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The characteristics of eleven mutants of R-factor R57 constitutive for tetracycline resistance, selected and tested in *Escherichia coli* K12

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SUMMARY

Eleven mutants of R-factor R57 have been isolated which show constitutive expression of resistance to tetracycline (Tc). These derepressed (Tdr) mutants all gave a much greater resistance to Tc and to its analogue, minocycline, than could be obtained by optimal induction of cells carrying the wild-type (T^+) determinant. Cells carrying each of the Tdr mutants together with T^+ of either R6-S or of a plasmid found in *Escherichia coli* mil9 showed inducible Tc resistance, indicating that the Tdr mutants were all recessive, i.e. of repressor-negative type. Tdr1 was not recessive to the T-determinant of RP1, suggesting that the repressor gene products of the T-determinants in R57 and RP1 have different specificities.

1. INTRODUCTION

R-factor mediated resistance to the tetracyclines in coliform bacteria acts by producing a specific decrease in permeability of the cell membrane to this group of antibiotics; the resistance is inducible, since a short exposure of growing cells to a sub-inhibitory concentration of tetracycline causes a rapid increase in their resistance (Izaki, Kiuchi & Arima, 1966; Franklin, 1967; Robertson & Reeve, 1972). The model put forward to explain these results by Franklin (1967) and elaborated by Franklin & Cook (1971) and Robertson & Reeve (1972) assumes that the R-factor determinant contains both a resistance and a repressor gene: the former codes for one or more proteins which modify the cell membrane so as to reduce its permeability to the tetracyclines, while the repressor gene product restricts expression of the resistance gene. Inactivation of the repressor substance by complexing with tetracycline would then account for the inducible nature of the resistance, while challenge of uninduced cells with a high concentration of the drug would not allow induction since protein biosynthesis would be inhibited. This hypothesis is supported by the recent results of van Embden & Cohen (1973) and Levy & McMurry (1974). The latter found that *Escherichia coli* minicells, when incubated in the presence of a low concentration of tetracycline, synthesize an R factor-specific protein with a molecular weight of about 50000, which appears to be located in the minicell membrane.

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If the hypothesis is correct, it should be possible to select two types of R-factor mutant giving constitutive expression of full tetracycline resistance, those in which a change in the repressor gene leads to production of an inactive repressor, and those in which a change at the operator site of the resistance gene makes it no longer repressible. These may be referred to as i^- and o^c mutants, respectively, by analogy with the *lac* operon of *Escherichia coli* (Beckwith & Zipser, 1970), and these classes of constitutive (i.e. derepressed) mutants will be referred to as Tdr to indicate that they are derepressed for tetracycline resistance. Selection of Tdr mutants is difficult because chromosomal mutations to low-level tetracycline resistance occur at a rather high frequency and cause a marked increase in the level of resistance shown by R-factor carrying strains (Reeve, 1966). One partially constitutive R-factor mutant has been described by Franklin & Cook (1971), but it was not clear whether it was of o^c or i^- type. This paper describes an improved selection method which enabled us to obtain 11 Tdr mutants, all of which were fully constitutive. When each mutant was placed in the same bacterial host with each of two wild-type T determinants it was repressed, suggesting that they are all of the repressor-negative (i^-) type.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

RE26F$^-$ProA$^-$Trp$^-$His$^-$LacY$^-$Str^s (see Robertson & Reeve, 1972) and RE13F$^-$Met$^-$Str^s (W1485 from W. Hayes in 1961) are derivatives of *Escherichia coli* K12. HfrR4-Tmil is a strain of K12 carrying a T determinant (from a plasmid found in *E. coli* strain mil9) integrated into the chromosome close to *leu* (Hoekstra, Zuidweg & Kipp, 1973) and was supplied by W. P. M. Hoekstra, who labelled it HfrR4-*tet*$^+$. We shall refer to it below as HfrR4-Tmil in order to indicate the origin of the T determinant.

(ii) *R-factors*

R57(STSu)* was obtained from Naomi Datta in 1965, and originated from a strain of *Salmonella typhimurium*; its compatibility group has not been determined, but it is *fi*$^-$ and is compatible with RP1 and R6-S. R57 was described by Reeve (1966) and Robertson & Reeve (1972), carries the reference number assigned by Naomi Datta, and is entirely unconnected with the R-factor later labelled R57 (Witchitz & Chabbert, 1971) and R57b (see Datta & Hedges (1972)).

RP1 (AKT) belongs to the P compatibility group (Grinsted *et al.* 1972) and was obtained from D. J. Stewart. R6-S(CT) is a derivative of R6 and belongs to the FII group (Foster & Shaw, 1973) and was obtained from T. J. Foster.

* Abbreviations: A, C, K, S, Su, T indicate R-factor determinants for resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracycline, respectively. T$^+$ and Tdr indicate wild-type and derepressed T-determinants. Tc, Tetracycline; Mc, minocycline; Tc20, tetracycline incorporated in the medium specified, at 20 μg/ml. The presence of an R-factor is indicated by parentheses, e.g. RE13(R1) and RE13(R1, R2) refer to strain RE13 carrying R1 and both R1 and R2, respectively. RE numbers refer to our own strains of *E. coli* K12.

(iii) *Media*

L-Broth was prepared as in Robertson & Reeve (1972). NB is Difco nutrient broth, and NA is this broth solidified with 15 g Difco Bacto agar/l H_2O.

(iv) *Antibiotics*

Tetracycline (trade name Achromycin) and minocycline, in powder form, were gifts from Cyanamid of Great Britain and Lederle Laboratories. Chloramphenicol was a gift from Parke, Davis & Company, and other antibiotics were purchased commercially. Sulphonamide resistance was checked with Oxoid Multodisks.

(v) *Growth and challenge tests*

These were essentially as described by Robertson & Reeve (1972). For each test, a culture of the strain to be tested, growing in log phase in L Broth after overnight growth in the same medium, was at time 0 diluted 10 ml into four 100 ml metal-capped flasks containing 10 ml of warm L Broth, of which two also contained twice the inducing dose of antibiotic. At time 15 min, one induced and one uninduced flask received sufficient antibiotic dissolved in 1 ml warm L Broth to give the correct challenge dose, and the other two flasks received 1 ml warm L Broth alone. In some experiments the induced but unchallenged culture was omitted. The flasks were shaken in a water bath at 37 °C during the test, and growth was measured by optical density (550 nm, 1 cm light path) read at times 0, 20, 40, 60 and 80 min. The growth rate was calculated as the linear regression coefficient of ln OD on time over the period 20–80 min. The initial culture was diluted so as to give test cultures with OD about 0·1 at time 0.

After completion of the test, the cultures were streaked on NA to give single colonies, and usually 20 colonies each from the two challenged cultures were tested for their nutrient requirements and the antibiotic resistance present. Except where stated, these were correct for all the colonies tested.

3. RESULTS

(i) *Selection of constitutive mutants*

E. coli K12 RE26(R57) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine before selection was applied. Various modifications of the method of Franklin & Cook (1971) were first tried, and led after much labour to a single mutant, R57Tdr1. An improved method was developed when it was discovered that cells growing in log phase in broth carrying an R-factor rapidly lose viability for 60–90 min after addition of a high concentration of Tc to the culture. Fig. 1 shows the effects of adding Tc at a final 120 or 200 μg/ml to RE26(R57) growing in L Broth. In uninduced cultures, cell mass as measured by OD_{550} remained almost constant for 3 h, but viable count declined dramatically during the first 90 min after challenge, some 400-fold in the presence of 120 μg/ml, and about 3000-fold in the presence of 200 μg/ml of the antibiotic. After this time the viable count began

to increase again slowly, probably because a small fraction of the challenged cells had become induced to full Tc resistance instead of being killed. Fig. 1 also shows that induced cells only lose viability very slowly after challenge with Tc 200. Since cells of the same bacterial strain carrying the mutant Tdr1 were able to grow slowly immediately after challenge with 200 μg/ml Tc, it is clear that 90 min challenge of a population of growing cells with Tc 200 should give a marked enrichment of Tdr mutants, perhaps by several thousand times.

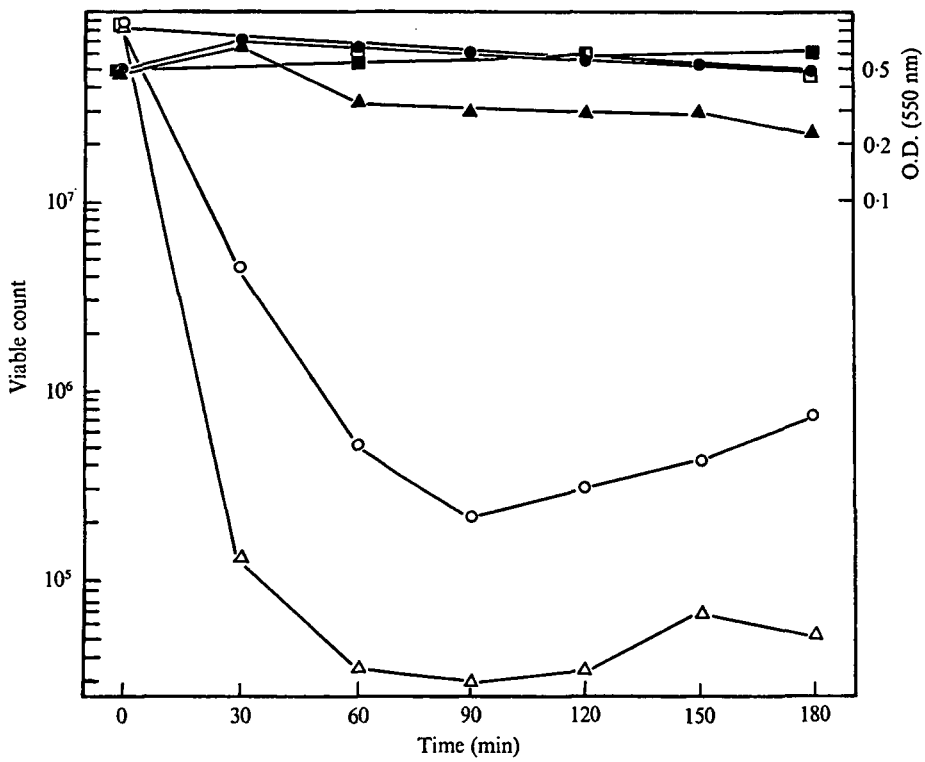

Fig. 1. Growth and viability of RE26(R57T+) after challenge with high concentrations of Tc. Cells growing in L Broth at 37 °C were challenged at time 0. The induced culture received 2 μg/ml Tc 15 min before challenge. Cell mass was measured by optical density at 550 nm, viability by plating on NA. Symbols:

Induction	Challenge	OD_{550}	Viable count
None	120 μg/ml Tc	●	○
None	200 μg/ml Tc	▲	△
2 μg/ml Tc	200 μg/ml Tc	■	□

After mutagen treatment, R26(R57) was therefore grown to log phase in L Broth, challenged with 200 μg/ml Tc for 90 min, then immediately diluted and plated for single colonies on NA. Colonies were picked with sterile tooth-picks onto NA master plates (100 per plate), incubated overnight and replicated on to both NA and NA + Tc20, and these plates were incubated overnight. The replicated colonies could grow well on both plate types, the function of the NA + Tc20 plates being to

produce colonies induced to maximum Tc resistance. Both the NA and the NA + Tc20 replica-plates were then themselves replicated on to minimal agar containing Tc at 200 μg/ml and incubated 48 h. Tdr colonies should do equally well on these plates whether replicated from NA or NA + Tc20, since their maximum resistance should be expressed without induction, while inducible colonies should grow better when replicated from the Tc plates. The results were not very clear-cut, but 52 colonies were isolated as potential Tdr mutants and were purified from the original master NA plates. Each was then grown overnight in NB and NB + Tc20, and both cultures were diluted 10^{-2} in saline and streaked across minimal agar containing Tc at 0, 100, 150 and 200 μg/ml. Ten mutants showed good growth on the Tc150 plates regardless of induction, and were retained for further tests. Each was constitutive for Tc resistance, and this property was retained when they were transferred to a fresh culture of RE26 via another K12 host, by selecting in each case for transfer of the S determinant. The mutation responsible for constitutivity cannot therefore be in the host chromosome but must be in an R-factor gene (chromosomal mutations can have a marked effect on the level of R-factor Tc resistance (Reeve, 1966)). These mutant R-factors have been labelled R57Tdr2–11.

(ii) *Characteristics of the Tdr mutants*

The simple growth and challenge tests devised by Robertson & Reeve (1972) make it possible to estimate the resistance of R-factor carrying cells to different concentrations of antibiotic in terms of the effect on growth rate. Fig. 2 summarizes the results of applying tests of this kind to strain RE26 carrying R57T$^+$ and R57Tdr1, respectively. Fig. 2(*a*) shows that cells carrying the T$^+$ determinant grew very slowly after challenge with Tc 80 unless previously induced with Tc2, when growth continued at about 40 % of the normal rate. Uninduced cells carrying R57Tdr1, on the other hand, grew at almost the normal rate after challenge with

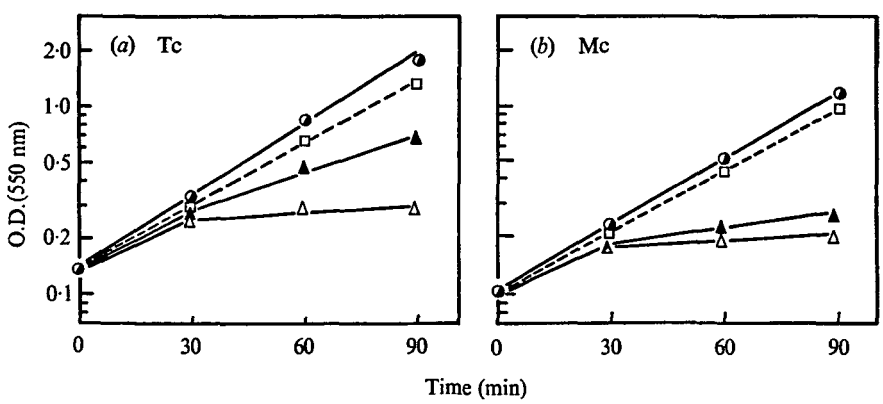

Fig. 2. Growth and challenge tests with (*a*) tetracycline and (*b*) minocycline on RE26 carrying R57T$^+$ (triangles) and R57Tdr1 (squares). White symbols: uninduced cultures challenged with 80 μg/ml Tc or 5 μg/ml Mc. Black symbols: cultures challenged after previous induction with 2 μg/ml Tc (on left), 0·1 μg/ml Mc (on right). Circles: unchallenged cells of each strain. Induction at time 0, challenge at time 15 min.

Tc80. Fig. 2 (*b*) shows that essentially the same result was obtained when the related antibiotic, minocycline (Mc) was substituted for Tc, using a challenge dose of 5 μg/ml Mc instead of 80 μg/ml Tc, except that Mc was a relatively ineffective inducer of increased resistance in T^+ cells. 5 μg/ml Mc had very little effect on the growth rate of cells carrying R57Tdr1. The effect of minocycline (7-dimethylamino-6-demethyl-6-deoxytetracycline) on *E. coli* carrying a T^+ R-factor has been examined by Robertson & Reeve (1972).

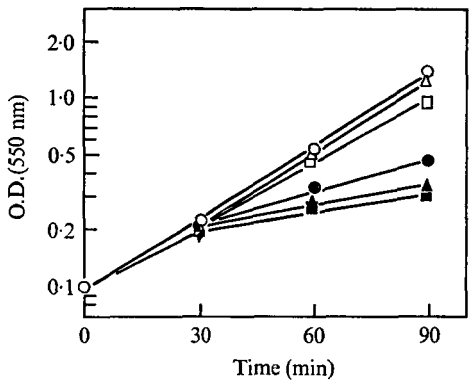

Fig. 3. Growth and challenge test on RE26(R57Tdr1). White symbols: unchallenged. Black symbols: challenged with 200 μg/ml. Inducer dose: circles, none; triangles, 40 μg/ml Tc; squares, 80 μg/ml Tc. Induction at time 0, challenge at 15 min.

Further tests are summarized in Fig. 3, which shows the effect of growing RE26-(R57Tdr1) in broth, inducing with Tc at 40 or 80 μg/ml and then challenging with 200 μg/ml Tc. The effect of challenging uninduced cells is also shown. Tc40 had almost no effect on growth rate of unchallenged cells, while Tc80 caused a slight fall in growth rate. Challenge with Tc200 caused a sharp drop in growth rate which was not at all alleviated by previous induction – in fact uninduced cells grew faster than induced cells after challenge. Lower inducing doses were found to be equally ineffective, and it is clear that R57Tdr1 gives completely constitutive resistance to Tc. Similar tests showed that resistance to Mc is likewise constitutive.

A number of such tests have been used to produce the resistance profiles of RE26(R57Tdr1) against Tc shown in Fig. 4(*a*) and against Mc (Fig. 4*b*), each profile being compared with those of uninduced and induced cells of RE26 carrying R57T^+. In each case the profile shows the growth rate (cell doublings per hour) during the 60 min after challenge, plotted against the challenge dose. The most striking fact brought out by these comparisons is that R57Tdr1 confers a much higher resistance to both antibiotics than can be achieved by induction of cells carrying the wild-type R57T determinant. The superiority of R57Tdr1 is particularly marked against Mc, due to the small increase in resistance of R57T^+ cells after induction by Mc. The difference in the horizontal scales for concentration of Tc and Mc should be noted, Mc being effective at a much lower concentration than Tc against R-factor carrying cells.

From Fig. 4 we can derive a numerical index of resistance by reading off the concentration of antibiotic needed to reduce growth by 50 %. Indices for the two R-factors and for both antibiotics are given in Table 1, and show that cells carrying R57Tdr1 need 2·5 times as much Tc, or 5 times as much Mc, to produce the same

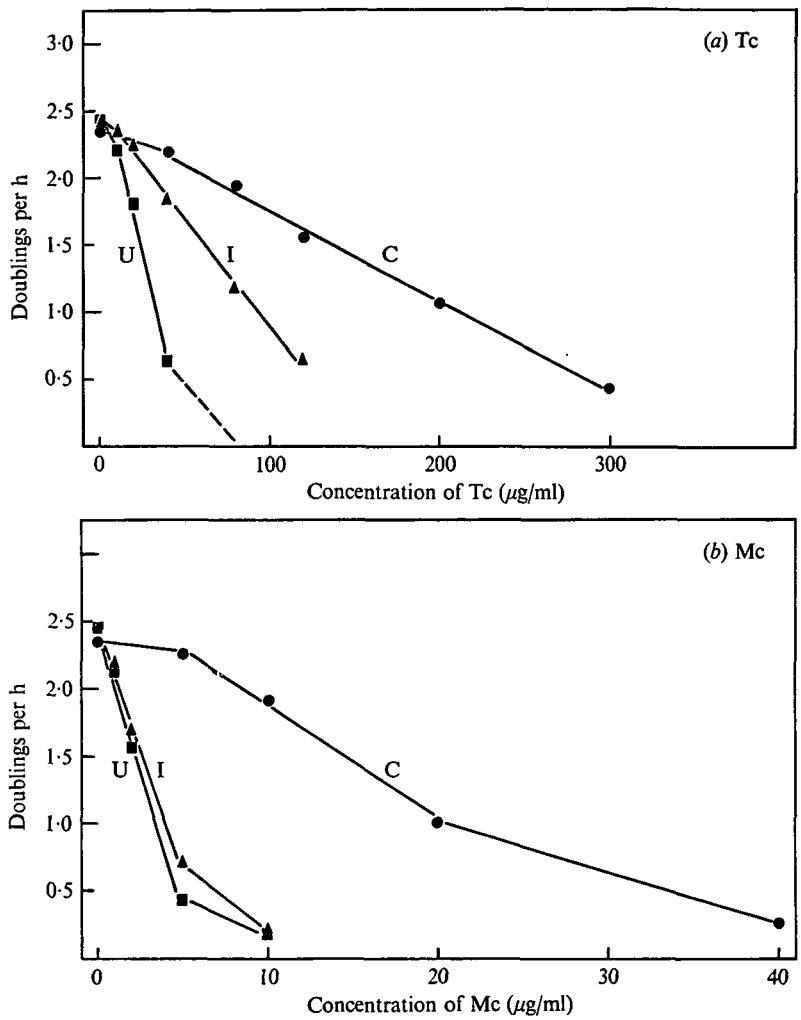

Fig. 4. Resistance profiles of RE26(R57Tdr1) uninduced cells (C), RE26(R57T+) induced cells (I) and uninduced cells (U), against tetracycline (upper graph) and minocycline (lower graph). The growth rate after challenge is plotted in units of doublings/h against the challenge dose.

degree of growth inhibition as cells carrying R57T+ which have been induced to give maximal resistance.

Tests have not been carried out in such detail on the other R57Tdr mutants, but several tests on each indicate that they are all completely constitutive for resistance, that all give much greater resistance than induced R57T+ cells, and that

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their resistance profiles are roughly the same as that of R57Tdr1 against Tc. Their ability to confer resistance against Mc has not been tested.

(iii) *Combination of Tdr and T+ determinants in the same host cell*

To test for dominance of a Tdr mutant over the T+ determinant, it is necessary to put R-factors carrying the two determinants in the same host cell, perform the

Table 1. *Concentrations of antibiotic which reduce growth rate by* 50%

	Drug concentration (μg/ml)	
R factor in RE26	Tc	Mc
R57T+, induced cells	75	3·6
R57Tdr1, uninduced cells	185	18
Ratio Tdr1/T+	2·5	5

dominance test and then show that each R-factor retains its identity on transfer to another host. Two compatible R-factors carrying different T determinants had to be used for these tests, since we did not have the T+ determinant of R57 integrated into another R-factor; and this, as we shall see, raises questions about the interactions between T determinants of different origin.

(*a*) *Tests with RP1*

We first used RP1, which carries the determinants A, K and T and was found to be compatible with R57 (which carries S, T and Su). RP1 was introduced into RE26, RE26(R57Tdr1) and RE26(R57Tdr5) by selection for the nutrient requirements of RE26 and for K to obtain RE26(RP1), and for KS to get the doubly infected strains. In the latter case clones carrying AKSSuT were isolated. Growth and challenge tests on RE26 carrying RP1 alone or in combination with R57Tdr1 or R57Tdr5 (as judged by the resistance characters present) are shown in Figs. 5(*a–c*), the inducing and challenge doses in each case being 2 and 20 μg/ml Tc. Uninduced RP1 gives a low level of resistance to Tc, since growth was almost stopped by 20 μg/ml, but after induction the challenge dose had hardly any effect on growth. Figs. 5(*b*, *c*) suggest that the mutation carried by R57Tdr1 is dominant and that of R57Tdr5 is recessive, to T+ carried by RP1, since they give constitutive and inducible resistance, respectively. However, an alternative explanation of these results would be that one or both of the doubly infected strains had lost one of the two T determinants (T+ from the first combination, Tdr5 from the second). To test these possibilities, both double-R carrying strains of RE26 were mated to the Met− strain RE13 and selection was made for transfer of S and K, respectively, to obtain recipients carrying each R-factor separately. In the case of the (R57Tdr1, RP1) combination, selection for S gave 86% transfer of ST without AK and 14% AKST colonies (the latter possibly the result of two separate transfer events), while selection for K gave 100% AKT colonies. Tests on a small number of these clones showed that ST clones also carried Su and were T-constitutive, while AKT clones

did not carry Su and were T-inducible. A single AKST clone tested carried Su and was T-constitutive like the donor. These results indicate that both Tdr1 and T^+ were present in RE26(R57Tdr1, RP1) and that the mutation carried by R57Tdr1 was dominant to T^+ or RP1.

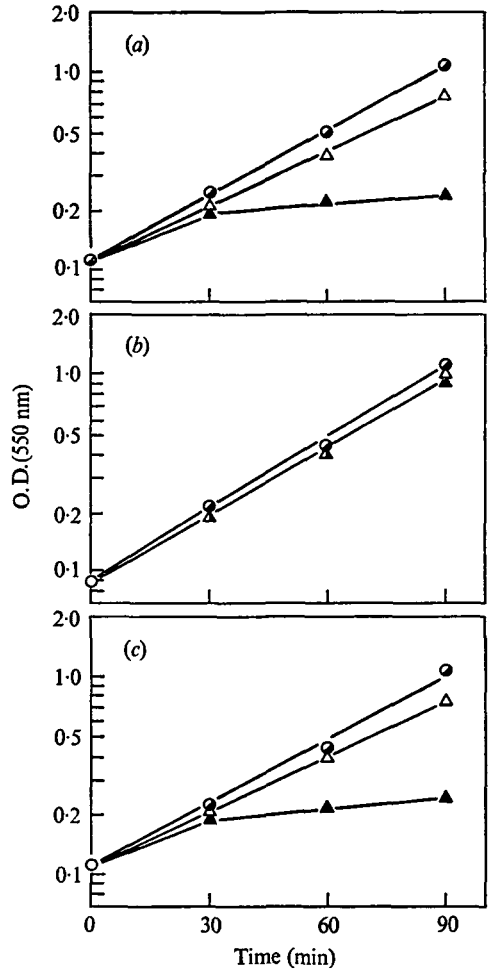

Fig. 5. Growth and challenge test on (*a*) RE26(RP1), (*b*) RE26(RP1, R57Tdr1), (*c*) RE26(RP1, R57Tdr5). ●, Untreated control; ○, induced with 2 μg/ml Tc, no challenge; ▲, no induction, challenge 20 μg/ml Tc; △, induced with 2 μg/ml Tc, challenge 20 μg/ml Tc. Induction at time 0, challenge at time 15 min.

The transfer test on RE26(R57Tdr5, RP1) failed to separate the two R-factors, since selection for S gave clones which had either lost A, K and both T determinants, or carried S, T and A with or without K. Selection for K gave clones which were predominantly AKST. Further tests on a few clones showed that Su was present when S was present and that ATK and ASTKSu clones were T-inducible. These results suggest that recombination had occurred in RE26 between

R57Tdr5 and RP1, with loss of Tdr5, and that the recombinant R-factor(s) fragmented on transfer to RE13. We cannot therefore assume that Tdr5 was present in the bacterial strain tested in Fig. 5*c*, or that it is recessive to T^+ of RP1.

(*b*) *Tests with R6-S*

In view of the difficulties experienced with RP1, we decided to carry out a new set of tests in which the R57Tdr mutants were put together with R6-S, which carries the determinants C and T, T giving high-level inducible resistance to Tc

Table 2. *Growth and challenge tests on RE26(R6-S, R57Tdr) lines*

Tdr	Growth rate (d/h)*	Effect of challenge on growth rate	
		IC (%)†	(UC/IC) (%)‡
1	2·74	74	66
2	2·87	69	68
3	2·66	71	32
4	2·76	71	45
5	2·66	64	48
6	2·56	70	71
9	2·87	67	63
10	2·78	71	62
11	2·76	73	60

All tests were performed as described under Methods, on RE26 successively infected with a Tdr mutant of R57 and with R6-S. The induction and challenge concentrations were 2 and 80 μg/ml Tc. One test was made on each R-factor combination except those containing Tdr4 (2 tests) and Tdr5 (3 tests) for which average values are quoted.

* Growth rate is number of mass doublings/h of unchallenged cells, averaged for uninduced and induced cultures.

† Growth rate of induced challenged cells (IC) as percentage of growth rate of challenged cells.

‡ Induction effect: growth rate of uninduced challenged cells (UC) as percentage of that of induced challenged (IC). This index would be 100 % for constitutive resistance.

similar to that reported for R57 (Robertson & Reeve, 1972). R6-S was therefore introduced into RE26 carrying each R57Tdr mutant, and growth and challenge tests were carried out on all but two of the doubly infected strains. For these tests induction and challenge concentrations of 2 and 80 μg/ml Tc were used, and the results are summarized in Table 2, which gives the growth rate of unchallenged cells and the effects of challenge on induced and uninduced cells, expressed as the relative growth rate of induced cells after challenge (IC %) and the growth rate of uninduced challenged cells as a percentage of that of induced challenged cells (UC/IC) %. The latter index should be 100 % for constitutively expressed resistance.

Clearly all nine Tdr mutants tested in this series have either been lost or show inducible resistance in the presence of the T^+ determinant of R6-S, induced cells growing at about 70 % of normal after challenge, while uninduced challenged cells grow at about 60 % of the rate of induced challenged cells. Challenged cells carrying any of the Tdr mutants alone were found to grow at virtually the normal rate,

whether induced or not. All the doubly infected host strains were mated to another K12 strain, and in each case selection for S and C separately produced clones which were, respectively, $(STSu)^r$ T-constitutive and $(CT)^r$ T-inducible, without combinations of the two R-factors being obtained. This shows that the two R-factors retained their identity in the host strain and that both T determinants were present together. We must conclude, then, that the nine Tdr mutants tested were recessive to the T^+ of R6-S, and are therefore of the repressor-negative or i^- type. The fact that Tdr1 was apparently dominant to the T determinant of RP1 will be considered later.

Table 3. *Tests on Tdr mutants in the presence of R6-S and Tmil*

Tdr mutant	Tdr + R6-S in RE13		Tdr + Tmil in HfrR-4	
	IC(%)	UC/IC(%)	IC(%)	UC/IC(%)
1	56	19	69	38
2	59	29	83	16
3	63	29	72	25
4	62	35	72	18
5	60	32	69	43
6	62	26	69	18
7	68	33	74	38
8	69	69	67	54
9	61	44	72	45
10	62	25	61	32
11	57	22	72	64

Growth and challenge tests as described under Methods. Inducing dose 2 μg/ml Tc, challenge dose 160 μg/ml Tc for tests with R6-S, 140 μg/ml Tc for tests with Tmil. All means quoted are averages of three tests except for Tdr mutants 3, 5, 6 and 7 of the (Tdr + R6-S) series which are based on 5, 4, 2 and 2 tests, respectively. Tmil is the T-determinant from *E. coli* mil9 integrated into the chromosome of HfrR4 near *leu* (Hoekstra *et al.* 1973). Data on (Tdr + Tmil) from Catherine McTavish (unpublished).

A more extensive series of tests was also carried out on the K12 strain RE13 carrying R6-S together with each of the eleven R57Tdr mutants, replicate tests on the same combination being made on different dates in each case. The doubly infected hosts were checked for the presence of all the resistance determinants, both before and after each test, and no segregation was observed. The level of Tc resistance appeared to be higher in RE13 than in RE26, and a challenge dose of Tc 160 was used, the induction dose being Tc2 as before.

These data will be considered alongside data on the behaviour of each Tdr mutant in the presence of another wild-type T-determinant (Tmil), derived from *E. coli* strain mil9 and integrated into the chromosome of *E. coli* K12 strain HfrR4 close to the *leu* locus (Hoekstra *et al.* 1973). The new host strain, HfrR4-Tmil gives a pattern of inducible high-level Tc-resistance similar to those of R6-S and $R57T^+$ in RE13. HfrR4-Tmil was infected with each of the Tdr mutants and growth and challenge tests were made on three separate clones carrying each mutant. This work was carried out by Miss Catherine McTavish (Honours Thesis, University of Edinburgh, Genetics Dept., unpublished), to whom we are indebted for the data.

The results of both series of tests are shown in Table 3, which gives the two indices, IC % and (UC/IC) % for each Tdr mutant in combination with both the wild-type T determinants. All Tdr mutants have become inducible in the presence of either R6-S or Tmil as is shown by the fact that the index (UC/IC) % is well below the 100 % expected for constitutive expression. The recessive behaviour of the mutants is further shown by the fact that the IC % values for (Tdr + R6-S) range round about 60 % whereas they are close to 100 % when any Tdr mutant alone is present in RE13 (unpublished observations).

Table 4. *Analysis of (Tdr + R6-S) and (Tdr + Tmil) tests*

		IC (%)		(UC/IC) (%)	
Source of variance	Degrees of freedom	Mean square	F	Mean square	F
Between Tdr mutants	10	19·8	—	338	3·13**
Mutant × T^+ interaction	10	31·9	—	147	1·36
Error	45	34·8		108	

To take into account the variation in number of tests performed on the (Tdr + R6-S) combinations a weighted analysis of variance was made, i.e. if x_{1r} and x_{2r} are the means for Tdr mutant r tested with R6-S and Tmil, respectively, based on n_{1r} and n_{2r} tests, and $w_r = n_{1r}n_{2r}/(n_{1r}+n_{2r})$, then the sum of squares 'between Tdr mutants' is calculated as $\Sigma\, w_r(x_{1r}+x_{2r})^2 - [\Sigma\, w_r(x_{1r}+x_{2r})]^2/\Sigma\, w_r$, while substitution in this expression of $-x_{2r}$ for $+x_{2r}$ gives the 'mutant × T^+' interaction. The error variance is the variance between replicates.
** $P < 0{\cdot}01$.

The level of (UC/IC) % shows considerable variation in Table 3, ranging from 19–69 % in the R6-S series and 16–54 % in the T(mil) series. The replication used in these experiments (a total of 67 tests on the 11 Tdr mutants) makes it possible to test the statistical significance of this variation, as shown in Table 4. For this analysis the individual index values were converted to degrees (Fisher & Yates, 1957, table x) and a 'between mutants' and an 'interaction' term were separated, the latter measuring differences in the behaviour of each Tdr when tested in the presence of the two T^+ determinants.

Table 4 shows that there is no significant variation in the IC % index, either between mutants or in the [mutant × T^+] interaction. For the induction index, (UC/IC) % there is again no significant interaction variance, but a statistically significant variation between the 11 Tdr mutants was found. Thus, although these mutants are all expressed inducibly in the presence of either T^+, and so are more or less recessive, the level of recessivity varies. Reference to Table 3 suggests that this variation is mainly due to the higher level of (UC/IC) % for Tdr8, which appears to behave as if it was incompletely repressed by either T^+.

A final comparison is given in Table 5, which shows the IC % and (UC/IC) % indices for RE13 carrying R6-S or R57T^+ alone (average values for the two R-factors are given since they differed very little), for RE13 carrying both R-factors together; and the average values for the combination of R6-S with the eleven R57Tdr mutants, based on the data of Table 3. The comparisons of interest are

tested for significance in the rest of the table. Taking first the two wild-type R-factors, we see that they do not differ significantly in either index, but when combined together in the same host they give an increase in IC % (from 39 to 58 %) and a decrease in (UC/IC) % (from 25 to 14 %), both differences being statistically significant. This means that the growth rate of uninduced challenged cells, obtained by multiplying the two indices together, is about the same (8–10 %) when either or both T^+ determinants are present, but induction gives greater resistance (higher IC %) when both determinants are present. This is what would be expected if neither T^+ determinant alone can produce sufficient 'resistance protein' to saturate the membrane sites available, so that the presence of both together raises the maximal resistance which can be achieved by induction.

Table 5. *R6-S, R57, R6-S + R57 and R6-S + R57Tdr in RE13*

		Average effect of challenge	
R-factor(s) in RE13	No. of tests	IC (%)	(UC/IC) (%)
R6-S or R57 alone	3 on each	39	25
R6-S + R57	5	58	14
R6-S + R57Tdr	34	62	32

Analysis of variance

	Degrees of freedom	Mean squares	
		IC (%)	(UC/IC) (%)
(1) R6-S and R57			
Single versus double infection	1	338*	200*
R6 versus R57 single infections	1	14	2
Error	8	47	37
(2) (R6-S + R57) versus (R6-S + R57Tdr)			
T^+ versus Tdr	1	16	762*
Error	27	38	117

The inducing and challenge doses in all tests were 2 and 160 μg/ml Tc. The variance analysis was performed on variates transformed to degrees.

* Significant at $P = 0{\cdot}05$.

When (R6-S + R57) was compared with (R6-S + R57Tdr) (averaged over the 11 mutants), for the two indices, the presence of a Tdr mutant in place of $R57T^+$ had no significant effect on IC % but gave a marked and significant increase in (UC/IC) % (from 14 to 32 %), which is just what would be expected if there were two resistance genes together with one repressor gene in the bacterial host. Thus the Tdr mutants are not completely recessive to the T^+ determinant of R6-S, since they give a higher basal level of Tc-resistance than the (R6-S + $R57T^+$) combination.

4. DISCUSSION

Our aim was to test the current model for the mechanism of R-factor mediated Tc-resistance by selecting mutants giving constitutive expression of resistance, with the expectation that these would include mutants of both classes (i^- and o^c) predicted by the model. Eleven mutants were isolated in R-factor R57, and all

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gave a much higher level of resistance than could be obtained by optimal induction of cells carrying the wild-type R-factor. When each of these Tdr mutants was placed together with a wild-type T determinant (T^+ from either R6-S or mil9) in the same cell, in every case the doubly infected strain gave the wild-type inducible expression of Tc-resistance, showing that each Tdr mutant was of the repressor-negative (i^-) type. Thus we have not yet obtained both the expected mutant classes. This could mean either that the model is wrong, or simply that our selection procedure favours the i^- as against the o^c class of mutant, as would be the case if o^c mutants were only partially constitutive or gave a lower resistance than i^- mutants, or occurred at a much lower frequency. Only further selection could settle this question.

A third T^+ determinant, that of RP1, was also tested against Tdr1 and Tdr5 and the combination was constitutive for Tdr1, but was not tested effectively for Tdr5 because of apparent loss of the Tdr mutant from the doubly infected host. We have not tested RP1 in combination with the other Tdr mutants because of uncertainty in interpreting the results with Tdr5. However, the fact that R57Tdr1 and RP1 could be recovered separately from cells infected with the two R-factors, after the growth and challenge test, indicates that Tdr1 was expressed constitutively in the presence of T^+ of RP1. This result could be explained on the basis of the current model of the inducibility system by the hypothesis that the repressor protein coded by T^+ of RP1 is unable to repress the R57 T determinant. This hypothesis is not unlikely in view of the different origins of the two R-factors, since R57 was first found in *Salmonella typhimurium* (Naomi Datta, personal communication) while RP1 originated from *Pseudomonas aeruginosa* (Grinsted *et al.* 1972). R6-S (Foster & Shaw, 1973) and the T-determinant in HfrR4-Tmil (Hoekstra *et al.* 1973) both originated from strains of *Escherichia coli*.

Although each Tdr mutant was repressed for Tc resistance by the T^+ of R6-S, the latter was not completely dominant. In fact (R6-S + R57Tdr) was superior to (R6-S + R57T^+) in the resistance of uninduced cells, while both combinations gave a higher resistance in induced cells than either R6-S or R57T^+ alone. There was also significant variation in the extent to which the constitutive expression of the different Tdr mutants was repressed by a wild-type T. As far as the T^+ determinants are concerned, our results do not agree with those of Foster & Walsh (1975), who could detect no difference in the level of resistance given by one and two T^+ determinants. It is not clear how far this reflects differences in technique, in the host strain used in the tests, or in the use of oxytetracycline by Foster & Walsh and tetracycline in our tests.

The sharp rise in maximum resistance given by the Tdr mutants, estimated in Table 1 to be 2·5× for tetracycline and 5× for minocycline, should be noted. Since chromosomal mutations having only a small effect on their own can also cause a dramatic rise in R-factor mediated resistance (Reeve, 1966), one must expect that coliform bacteria carrying a T-determinant will be well able to protect themselves against the potent new tetracycline antibiotic (minocycline), should it be used on a large scale, by employing one of these two classes of mutation.

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