affected the cell's response to these four basic dyes but from the results obtained with methylene blue it would appear that this mutation alters the growth rate. Slight differences in the growth rates of RE26 and RE26strB are detectable in some of the earlier experiments. (See Figure 16).

TABLE XIV

Growth of RE26 and RE26*strB* in L-broth + basic dyes

Dye (μg/ml)		RE26	RE26*strB*
ACRIFLAVINE	1	+	+
	5	+	+
	10	+	+
	25	+	-
ACRIDINE ORANGE	5	+	+
	10	+	+
	50	+	+
	100	+	+
METHYLENE BLUE	1	+	+
	5	+	+
	10	+	+
	25	+	+
	100		
EOSIN-Y	0.1	+	+
	0.25	+	+
	0.5	+	+
	1.0	+	+

The tubes containing L-broth + dye at the concentrations stated were scored after overnight incubation as turbid or clear. The original inoculum was approximately 10^4 cells.

The basic dye, crystal violet, also provides a means of detecting alterations in the cell envelope. Nordström et al (1970) detected an alteration in response to crystal violet in a strain of E.coli which carries a mutation giving resistance to 20μg/ml Ampicillin.

Each of the str-r mutations was examined for survival on L-broth agar containing crystal violet. The viable count method for determining antibiotic resistance levels was used. The results are given in Table XV which gives $L.D_{50}$ of crystal violet for the mutant and parent strains.

Response of the mutants to crystal violet is variable. The most common group of mutants is unaltered with respect to resistance to crystal violet. In general the mutants are more sensitive to crystal violet than the parent strain. RE26strB proved exceptional in being sensitive to Novobiocin and is again exceptional in its sensitivity to crystal violet; the $L.D_{50}$ of the strain is less than 5μg/ml. The presence of strB in RE26 (R46) also reduces $L.D_{50}$ of the strain for crystal violet.

Only two of the mutants selected in RE26 (R46) have a reduced $L.D_{50}$ compared with the parent strain, the remainder have an increased survival on crystal violet L-broth agar.

The results with crystal violet are suggestive of an alteration in the outer layers of the cell, although it is not possible to say exactly what this alteration is the streptomycin resistant mutants do seem to affect the permeability of the cell.

In addition to increasing the cell's resistance to crystal violet the Ampicillin resistant mutant described by Nordström et al

TABLE XV

Sensitivity of Mutants to Crystal Violet

$L.D_{50}$ Crystal Violet (μg/ml)	No. of mutants	Mutant designation
<5	1	RE26strB
5	3	str27; str2/46; strB/46.
10	4	str23; str26; str34; str7/46.
15	2	RE91; RE26(R46)
20	6	str22; str25; str35; str4/46; str10/46; RE91str6.
30	15	RE26; str21; str24; str28; str29; str30; str31; str32; str33; str36; str37; str38; str39; str40; str1/46; str3/46; str5/46; str6/46; str8/46; str9/46

$L.D_{50}$ is the concentration of crystal violet which allows 50% survival of each particular mutant. It was obtained from the viable count method of resistance level determination.

(1970) was more susceptible to the killing effect of Ethylene-diaminetetraacetate (EDTA). The immediate effect of EDTA on cells is the release of lipopolysaccharide. EDTA is a chelating substance which combines with divalent metal ions e.g. Mg^{2+} or Ca^{2+}. Either EDTA combines with the metal ions which bind the lipopoly-saccharide to other cell wall components or it removes the metal ions which inhibit the action of autolytic enzymes normally present, though inactive in the cell walls. (Gray and Wilkinson 1965b).

The procedure for testing the response of cells to EDTA is described in Methods (xii). The results are expressed as the ratio of the number of viable cells at time x to the number of viable cells at time zero. Table XVI lists the "killing effect" of EDTA for the various mutants at (0 + 30)minutes) and (0 + 120) minutes. Again strB is exceptional in being the only mutant which is more resistant to the killing effect of EDTA than the parent strain. The effect is however, reversed when the mutants of RE26 (R46) are tested.

The response to EDTA is not decided by the chromosomal location of the mutant in spite of the fact that RE91 is more resistant to the action of EDTA than RE91str6. However the results do suggest that none of the twenty independent str-r mutants is identical to strB.

The results with EDTA support the hypothesis that mutation to low level streptomycin resistance involves an alteration in the cell envelope.

Roth et al (1960) have shown that incubation of strepto-mycin sensitive cells in 60μg/ml streptomycin in minimal medium

TABLE XVI(a)

Effect of E.D.T.A. on Cell Survival

Mutant	Proportion of Viable Cells			
	(0 + 30')	(0 + 60')	(0 + 90')	(0 + 120')
Str21	0.02	0.001	0.001	0.001
Str22	0.01	0.01	0.01	0.01
Str23	0.025	0.015	0.015	0.01
Str24	0.037	0.025	0.025	0.015
Str25	0.03	0.015	0.01	0.01
Str26	0.03	0.016	0.016	0.016
Str27	0.012	0.002	0.002	0.002
Str28	0.02	0.006	0.007	0.007
Str31	0.185	0.11	0.10	0.05
Str32	0.01	0.01	0.006	0.004
Str33	0.09	0.08	0.06	0.04
Str34	0.085	0.08	0.05	0.05
Str35	0.01	0.01	0.007	0.007
Str36	0.011	0.01	0.005	0.005
Str37	0.02	0.02	0.016	0.01
Str38	0.001	0.001	0.001	0.001
Str39	0.001	0.001	0.001	0.001
Str40	0.001	0.001	0.001	0.001
StrB	0.66	0.65	0.6	0.5
RE26	0.26	0.24	0.21	0.17

The proportion of viable cells was calculated as follows:

$$\frac{\text{Viable count at time X}}{\text{Viable count at time 0}}$$

TABLE XVI(b)

Effect of E.D.T.A. on Cell Survival

Mutant	Proportion of Viable Cells			
	(0 + 30')	(0 + 60')	(0 + 90')	(0 + 120')
Str1/46	0.29	0.21	0.08	0.04
Str2/46	0.75	0.37	0.27	0.14
Str3/46	0.34	0.20	0.15	0.10
Str4/46	0.51	0.44	0.38	0.22
Str5/46	0.44	0.24	0.24	0.17
Str6/46	0.50	0.32	0.27	0.16
Str7/46	0.44	0.28	0.14	0.12
Str8/46	0.82	0.64	0.38	0.28
Str9/46	0.62	0.48	0.34	0.22
Str10/46	0.70	0.27	0.20	0.11
StrB/46	0.50	0.30	0.20	0.08
26/46	0.22	0.16	0.13	0.08

The proportion of viable cells was calculated as follows:

$$\frac{\text{Viable count at time X}}{\text{Viable count at time 0}}$$

causes the excretion of mucleotides into the growth medium. The presence of the nucleotides is detected by an increase in $O.D_{260}$ of the medium.

60μg/ml of streptomycin is bacteriostatic for RE26 and RE26strB but an experiment was carried out to determine whether $O.D_{260}$ of the two strains was the same after incubation in this concentration of antibiotic. The experimental procedure has been described in Methods (xiv). Over a period of 150 minutes $O.D_{260}$ of RE26 rose from 0.33 to 0.64, during the same time $O.D_{260}$ of RE26strB rose from 0.34 to 0.52. The difference between the two strains is not very large but does suggest that RE26strB is excreting less 260-absorbing material into the culture medium.

(ix) Inoculum Effect

Demonstration of an inoculum effect for an antibiotic resistant mutant is usually taken as evidence that an enzyme mediated resistance mechanism is operating (Harwood and Smith 1969). Thus the str-r mutants isolated and described would not be expected to exhibit such an effect. Table XVII illustrates the pattern of resistance obtained with different inocula of the str-r mutants in different concentrations of streptomycin.

The results in Table XVII were obtained by overnight incubation of 1ml broth samples of each str-r mutant started with four different inocula for each streptomycin concentration. The tubes were scored as turbid or clear after overnight incubation. In Table XVII growth of a particular mutant in any of the streptomycin concentrations is represented by a solid line. A broken line indicates no growth of the mutant in that concentration of streptomycin

TABLE XVII

Inoculum Effect of Mutants

Mutant	5μg/ml Str.				10μg/ml Str.				20μg/ml			
	10^{-1}	10^{-2}	10^{-4}	10^{-6}	10^{-1}	10^{-2}	10^{-4}	10^{-6}	10^{-1}	10^{-2}	10^{-4}	10^{-6}
str 21	—	—	—	—	—	—	—	—	—	—	—	—
str 23	—	—	—	—	—	—	—	—	—	—	—	—
str 24	—	—	—	—	—	—	—	—	—	—	—	—
str 27	—	—	—	—	—	—	—	—	—	—	—	—
str 28	—	—	—	—	—	—	—	—	—	—	—	—
str 31	—	—	—	—	—	—	—	—	—	—	—	—
str 36	—	—	—	—	—	—	—	—	—	—	—	—
str 37	—	—	—	—	—	—	—	—	—	—	—	—
str 38	—	—	—	—	—	—	—	—	—	—	—	—
91str6	—	—	—	—	—	—	—	—	—	—	—	—
strB	—	—	—	- -	—	—	—	—	—	—	- -	- -
str 26	—	—	- -	- -	—	—	—	—	—	—	- -	- -
str 32	—	—	—	—	—	—	—	- -	—	—	- -	- -
str 22	—	—	—	—	—	- -	- -	- -	—	- -	- -	- -
str 39	—	—	—	—	—	- -	- -	- -	—	- -	- -	- -
str 25	—	—	- -	- -	—	—	- -	- -	—	—	- -	- -
str 40	—	—	—	—	—	—	—	—	—	—	—	—
RE 26	—	—	- -	- -	—	—	- -	- -	—	- -	- -	- -

The sizes of inocula are indicated by the dilution of a culture $O.D_{590}$ 0.2 (approx. 2×10^8 cells/ml)

Solid lines indicate growth

Broken lines indicate no growth

Tubes were scored after overnight incubation

For each mutant and each streptomycin concentration four different initial inocula are used: the highest is approximately $2x10^7$ cells /ml, then $2x10^6$ cells/ml, followed by $2x10^4$ cells/ml. and finally $2x10^2$ cells/

The theory of the inoculum effect is that the larger the inoculum the greater is the concentration of antibiotic inactivating enzyme present. This results in the reduction of the effective concentration of antibiotic in the culture medium and allows less resistant cells in the population to grow.

Strictly speaking the effect observed with these str-r mutants is not an inoculum effect in the sense described above. Pearce and Meynell (1968) have described how a strain carrying one streptomycin resistance determinant apparently mutates very readily to a higher level of streptomycin resistance. The reason which they give for this is that the first resistance determinant allows the population to reach a density where one of the very frequent mutations to low level resistance occurs. The combined effect of the two resistances enables the strain to grow at a much higher level of antibiotic. A similar situation could be operating in this experiment and would thus give the appearance of an inoculum effect.

Evidence that this is in fact happening comes from the behaviour of RE26 in the broth cultures of different streptomycin concentration. Resistance to 5μg/ml of the antibiotic only occurs with an inoculum of $2x10^6$ cells, and it is with this size of inoculum that the mutants generally appear to have increased resistance. The ability of RE26 to grow in 20μg/ml streptomycin may be due to a combination of factors. The streptomycin sensitive cells

may absorb streptomycin thus reducing the effective concentration of antibiotic in the culture medium. As a result of this a single mutation may enable the strain to grow in the reduced concentration of streptomycin.

4. DISCUSSION

Streptomycin resistance in E.coli K12 has been described extensively in the literature beginning with the work of Demerec in 1948. Later workers have dealt specifically with the high level streptomycin resistance and have shown that this is due to mutation at a single locus designated strA. In addition to high level resistance, Demerec also described a type of intermediate streptomycin resistance which was the result of more frequently occurring mutations.

The streptomycin resistant mutants isolated and investigated here are of the latter group. The frequency with which the mutants occurred and the resistance levels obtained are good evidence for this. Failure to isolate mutants resistant to a high level of streptomycin in the course of the selection experiments could be due to one of two possible reasons. The mutation frequency for the strA locus is 5×10^{-9}, thus with the number of cells plated in the selection experiments a strA mutant would be very unlikely to occur. The second reason is connected with the concentration of streptomycin used in the selection experiments. The survival curve drawn up by Demerec (1950) illustrates the differences in survival rates of E.coli K12 for increasing concentrations of streptomycin. This suggests different cell organelles are affected by different concentrations of streptomycin. The ribosomes (involved in resistance of the strA type) may be altered only by higher concentrations of streptomycin than that used in these selection experiments.

The mutants selected are also interesting in that some fail to grow on the concentration of streptomycin used for their selection. A possible reason for this arises from the work of Dubin et al (1963). These workers have described the effects of incubating streptomycin-sensitive cells in the presence of streptomycin. One of these effects is the uptake of streptomycin by the cells. Hancock has also correlated streptomycin sensitivity with uptake of the drug. Thus it is possible that the effective plate concentration of streptomycin on the selective plates is reduced as a result of absorption of streptomycin by sensitive cells.

One str-r mutant, RE26*strB* is the result of mutation in a chromosomal gene located between *proA* and *proB* at 8.0 minutes on the linkage map of *E.coli* (Taylor and Trotter 1967 and Figure 3). A second mutant in RE91 has a different and unknown location on the chromosome.

The map location of *strB* provides a diagnostic feature for screening a number of independent str-r mutants. Twenty of these mutants in RE26 were analysed for co-transudction with *proA*.

Over a number of experiments the results were fairly consistent and it was possible to classify the mutants into groups. One of the groups showed no co-transduction with *proA* and these mutants may or may not be identical with the mutant strain RE91*str6*. The remainder of the str-r mutants were co-transducible with *proA* to varying degrees. Only two of the mutants have a co-transduction frequency with *proA* which could give them a location similar to *strB*. The other mutants may be located in the same region though in the absence of data concerning their co-transduction with *proB* it is impossible to state this with any certainty.

From the mapping data it is evident that the str-r mutants involve at least two, and possibly more, loci. Thus there is some evidence that the genetic basis of low level streptomycin resistance may be comparable with that described by Cavalli and Macaccaro (1952) and Reeve (1967, 1968) for chromosomal chloramphenicol reistance. The first two authors have described the combination of separate low level resistance mutations to give an organism with a higher level of resistance.

A similar combination of low level streptomycin resistance has been described, not in E.coli, but in the closely related S. typhimurium by Watanabe and Watanabe (1959). These authors have shown the existence of different loci responsible for intermediate streptomycin resistance in S. typhimurium. Combination of two of these different genes results in an organism with higher streptomycin resistance.

Combination of the apparently different resistance genes described here has not been done but there is indirect evidence from the inoculum effect of the mutants that combination of two str-r mutants produces an organism with higher resistance. None of the mutants is resistant to more than 5.0μg/ml streptomycin but with a sufficiently large initial inoculum it is possible for them to grow in L-broth containing 20μg/ml streptomycin. The probable reason for this effect is the high frequency with which str-r mutations occur. If the initial inoculum is large enough (greater than 10^6 cells/ml) such mutations will occur. Thus the growth of the mutant strains in the high concentrations of streptomycin is possibly due to the presence of a second mutation in a strain which already carries an str-r mutation.

Mutation may occur at any of the base pairs in a gene. However some of these mutations may be lethal for the organism and the mutants which survive will represent only a small proportion of the total number of possible mutations. This situation is most likely to occur in genes which code for an indispensable cell organelle e.g. a ribosome. In other genes any of the alterations in the base pairs may affect the end product of the gene only slightly and will be expressed in the mutant organisms. Such a situation may exist in the genes coding for components of the cell membrane. Thus the str-r mutants which have been selected may represent slight alterations in the membrane of the bacterium. The frequency of these mutants is indicative of many mutable sites within the gene or genes whose effects are always expressed phenotypically. Thus the common str-r phenotype of the mutants could be the result of mutations at different sites in some of the genes which code for membrane structure or function. This hypothesis is supported by the co-transduction experiments and the frequency of the mutations.

Reeve (1966) has demonstrated interaction between low level chromosomal chloramphenicol resistance and R-factor resistance determinants for chloramphenicol and Tetracycline. Introduction of a streptomycin resistant R-factor into RE26<u>strB</u> resulted in a strain with a high level of streptomycin resistance. A similar phenomenon has been described by Gundersen (1963) and Ginoza and Painter (1964). Both groups of workers have described the high level streptomycin-resistant variants obtained as being the result of a "mutator gene" present on the R-factor. Gundersen (1963) demonstrated that the "mutator" effect was specific for a streptomycin

resistance gene in the trp region of the chromosome.

Pearce and Meynell (1968) have described a phenomenon similar to those of Gundersen (1963) and Ginoza and Painter (1964). Their hypothesis explains high level streptomycin resistance on the basis of synergism between two resistance mechanisms. The presence of the first resistance (R-factor streptomycin resistance) allows the population to reach a density on a high concentration of streptomycin at which one of the relatively frequent str-r mutations can occur. Additional evidence for this hypothesis was obtained when increased streptomycin resistance was selected in RE26(R46). The frequency with which mutants arose on selective plates was very similar to that with which resistant mutants appeared on 5μg/ml streptomycin. This fact also suggests that one is selecting for chromosomal resistance - an assumption which was borne out after the R-factors had been transferred from the mutant strains into a streptomycin sensitive strain.

The synergism between strB and R-factor streptomycin resistance does not extend to other R-factor resistance determinants. In the presence of strB the expression of R-factor Ampicillin, Tetracycline and Chloramphenicol resistances is reduced. It has been shown for Tetracycline resistance that the reduction in resistance is due to strB alone and not the combination of extra-chromosomal and chromosomal streptomycin resistances. Tetracycline resistance mediated by R57, an R-factor which carries only Tetracycline resistance, is very much reduced in the presence of strB.

Similar effects have been observed with other mutants (Figure 7). RE91str6 also has a similar effect on Ampicillin and Tetracycline resistances.

When examined more closely by growth rate experiments in L-broth the interaction between the streptomycin resistance and Tetracycline resistance seems to involve a decrease in the growth rate of the strain in the presence of Tetracycline. It is difficult to determine whether the induction mechanism is affected. In one instance, the combination of strB and R57 (Figure 12), there is only partial induction to high level Tetracycline resistance.

Franklin (1967) has explained the induction of high level Tetracycline resistance in terms of an enzyme system which is induced by low concentrations of Tetracycline and acts upon the cell membrane to prevent further entry of Tetracycline. If low level streptomycin resistance interferes with the induction mechanism it could be due to the entry of too much Tetracycline into the cell in the first instance. Another possible explanation is that the streptomycin resistance involves an alteration in the permeability of the cell which makes the enzyme mediated impermeability of the cell to Tetracycline less effective.

The discussion has been partly resolved by examining the response of str-r mutants to Tetracycline. In the twenty-two mutants tested only two showed no reduction in growth rate when challenged with Tetracycline in L-broth. It therefore seems that mutation to low level streptomycin resistance produces "collateral sensitivity" to Tetracycline. This term was used by Szybalski and Bryson (1952) in their study of microbial cross-resistance to a number of antibiotics. Thus the effect of streptomycin resistance on R-factor Tetracycline resistance must be due

to the "collateral sensitivity" of these mutants to Tetracycline.

Among the mutants tested, str23, was exceptional in that it did not affect the Tetracycline resistance of R46 (Figure 7). When grown in L-broth containing Tetracycline this mutant undergoes only a very slight reduction in growth rate, less than RE26 - the parent strain.

Of the ten mutants of RE26 (R46) which were selected for increased streptomycin resistance none had unaltered Tetracycline resistance. It is interesting that these mutants vary in their expression of Ampicillin resistance (Table XIII). The most common group of mutants has increased Ampicillin resistance.

As stated above the increased streptomycin resistance of these mutants is due to chromosomal mutations. Thus there is now evidence that not all mutants which are responsible for low level streptomycin resistance reduce R-factor Ampicillin resistance.

The interaction between Tetracycline resistance and the str-r mutants provides a strong argument in favour of a permeability resistance mechanism operating in these mutants. Sensitivity to Tetracycline is correlated with absorption of the drug (Arima and Izaki, 1963). Thus increased sensitivity to Tetracycline is likely to involve increased absorption of the antibiotic. It has been mentioned previously that streptomycin sensitivity is also due to uptake of the drug (Hancock 1961, Dubin et al 1963). It is therefore possible that whatever permeability effect prevents entry of streptomycin into the cell allows Tetracycline to enter more freely into the cell.

R-factor Ampicillin resistance is due to the production

of the enzyme β-lactamase. It is rather difficult to see how a permeability mutant could affect the expression of an enzyme mediated resistance mechanism. However the alteration in the cell membrane, which is presumed to exist in the mutants, could affect the enzyme such that it does not function at optimum efficiency. Some of the mutants seem to provide more favourable environment for the enzyme's function as evidenced by the increased Ampicillin. resistance in some of the RE26 (R46)mutants.

The results of the mapping experiments are consistent with a minimum of two genes being involved in low level streptomycin resistance. With the exception of strB, all the mutant strains and the parent strains are resistant to novobiocin. This property of novabiocin resistance which is common to mutants mapping in more than one region of the chromosome, suggests that those mutants located close to proA differ in their response to novobiocin. Thus there may be a minimum of three genes responsible for str-r: strB 91str6 and other mutants which map near proA.

RE26strB again proves exceptional when the mutants are examined for their response to Ethylene-diaminetretraacetate. All the independent mutants of RE26 and RE91str6 were more sensitive to E.D.T.A. than the parent strains. RE26strB and the mutants in RE26 (R46) were more resistant to E.D.T.A. than the parent strains. The action of E.D.T.A. on cells is to remove lipopolysaccharide from the surface of the cell, prolonged incubation leads to cell death. It is reasonable to assume that a strain is more resistant to E.D.T.A. by virtue of an alteration in the surface layers of the cell which prevents removal of lipopolysaccharide.

A third test provides some evidence for the altered permeability of these mutants. The basic dye crystal violet has been used to detect the presence of mutants with altered cell envelopes, (Nordström et al 1970). Some of the mutants do not have an altered $L.D_{50}$ of crystal violet but the majority are more sensitive than the parent strains. Again RE26strB proves exceptional in being very sensitive to crystal violet. The percentage survival of RE26strB on 5μg/ml of the dye in L-broth agar was zero. This very marked reaction to a basic dye is in contrast to the apparent lack of difference between RE26 and RE26strB when incubated in four other basic dyes. The only difference observed in that series of experiments was the slowness with which RE26strB produced reducing substances to decolourise methylene blue compared with RE26.

Permeability alterations in the cell seem to be the most likely resistance mechanism operating in these mutants. Whatever the permeability effect in RE26strB it prevents to a small degree the excretion of 260- absorbing material into the culture medium as a result of incubation in the presence of streptomycin.

The genetic basis of str-r resistance has been discussed above and the variations in the properties of the independent mutants reflect the possible variation in the precise location of the genetic alterations underlying these mutants. In addition to the common str-r phenotype the mutants are more sensitive to tetracycline but vary in their response to the action of E.D.T.A. and the effect which they have on the expression of R-factor Ampicillin resistance. Resistance of the mutants to Crystal Violet is another variable property as was the resistance to Novobiocin.

Str-r and collateral sensitivity to Tetracycline is the result of mutation in at least two different loci as shown by mapping experiments. However when other properties of these mutants are tested other differences come to light. Mapping data from the twenty independent str-r mutants of RE26 suggest that a number of different loci mapping close to proA may be involved in str-r. Thus it is possible that there is a complex locus near proA which codes for membrane structure and/or function. Each of the mutations by virtue of the selection procedure is str-r but they differ in other properties.

Therefore it is possible to state that str-r resistance in E.coli K12 is the result of mutation at more than one locus and as such may be described as a polygenic system. Each mutation produces a small effect and in combination they can act additively to produce more highly resistant organisms as evidenced by the inoculum effect. It may not be possible to combine a number of the different genes because the resultant effect may prove lethal as would be expected if the mutations involve permeability changes in the cell.

The data, in addition to showing how one can select permeability mutants in E.coli with low concentrations of streptomycin, provide some interest from the point of antibiotic therapy. The concept of two antibiotics being more effective than one is common knowledge in chemotherapy - the presence of one antibiotic makes the second more effective in its attack. However with str-r mutants one has the situation where pre-treatment with streptomycin at the

correct concentration could make it possible to treat a Tetracycline resistant infection with Tetracycline.

Admittedly this is very much in the realm of theoretical speculation but is interesting when one considers that it is possible to alter the resistance pattern of an R-factor carrying bacterium by making the bacterium resistant to a low concentration of streptomycin.

5. SUMMARY

Mutants of E.coli K 12 resistant to a maximum of 5.0μg/ml streptomycin occur with a frequency of 10^{-6}. This type of low level resistance is due to mutation of at least two and possibly more different loci.

One gene, designated strB, is located at 8.0 minutes on the E.coli linkage map. Another mutation, in RE91, and five in RE26 show no linkage with proA, one of the criteria used to locate strB. Other independent mutations in RE26 show some linkage with proA but it is impossible to say with any certainty that these are due to mutations of $strB^{+}$.

Combination of R-factor resistance determinants with low level streptomycin resistance reveal that both streptomycin resistance mechanisms act synergistically. In contrast the presence of strB and other mutants interferes with the expression of R-factor Tetracycline resistance and also to some extent with extrachromosomal Ampicillin and Chloramphenicol resistances. A similar effect is observed when low level streptomycin resistance is selected in R-factor carrying strains. Selection for increased streptomycin resistant R-factor is interesting in that the effect of these mutants on the Ampicillin resistance of the R-factor is more variable.

Certain physiological properties suggest that the mutants have an altered permeability in comparison with the sensitive parent strains. This hypothesis is supported by the interaction of the mutants with R-factor Tetracycline resistance.

6. APPENDIX

Preparation of proB mutants

The purpose of selecting a *proB* mutant in *E.coli* K12 was to provide a second reference point for the location of the gene in RE26*str16* which was responsible for the str-r phenotype of the strain. Use of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) increases the general mutation rate and therefore the yield of any particular type of mutant selected. The procedure used was that described by Adelberg, Mandel and Chen (1965).

RE91 was treated with NTG at a final concentration of 100μg/ml in TM buffer for 15 minutes. After removal of NTG by washing with TM buffer the cells are incubated overnight in a high concentration of proline. Next morning the cells were washed and resuspended in proline-free medium prior to penicillin treatment to select for the non-growing proline-requiring mutants. After removal of penicillin the culture was incubated to a visible turbidity and samples from this were plated on proline-supplemented minimal medium to give 50-100 colonies per plate.

After 2 days incubation these plates were replicated onto minimal medium lacking proline: fifty seven proline requiring mutants were obtained by the NTG treatment. Purification and retesting of these mutants produced forty one pro^- mutants of RE91.

Three loci are involved in proline synthesis in *E.coli* K12 It is possible to distinguish *proA* mutants from *proB* and *proC*

mutants because the former mutant is crossfed by *proB* and *proC* mutants. Cross feeding experiments using RE226 and RE26 eliminated proA mutants (see Table IA).

The remaining nineteen mutants were either *proB* or *proC* mutants and were distinguished from each other by transduction with P1 donor phage grown on RE226. This strain is a *proC* mutation and consequently donor phage grown on it will only transduce to proline independence those mutants which are *proB* (see Table IIA). Fourteen *proB* mutants were obtained by this test and one was selected for the mapping experiments with RE26*str16*.

TABLE IA

Crossfeeding property of NTG induced Pro^- mutants of RE91

Crossfeeding	no. of mutants
Crossfed by RE226 (*proC*)	20
Mutants which crossfeed RE26 (*proA*)	21

TABLE IIA

Transduction Analysis of Pro^- mutants of RE91 which crossfeed RE26.

Transduction to Pro^+ by RE226.	no. of mutants
Yes	11
No	8

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ABSTRACT OF THESIS

Name of Candidate LILIAN MARY ROBERTS

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Title of Thesis A study of mutations affecting antibiotic resistance in Escherichia coli.

Mutants of E.coli K12 resistant to a maximum of 5.0μg/ml streptomycin occur with a frequency of 10^{-6}. This type of low level resistance is due to mutation of at least two and possibly more different loci.

One gene, designated strB, is located at 8.0 minutes on the E.coli linkage map. Another mutation, in RE91, and five in RE26 show no linkage with proA, one of the criteria used to locate strB. Other independent mutations in RE26 show some linkage with proA but it is impossible to say with any certainty that these are due to mutations of $strB^{+}$.

Combination of R-factor resistance determinants with low level streptomycin resistance reveal that both streptomycin resistance mechanisms act synergistically. In contrast the presence of strB and other mutants interferes with the expression of R-factor Tetracycline resistance and also to some extent with extrachromosomal Ampicillin and Chloramphenicol resistances. A similar effect is observed when low level streptomycin resistance is selected in R-factor carrying strains. Selection for increased streptomycin resistant R-factor is interesting in that the effect of these mutants on the Ampicillin resistance of the R-factor is more variable.

Certain physiological properties suggest that the mutants have an altered permeability in comparison with the sensitive parent strains. This hypothesis is supported by the interaction of the mutants with R-factor Tetracycline resistance.

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