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Evidence that there are two types of determinant for tetracycline resistance among R-factors

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SUMMARY

A series of derepressed mutants of the tetracycline resistance (T) determinant in R-factor R57 have been found to be repressor-negative and recessive to the T determinant in R6. It is shown that these (Tdr) mutants are dominant to the inducible T determinant in RP1, indicating that the T determinants in R57 and RP1 code for different repressors of the resistance gene. The same Tdr determinants are unstable in cells carrying both the R57 mutant and RP1, probably due to selection against the dominant Tdr gene because it depresses the growth rate of the host cell compared with its T^+ homologue. It is suggested that the T determinants giving high-level resistance in R57, R6 and R100 form one homologous group, probably disseminated by the transposon Tn10, while T determinants giving a much lower level of resistance, such as that in RP1, form a separate group, which may include those in R46, and R199. It is proposed that the gene responsible for tetracycline resistance should be designated *tetA* and the repressor gene *tetI*. The R57 Tdr mutants then have the genotype $tetI^- tetA^+$.

1. INTRODUCTION

R-factor mediated resistance to the tetracyclines in the Enterobacteriaceae is an inducible system depending on at least two genes: a resistance gene coding for the TET protein and a repressor gene whose product represses biosynthesis of the TET protein unless tetracycline, which inactivates the repressor, is present (Yang, Zubay & Levy, 1976). On this model, mutations to constitutive high-level resistance should be of two types, recessive or repressor-negative, and dominant or non-repressible. Reeve & Robertson (1975) selected eleven such derepressed (Tdr) mutants of the T-determinant in R57, and found them to be fully constitutive and to give a much higher level of resistance than could be obtained by inducing the wild-type factor. These mutants all behaved as recessive in the presence of two other determinants, those of R6-S and *Escherichia coli* mil9, indicating that they are of the repressor-negative type; however, tests with RP1 gave anomalous results in that one mutant (Tdr1) was dominant to the inducible T determinant of RP1, while a second (Tdr5) was either recessive to RP1 or was unstable in combination with RP1 so that it could not be tested effectively. In

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view of this apparent instability, the other Tdr mutants were not tested against RP1 at the time.

In this paper we present further evidence that the Tdr mutants of R57 are dominant to the T^+ in RP1, and conclude that the T determinants of the two R-factors differ in repressor specificity, and are, therefore, probably unrelated. The cause of the instability of the Tdr mutants when present in the same cell as RP1 is investigated.

2. MATERIALS AND METHODS

Bacteria: RE13, F^-MetB$^-$; RE15, F^-wild-type; RE26, F^-Pro$^-$Trp$^-$His$^-$Lac$^-$Str^s were described by Robertson & Reeve (1972). RE298 is a spontaneous Nal^r mutant of RE13. JC4046, *Fhis-323/his-504* was obtained from N. S. Willetts. The above strains are all derivatives of *Escherichia coli* K12.

R-factors: R57(STU),* Tdr mutants of R57, R6-S(CT) and RP1(AKT) are as described by Reeve & Robertson (1975). R6-S, a derivative of R6, belongs to the FII incompatibility group, RP1 (also called RP4) to the P group. R57 is *fi*$^-$ and is compatible with R6-S and RP1, but its compatibility group is not known.

Media: L broth, NB (nutrient broth), NA (nutrient agar) and M9 minimal agar were prepared as described previously (Reeve & Robertson, 1975). Amino acids were added at 50 μg/ml, antibiotics except tetracycline at 20 μg/ml and tetracycline at the concentrations stated in the text, as required. All experiments were conducted at 37 °C.

Transfer of R-factors was obtained by mixing donor and recipient cells on L broth agar, incubating overnight and spreading loopfuls on minimal agar, which contained S, C and K, respectively, to select for transfer of R57, R6-S and RP1. The donor strain was counter-selected with nalidixic acid or the different nutrient requirements of the two strains. Purified colonies were tested for antibiotic resistance by streaking on plates containing each antibiotic, or with Oxoid multodisks (30-44K). For double infection with R57 and another R-factor, R57 was transferred first and retained by selection on plates containing S.

Growth and transfer tests were as described by Reeve & Robertson (1975) except that the test cultures were started at time 0 as dilutions of overnight cultures to OD 0·025 (550 nm), the inducing and challenge doses were added at 30 and 45 min, and OD was read at 50, 100, 150, 200 min. The inducing dose was 2 μg/ml and the challenge dose 80 μg/ml of tetracycline except for tests on RP1, for which a challenge dose of 20 μg/ml was used. S, C and K at 20 μg/ml were present as required, to prevent loss of the corresponding R-factors when two were present together.

* *Abbreviations:* A, C, K, S, Su, T are used for the determinants giving resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracyclines, respectively, in an R-factor or other plasmid, and also for these antibiotics when incorporated into media. T^+ and Tdr indicate wild-type and derepressed tetracycline determinants. R^- bacteria are bacteria containing no R-factor.

RESULTS

(i) *Behaviour of R57Tdr and R6-S in the same host cell*

Reeve & Robertson (1975) found that eleven Tdr mutants of R57 were recessive to the T^+ determinant of R6-S: the combination always gave a T^+ phenotype, but in each case the Tdr determinant could be recovered by mating and was found to retain its identity. This shows that the Tdr mutants are all of repressor-negative type, and that the TET gene of R57 is repressed by the product of the R6-S repressor gene.

Table 1. *Character of T determinant transferred from RE26(R57Tdr, R6-S) to JC4046, by conjugation*

	No. of isolates*	
Selection on:	T^+	Tdr
CT	9	0 all S^-
ST	0	10 all C^-
STC	12	9
Donor strains (STC)	9	0

One or more mating tests were made with Tdr1–9. RE26 carrying each Tdr was infected with R6-S, and the host strain was then mated to JC4046. Selection was on minimal plates containing the antibiotics C, T, S as indicated.

* T^+, inducible; Tdr, constitutive resistance.

The combination of R57Tdr and R6-S was quite stable in the host cell, but recombination between these two R-factors, with loss of one T determinant, could easily be obtained by mating the host to a recipient R^- strain and selecting for the simultaneous transfer of both R-factors. RE26 carrying each of the Tdr mutants 1 to 9 and R6-S was mated to the K12 strain JC4046, and selection was made on minimal agar containing CT, ST and STC, to select for transfer of R6-S, R57Tdr, and both factors together. Clones growing on each medium were purified and tested, with the results shown in Table 1. The donor strains were all T^+, as expected, and they transferred R6-S on CT plates and R57Tdr on ST plates, as shown by the T phenotype and the absence, respectively, of the S and C determinants. This shows that the two R-factors were not physically linked. Selection on STC plates, however, gave roughly equal numbers of clones of T^+ and Tdr phenotype (12 and 9). The latter, at least, must be the result of recombination between the two R-factors leading to loss of the dominant T^+ determinant.

Further analysis of these STC clones was not possible, since we did not at the time have a suitable second recipient strain. Instead, RE26(R57Tdr5, R6-S) was mated to RE13, STC recipients were selected and a T^+ and a Tdr clone were chosen for further study. Each was mated to RE26, and recipients were selected on plates containing S or C only, purified and tested for the presence of the three determinants, with the results shown in Table 2. The two parental STC clones of

RE13 were consistently T^+ and Tdr, respectively, in three separate tests. On mating them to RE26 (Table 2), selection for transfer of C or S always led to transfer of both these determinants, and the T determinant was present in all the clones from mating to donor 1, but was missing from about half the clones derived from mating to donor 2. Since both the parental R-factors, R57 and R6-S, are transferred at low efficiency, we can assume that the recipient clones referred to in Table 2 are each the result of a single transfer event. It then follows that the

Table 2. *Recombination between R57Tdr5 and R6-S*

(RE26(R57Tdr, R6-S) was mated to RE13 with selection on STC. Two RE13(STC) clones, T^+ and Tdr, were mated to RE26.)

		Analysis of progeny from RE13(STC) × RE26		
		No. of clones of RE26		
RE13 donor	Selection on	Tested	CS	T^r
1:T^+	C	40	40	40
	S	40	40	40
2:Tdr	C	40	40	17
	S	39	39	21

Selection was on minimal agar containing the nutrient requirements of the recipient strain and the antibiotics indicated by the symbols S, T and C. Progeny clones were purified twice on the selective agar and tested by streaking on antibiotic plates. Su was not tested.

two RE13 donors carry a single recombinant R-factor with an S, a C and a T determinant (the presence of Su from R57 was not tested). The Tdr determinant in donor 2 was lost during transfer in about half the progeny clones, presumably by a second recombinational event which does not lead to loss of C or S. Clearly, selection for transfer of a determinant of each R-factor in one event results generally in transfer of a single recombinant plasmid and not in transfer of the two separate plasmids. This method could be used to transfer a Tdr determinant from one R-factor to another.

(ii) *Behaviour of R57Tdr and RP1 in the same host cell*

These results are relevant to the behaviour of R57Tdr1 in the presence of RP1, and suggested further tests. Four Tdr mutants of R57 were transferred to the K12 strain RE298 (and gave consistently Tdr phenotypes), and these strains were then infected with RP1, and several isolates of RE 298 resulting from the double infection were tested for their T phenotype. For these tests, the two R-factors were retained by selection and purification on plates containing both S and C. As Table 3 shows, half the isolates proved to be Tdr and half were T^+, with some combinations giving both phenotypes in the small sample tested.

Four of these isolates were mated to RE26, and progeny clones growing on KT and ST (or K and S) plates were tested. These matings were made in liquid medium and samples were plated at 60 min intervals after an initial 10 min period. The

Table 3. *Tests on RE298 infected with R57Tdr and RP1*

R-factor combination	No. of isolates	
	Tdr	T+
RP1 + Tdr1	1*	5
RP1 + Tdr2	3	0
RP1 + Tdr3	2	2
RP1 + Tdr5	0	1*
RP1 + Tdr8	2	0

* Data from Reeve & Robertson (1975), the host strain in these cases being RE26.

Table 4. *Mating tests*: *RE298(R57Tdr, RP1) × RE26*

Colonies per plate at times given					Analysis of progeny clones	
					Numbers tested	Characters
Mating 1: Tdr donor from (Tdr2, RP1)						
Colonies	10	70	130	250 min		
On ST	0	1	13	30	4	All Tdr, [SSuT]^r
On KT	15	90	300	—	4	All T+, [AKT]^r
Mating 2: T+ donor from (Tdr1, RP1)						
Colonies	10	70	130	250 min		
On ST	0	2	15	20	4	All T+ (A, K, Su not tested)
On KT	107	67	500	—	4	All T+ (A, S, Su not tested)
Mating 3: T+ donor from (Tdr3, RP1)						
Colonies	10	70	130 min			
On S	0	9	137		40	39 [AKSSuT]^r, 1 [S, Su]^r
On K	400	—	—		40	40 [AKT]^r
Mating 4: T+ donor from (Tdr1, RP1)						
Colonies						
On ST	Numbers not counted				10	All [AKST]^r
On KT	Numbers not counted				10	9 [AKT]^r, 1 [AKTS]^r

Recipient strain was grown overnight in L broth, donor strain was grown overnight in L broth + SK, then diluted to OD_{550} 0·1 in L broth and grown without shaking to OD 0·4. For matings, 0·1 ml donor + 1 ml recipient were added to 10 ml warmed broth in a 100 ml flask at 37 °C. The flask was gently swirled to mix cells, then left at 37 °C without shaking. At times given, 0·1 ml samples were diluted into 1 ml saline, immediately vortexed, chilled, and 0·1 ml spread on 2–3 minimal plates carrying the nutrient requirements of the recipient strain (on which the donor could not grow) and the antibiotics shown. — indicates that there were too many colonies to count on the plates.

Progeny clones from the earliest selection times were purified twice on the selective plates and tested for T phenotype (matings 1 and 2 only), for resistance determinants with multodisks (matings 1 and 3) or by streaking across antibiotic plates (matings 2 and 4). Su was not tested in mating 4.

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results are summarized in Table 4, which gives the transfer frequencies (left-hand side) and the characteristics of the progeny clones analysed (right-hand side). In the first three matings RP1 (selected on K or KT plates) was always transferred much more efficiently than the R57 mutant (selected on S or ST plates). Mating 4 gave qualitatively the same picture. When some of the earliest clones obtained in these matings were purified and tested, the results depended on the T phenotype of the donor. In mating 1 the donor had received R57Tdr2 and RP1 and was Tdr in phenotype. Four clones selected on ST carried only the determinants of R57 (S, Su, T) and were of Tdr phenotype, while four clones selected on KT carried only A, K, and T and were of T^+ phenotype. So clearly the two R-factors retained their identity in the donor and could be recovered unchanged, and Tdr2 was dominant to the T^+ of RP1, thus confirming our previous test with Tdr1.

Table 5. *Mating tests: RE13(R57, RP1) × RE26*

Colonies of RE26 selected on	No. tested	No. with determinants: AKT	SSuT	AKSSuT	Level of T^r
K	45	45	0	0	All low
S	56	0	13	43	All high

Results of five matings of independent RE13(R57, RP1) isolates to RE26. Matings as in Table 5, plated on minimal agar containing K or S only and growth requirements of RE26. Recipient clones were purified on the same medium before testing. T^r level was determined by presence of zone of inhibition round disk of tetracycline: no inhibition shown by RE26(R57), RE26(R57, RP1) donors, and all progeny clones carrying S and Su; 1 mm inhibition zone shown by all AKT progeny clones.

In matings 2–4 the donor strain was T^+ in phenotype, and the R57Tdr factor could not be rescued from the combination of Tdr1 and RP1 (matings 2 and 4), or from that of Tdr3 and RP1 (mating 3). Mating 2 gave only T^+ clones, whether selection was on ST or KT; matings 3 and 4, in which the T-phenotype of the progeny clones was not tested, gave RP1 recovery without any determinants from R57 in 49 of the 50 clones tested (and one clone in which S and possibly Su was also present), but selection on S or ST produced no clones carrying S, Su and T alone. In fact 49 clones selected on S or ST carried A, K, S, Su and T (Su was not tested in 10 of these, but has not been found to separate from S), and one carried S, Su alone.

These results can only be explained by the hypothesis that the Tdr mutants are dominant to T^+ of RP1 but when the two determinants are present in the same cell there is a strong tendency for the Tdr determinant to be lost, either through recombination between the two plasmids or by simple loss of Tdr from R57 to give an S, Su factor. S was used to retain R57 when the two R-factors were put together, so complete loss of R57 could not occur. The results of matings 2–4 given in Table 6 are most easily explained by the hypothesis that the donor strain carried RP1 and a derivative of R57 containing only S and Su, the former being transferred with much higher efficiency than the latter so that nearly all recipients

of S, Su would also have received RP1; but it is possible that some donor cells carried a single recombinant plasmid with A, K, S, Su and T^+ on it, and recombination between the two R-factors may have been responsible for the original loss of Tdr.

Table 6. *Growth rates of RE26 carrying R57T^+ and R57Tdr mutants*

	L broth		Minimal medium	
R-factor	Rate	% of T^+	Rate	% of T^+
T^+	2·825	100	1·068	100
Tdr1	—	86	—	75
Tdr2	—	84	—	74
Tdr3	—	79	—	72

Averages of two growth tests in media containing S at 20 μg/ml to retain R-factor. Growth rate is measured as increment in $\log_e$ OD_{550} per 100 min.

The instability of the combination of RP1 with an R57Tdr factor, compared with the stability of the combination of each R57Tdr with R6-S, remains to be explained. This instability could arise from a property of R57 itself, in relation to RP1, in which case it should occur when R57T^+ is present with RP1, or it could be a consequence of the Tdr mutation. To test the former hypothesis, RP1 was transferred into RE13(R57T^+) and five different isolates were mated to RE26. Clones of RE26 from these matings selected on K and S plates, respectively, were purified and tested, with the results summarized in Table 5. Growth and challenge tests were not made, but it had been discovered that the T determinants of R57 and RP1 could be recognized by multodisk tests using a thin seeding of the test plates, since RP1 consistently gave a small zone of inhibition round the tetracycline disk while R57 and R57 + RP1 gave no such zone.

Table 5 shows that selection on K gave only RP1 transfer, while selection on S gave either R57 transfer alone (13 out of 56 clones), or transfer of both factors (43 A, K, S, Su, T clones showing the higher level of tetracycline resistance characteristic of R57). After 10 min mating, selection on K (for RP1 transfer) gave about 800 times as many transfers as selection on S (for R57 transfer); so it seems probable that the S clones carrying determinants of both factors had resulted from the separate transfer of each. Since the T of R57 was present in these clones, we conclude that the instability of R57Tdr in the presence of RP1 is not a property of R57 itself, but depends on the Tdr mutation.

One possible cause of this instability is that the Tdr mutations reduce the growth rate of their host bacteria because of the constitutive production of the TET protein. In the (Tdr, RP1) combinations, selection on SK would retain determinants of both factors, but cells which had lost Tdr would be at an advantage and would be favoured by selection; on the other hand, the T determinants of R57 would be repressed and so would not affect growth rate in both the (Tdr, R6-S) and (R57T^+, RP1) combinations, which would therefore remain stable. That this is the probable explanation is shown by Table 6, in which the growth

rates are compared for RE26 carrying R57T$^+$ and three Tdr mutants. Tests were made in L broth and in minimal glucose medium, and the three Tdr mutants reduced growth rate consistently by about 17% in the former and 26% in the latter medium. This difference, it may be noted, was not affected by including tetracycline at 2 μg/ml in the medium to induce the T$^+$ determinant. Thus the effect of the Tdr mutants on growth rate is the probable cause of their instability in the presence of RP1. Since the R57Tdr mutants have not shown any tendency to lose the T determinant when present alone in a host cell, it seems probable that recombination with RP1 is a contributing factor in this instability.

DISCUSSION

We have found that one group of T determinants, those in R57, R6 and *E. coli* mil9, mediate high-level resistance to tetracycline and show homology in repressor specificity. In this group we can obviously include R100-1, which shows almost complete DNA homology with R6 (Sharp, Cohen & Davidson, 1973) and high-level resistance (Levy, 1975, who refers to it as 222). The T determinant in R100-1 is contained in a transposon, Tn10 (Cohen, 1976), so it is possible that all the T determinants giving high-level resistance carry this same determinant or Tn10. Among those we can provisionally include in this group are the determinants in R82 and Klebsiella V9A (Robertson & Reeve, 1972).

A second group is represented by RP1 (also called RP4), which gives a much lower but still inducible resistance to tetracycline (Reeve & Robertson, 1975) and codes for a different repressor. It remains to be seen whether other R-factors giving low-level resistance, such as R46 and R199 (Robertson & Reeve, 1972) contain the same determinant as RP1. Clearly we cannot explain the difference between the T determinants of the two types as due simply to a lower level of expression in RP1 or a difference in the number of plasmid copies in the cell.

Selection for joint transfer of two compatible R-factors from the same host bacterium does not appear to have been tested systematically. When the two plasmids R6-S and R57Tdr were in the donor cell, either could be recovered alone by mating, but selection for the simultaneous transfer of both factors allowed isolation of a recombinant plasmid carrying determinants from both R-factors. This result may be of value in obtaining recombinants or transpositions between other pairs of compatible factors, provided that both transfer at low efficiency. The same method would be unsuccessful with R57 and RP1 in the donor strain, since most clones receiving R57 would also be infected with the much more efficiently transferred RP1.

Tdr determinants of R57 were so unstable in the presence of RP1 that it was difficult to prove that the resistance gene of R57 was dominant to (i.e. not repressed by) the T$^+$ of RP1. But this dominance is firmly established by the facts that it has been observed in four different Tdr, RP1 combinations, that when the combination shows the Tdr phenotype both parental T phenotypes can be

recovered, and that the Tdr parental phenotype cannot be recovered when the combination has the T^+ phenotype.

The cause of the instability appears to be selection favouring loss of the Tdr gene from the combination because host cells expressing the Tdr phenotype show a reduced growth rate compared with T^+ cells. The mechanism of this loss is unknown, but it did not involve complete elimination of the R57 plasmid, since its S determinant was retained in the parental cultures by the presence of streptomycin. Recent work on the extensive role of translocating genetic elements in the evolution of R-factors (Cohen & Kopecko, 1976; Kopecko, Brevet & Cohen, 1976) suggests several possibilities. The Tdr determinant could either be lost by excision from R57, or could even be inactivated by insertion of IS3 in the same way as led to the conversion of R6 into R6-5. Alternatively, R57 may carry an SSu transposable element similar to TnS with TnA excised from it. This element could then be transposed into RP1 followed by loss of the remainder of R57. The data in Table 4 suggest that at least two of these processes occurred.

The two genes so far recognized on the T determinant have not yet been given designations. Following the recommendations of Novick *et al.* (1976), they should be named *tet* genes, and we propose that the gene responsible for resistance be named *tetA* and the repressor gene *tetI*. Our Tdr mutants of R57 all have the genotype $tetI^-$, $tetA^+$, and we have found that the *tetI* genes of RP1 and R57 code for proteins which differ at least to the extent that the *tetI* gene of RP1 will not repress *tetA* of R57. It follows that the operator regions of the two *tetA* genes must also differ. Whether the *tetA* genes of the two R-factors code for identical proteins remains to be tested.

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