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Phenotypic characterization of R-factor tetracycline resistance determinants

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SUMMARY

The tetracycline-resistance determinants of R-factors from different compatibility groups have been tested in *Escherichia coli* K12 and phenotypically classified into two major classes. Class I determinants confer high-level resistance to tetracycline (> 100 μg/ml) and moderate resistance to minocycline (5–25 μg/ml) while those of Class II gave moderate resistance to tetracycline (50–70 μg/ml) and low resistance to minocycline. Each class was subdivided because of variation in resistance profiles and in the abilities of tetracycline and minocycline to induce increased resistance. Strains carrying two compatible Tet^r R-factors of the same or different phenotypic groups did not show increased tetracycline resistance.

1. INTRODUCTION

Tetracycline antibiotics are accumulated in *Escherichia coli* by an energy-dependent process (Izaki & Arima, 1963; Franklin & Godfrey, 1965). However, positive evidence that tetracycline binds to a membrane-located permease is lacking, since neither saturation nor competitive inhibition kinetics have been demonstrated (Franklin & Higginson, 1970; Reynard & Nellis, 1972). The presence of an R-factor bearing a tetracycline-resistance determinant prevents the cells from accumulating the antibiotic (Franklin, 1967). High-level resistance in *E. coli* R^+ is induced by exposing the culture to a sub-inhibitory concentration of tetracycline (Franklin, 1967). Further details about the mechanism of resistance are lacking except that osmotic shock transiently reduces resistance, suggesting that the effector is loosely associated with the cytoplasmic membrane or is located in the periplasm (Franklin & Foster, 1971).

Two R-factor-determined tetracycline resistance phenotypes have been identified by tetracycline and minocycline minimum inhibitory concentration determinations (Scavizzi, 1972). Minocycline is a new semi-synthetic tetracycline with greater activity than other tetracyclines against tetracycline-resistant R^+ enteric bacteria (Jarolman, Hewel & Kain, 1970). The TetA phenotype was assigned to factors determining moderate resistance to tetracycline and little resistance to minocycline, while TetB describes high-level tetracycline resistance and moderate minocycline resistance. Robertson & Reeve (1972) confirmed this grouping with

growth and challenge tests in broth cultures, and also found that minocycline did not induce resistance as efficiently as tetracycline.

This paper describes the induced and uninduced tetracycline and minocycline resistance levels conferred by a selection of R-factors from different compatibility classes and examines the resistance levels in bacteria carrying two compatible R-factors.

2. MATERIALS AND METHODS

(*a*) *R-factors.* The R-factors used are listed in Table 1. The bacterial host was *Escherichia coli* K12 J5-3 *pro-1 met-2 nal*^{r}.

(*b*) *Antibiotics.* Oxytetracycline hydrochloride and minocycline were gifts from Lederle Laboratories. The abbreviations Otc and Mc are used for these antibiotics in the text. Other drugs were ampicillin (Beecham), chloramphenicol (Parke Davis), and streptomycin (Glaxo), which were gifts from the manufacturers.

Table 1. *R-factors*

R-factor	Fertility inhibition	Compatibility group	Resistance* determinants	Source
R100–1	–	FII	Tet^r, Chl^r, Str^r, Sul^r	Egawa & Hirota (1962)
R6–S	+	FII	Tet^r, Chl^r	Foster & Shaw (1973)
R64	–	I	Tet^r, Str^r	Lawn *et al.* (1967)
R62	+	I	Tet^r, Str^r, Amp^r	Romero & Meynell (1969)
RP1	–	P	Tet^r, Kan^r, Amp^r	Grinsted *et al.* (1972)
R69	–	7	Tet^r, Amp^r	Chabbert *et al.* (1972)
R245	–	N	Tet^r	Hedges (1972)
R205	–	N	Tet^r, Amp^r, Sul^r	Hedges (1972)
R46	–	N	Tet^r, Amp^r, Str^r, Sul^r	Hedges (1972)
R48	–	N	Tet^r, Amp^r, Str^r, Sul^r,	Hedges (1972)
N3	–	N	Tet^r	Hedges (1972)
R390	–	N	Tet^r, Chl^r, Amp^r, Str^r, Sul^r	Foster & Shaw (1973)
RM2	+	FII	Tet^r, Str^r, Sul^r	E. Moorhouse†
RM4	+	FII	Tet^r, Str^r	E. Moorhouse†
RM16	+	FII	Tet^r, Str^r, Sul^r, Chl^r, Kan^r	E. Moorhouse†
RM22	+	FII	Tet^r, Str^r, Sul^r, Chl^r	E. Moorhouse†
RM34	+	?	Tet^r, Str^r, Sul^r, Amp^r, Chl^r	E. Moorhouse†

* Abbreviations for antibiotic resistance determinants are Chl (chloramphenicol), Str (streptomycin), Sul (sulphonamides), Tet (tetracyclines), Kan (kanamycin) and Amp (ampicillin).

† R-factors labelled RM were transferred from strains isolated by Dr E. Moorhouse (Royal College of Surgeons in Ireland) during a survey of clinical isolates for resistance to minocycline.

(*c*) *Resistance level determinations.* Nutrient agar plates (5 g Lab Lemco, 5 g NaCl, 20 g Difco Bacto agar per l. H_2O, pH 7·5) containing three overlapping series of doubling dilutions of Otc giving 300, 150, 75, 37·5; 250, 125, 62·5, 31·25 and 200, 100, 50, 25, 12·5, 6·25 μg/ml and Mc giving 50, 25, 12·5, 6·25, 3·12, 1·56; 40, 20, 10, 5, 2·5, 1·25, 0·625, and 30, 15, 7·5, 3·75, 1·87 μg/ml were inoculated with 10^4 dilutions of overnight broth cultures using a phage-typing apparatus. Up to 25 samples per plate were tested. There were approximately 10^3–10^4

organisms per inoculum in a 10 mm diameter zone of growth. Both uninduced and induced (grown in 1 μg/ml Otc) cultures were tested. After overnight incubation at 37 °C the highest concentration of antibiotic allowing visible growth was recorded as the resistance level.

(*d*) *Growth and challenge tests.* Resistance profiles in nutrient broth cultures were determined by a method similar to that described by Robertson & Reeve (1972). Starter cultures were grown overnight, either uninduced or induced with Otc (1 μg/ml) or Mc (0·2 μg/ml); 1–2 ml was added to fresh broth (50 ml in 250 ml flasks) containing inducing concentrations where appropriate and incubated at 37 °C in a Gallenkamp orbital shaker at 200 rev/min for 45–60 min to obtain exponentially growing cells. Fifteen minutes prior to challenge, Otc or Mc was added to cultures destined for short-term induction. When $E_{675\,nm}$ was between 0·1 and 0·2 the challenging concentration was added and the turbidity followed for 120 min.

(*e*) *Strains carrying two compatible Tet^r R-factors.* These were constructed by overnight mating followed by streaking on agar plates containing antibiotics selecting for the two plasmids (Table 3). The stability of these strains was tested by overnight growth in antibiotic-free broth before isolating single colonies on antibiotic-free agar which were replica-plated on agar selecting for either of the R-factors. All five strains were stable, and were tested for the presence of both tetracycline-resistance determinants by mating them to another strain of *E. coli* K12, identifiable by suitable genetic markers, and selecting for transfer of each R-factor. In all cases both R-factors could be recovered.

3. RESULTS

(i) *Resistance-level determinations by plating tests*

The tetracycline and minocycline resistance levels of R-factor bearing strains of *E. coli* K12 were measured by colony formation on antibiotic-containing agar (Table 2). Two phenotypic groups of resistance were distinguished. R-factors conferring a high level of resistance to Otc (> 100 μg/ml when induced) and moderate resistance to Mc (5–25 μg/ml when induced) were assigned to group I. Group II contains R-factors which conferred moderate resistance to Otc (< 100 μg/ml when induced) and low-level resistance to Mc (< 5 μg/ml).

R-factors determining high-level resistance to Otc could be separated into two further classes. The first (Ia) contains plasmids with the following resistance profile: Otc resistance (100–150 μg/ml uninduced; 200–230 μg/ml induced) and Mc resistance (12·5 μg/ml uninduced; 15–25 μg/ml induced). R-factors which conferred a lower level of resistance to both Otc and Mc than group Ia factors (Otc 40–80 μg/ml uninduced, 120–200 μg/ml induced; Mc 3–6 μg/ml uninduced and induced) have been assigned to group Ib. This group is heterogeneous since N3 and R245 conferred high level Tet^r when induced whereas the other Ib factors conferred a lower level of resistance (120–150 μg/ml induced, 40–75 μg/ml uninduced).

The second major phenotypic class of Tet^r factors contains plasmids which

determined moderate resistance to Otc (12·5–40 μg/ml uninduced; 50–70 μg/ml induced). One type of R-factor in this group (IIa) conferred a two-fold increase in Mc resistance compared to an R⁻ control, while others (IIb) were completely sensitive to Mc.

Table 2. *Tetracycline resistance phenotypes of R-factors in* Escherichia coli *K12*

Phenotypic group	R-factor	Resistance levels (μg/ml)			
		Oxytetracycline		Minocycline	
		Induced	Uninduced	Induced	Uninduced
Ia	R6–S	213	150	25	20
Ia	R100–1	213	108	22·5	15
Ia	R64	200	112	15	10
Ia	R69	233	125	25	20
Ia	RM4	200	100	25	15
Ia	RM2	200	116	22·5	13
Ia	RM16	200	142	20	12·5
Ia	RM35	213	158	22·5	13
Ib	N3	213	83	5·6	5·6
Ib	R245	200	83	6·2	6·2
Ib	RP1	150	75	4·3	4·3
Ib	RM22	118	41	3·1	3·1
Ib	RM34	118	46	2·9	2·9
IIa	R46	50	18	3·3	3·3
IIa	R48	70	43·5	3·4	3·4
IIa	R205	53	28	2·8	2·8
IIb	R62	75	37·5	0·93	0·93
IIb	R390	50	12·5	1·25	1·25
	R⁻	–	1·0	–	1·78

Resistance levels are the arithmetic means of three independent experiments. Induced and uninduced resistance levels refer to the state of the cultures when inoculated onto the plates. Induced cultures were grown overnight in the presence of Otc (1 μg/ml) for both Min and Otc resistance determinations. It is likely that some induction of uninduced cultures occurs on the surface of the agar allowing colonial growth at higher drug concentrations than those that inhibit growth in broth cultures (Fig 1–4).

(ii) *Growth and challenge tests*

The ability of Otc and Mc to induce R-factor tetracycline resistance was compared in growth and challenge tests in broth (Figs 1–4). When logarithmically growing cultures harbouring group Ia factors were challenged with 100 μg/ml Otc or 7·5 μg/ml Mc, growth of the culture was inhibited, whereas previous exposure to either Otc (1 μg/ml) or Mc (0·2 μg/ml) allowed continuation of exponential growth, albeit at a slower rate than the unchallenged control (Fig. 1). In general, induction for 15 min with either Otc or Mc was less effective than longer (overnight) induction. Also, 15-min induction with Mc was less effective than Otc, whereas longer exposure to Mc was generally as effective as longer exposure to Otc. Thus Mc is less effective in inducing resistance in the short term, and is also able to inhibit growth of both uninduced and induced cultures more

effectively than Otc. This confirms and extends the results of Robertson & Reeve (1972). R-factors with this phenotypic property (group Ia) are fi$^+$ F-like plasmids of incompatibility group FII, except for R64 (incompatibility group I) and R69 (incompatibility group 7) which are fi$^-$.

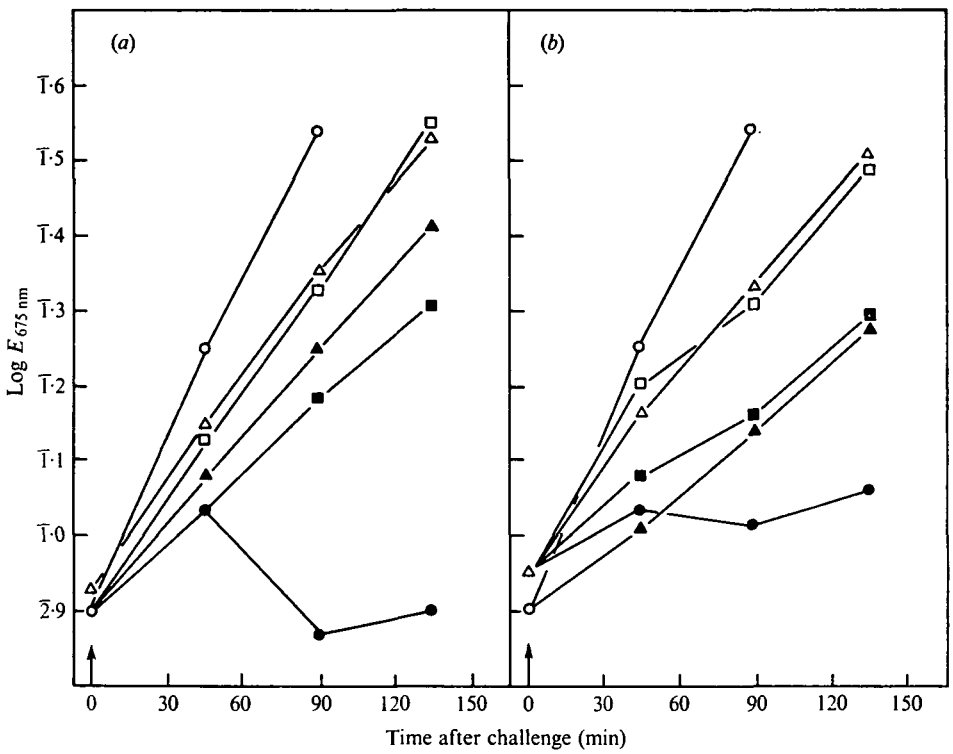

Fig. 1. Growth and challenge tests on *E. coli* J5-3 (R6-S). Cells growing in broth were challenged with (*a*) 100 μg/ml Otc or (*b*) 7·5 μg/ml Mc after no induction, or induction for 15 min or 16 h with 1 μg/ml Otc or 0·2 μg/ml Mc. Symbols in Figs 1–4:

Induction by		Challenge	Symbol
Otc	Mc		
—	—	—	○
—	—	+	●
15 min	—	+	▲
16 h	—	+	△
—	15 min	+	■
—	16 h	+	□

Growth and challenge tests with group Ib factors indicated variation in the ability of Mc to induce resistance (Figs 2, 3). N3, R245 and RM22 were similar to group Ia R-factors except that overnight Mc induction elicited a faster growth rate than Otc when the cells were challenged with either Otc or Mc (Fig. 2). Other group Ib factors differ because 15-min exposure to either Otc or Mc failed to induce resistance (Fig. 3).

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Class II Tet^r R-factors gave similar results to those class Ib plasmids that failed to be induced by Otc or Mc in 15 min prior to challenge by either Otc or Mc (Fig. 4). Longer exposure (60 min or overnight) to either antibiotic resulted in induction of the Otc and Mc resistance of class IIa R-factors, but only the Otc resistance of class IIb R-factors was induced. The latter plasmids were sensitive to Mc in plate resistance-level tests (Table 2). The properties of the Tet^r R factors described above are summarized in Table 3.

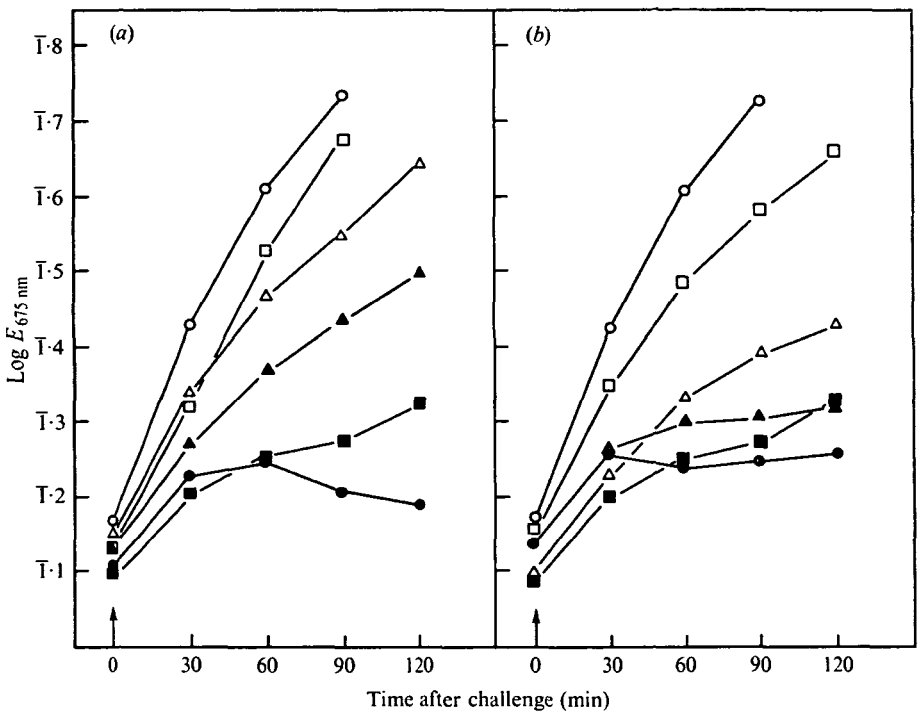

Fig. 2. Growth and challenge tests on *E. coli* J5–3 (R245). Cells growing in broth were challenged with (*a*) 75 μg/ml Otc or (*b*) 2 μg/ml Mc after no induction or induction for 15 min or 16 h with 1 μg/ml Otc or 0·2 μg/ml Mc. Symbols as in Fig. 1.

(iii) *Strains carrying two Tet^r R-factors*

The resistance levels of strains carrying two compatible R-factors are shown in Table 3 and Fig. 5. Both growth and challenge tests and resistance-level determinations demonstrated that Otc and Mc resistance were not increased. In the case of R6-S/RP1 and R6-S/R46 where a class Ia plasmid was present with a class Ib and IIa plasmid respectively, the resistance level was the same as that of R6–S alone. When uninduced the resistance of strains carrying two R-factors was often lower than that of the strain carrying a single class Ia R-factor.

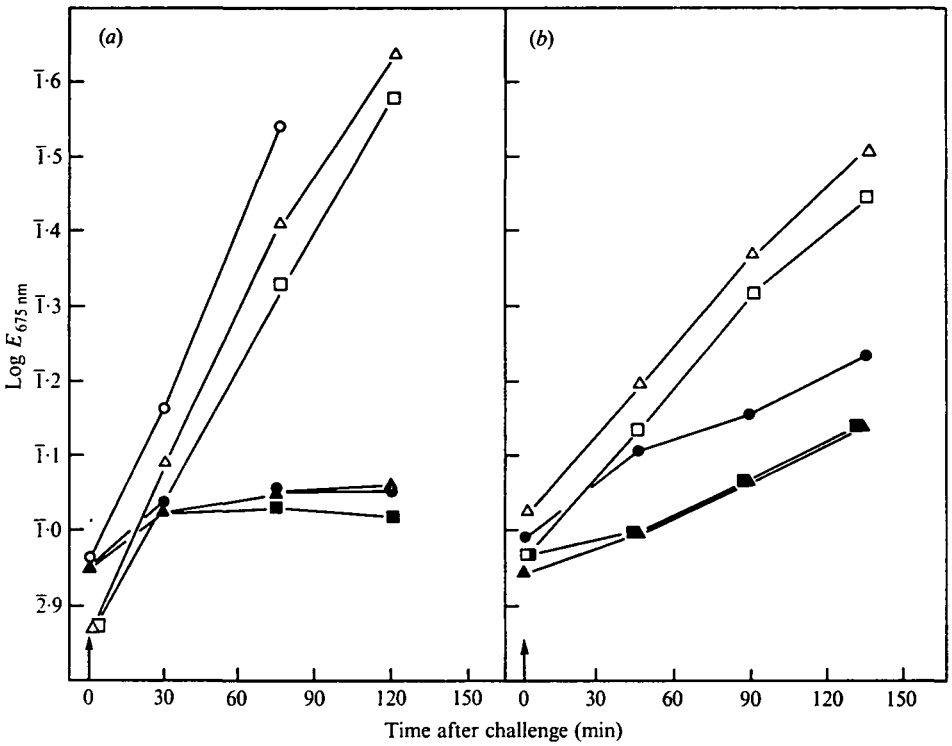

Fig. 3. Growth and challenge tests on *E. coli* J5–3 (RP1). Cells growing in broth were challenged with (*a*) 50 μg/ml Otc or (*b*) 2 μg/ml Mc, after no induction or induction for 15 min or 16 h with 1 μg/ml Otc or 0·2 μg/ml Mc. Symbols as in Fig. 1.

4. DISCUSSION

The classification of 17 R-factor determinants of tetracycline resistance based on the levels of resistance conferred to oxytetracycline and minocycline is described in this paper (the results are summarized in Table 3). Two distinct phenotypic classes were identified, agreeing with previous reports by Scavizzi (1972) and Robertson & Reeve (1972). Subdivision of these two classes was necessary because of variation both in the resistance levels and the abilities of Otc and Mc to induce resistance. Although 15-min exposure to Otc and Mc resulted in induction of class Ia and some class Ib determinants, maximum resistance was obtained only if cultures were induced for longer periods. Also, class II and some class Ib determinants were not induced by short-term exposure to either Otc or Mc. This appears to contradict the results of Robertson & Reeve (1972), who found substantial induction of R46 in 15 min. This discrepancy is most likely explained either by our use of oxytetracycline instead of tetracycline, or by the different bacterial hosts used. Another unexpected result was the marked effect of long-term Mc induction of the class Ib R-factors R245 and N3. Here the growth-rate after challenge with Otc and Mc was consistently greater than induction with Otc overnight or with either drug for 15 min. The possibility that overnight

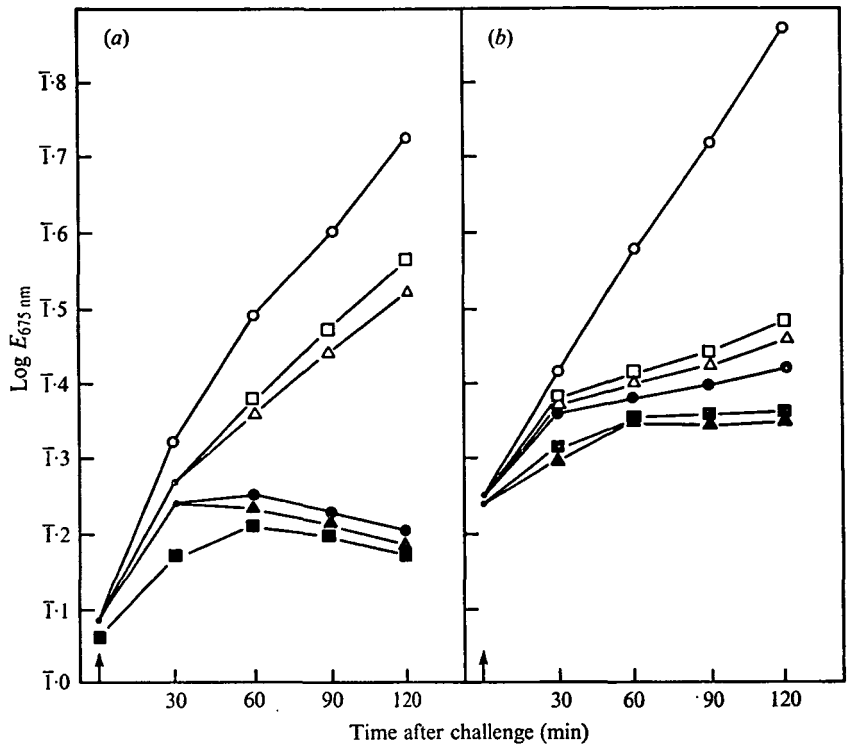

Fig. 4. Growth and challenge tests on *E. coli* J5–3 (R46). Cells growing in broth were challenged with (*a*) 20 μg/ml Otc or (*b*) 2 μg/ml Mc after no induction or induction for 15 min or 16 h with 1 μg/ml Otc or 0·2 μg/ml Mc. Symbols as in Fig. 1.

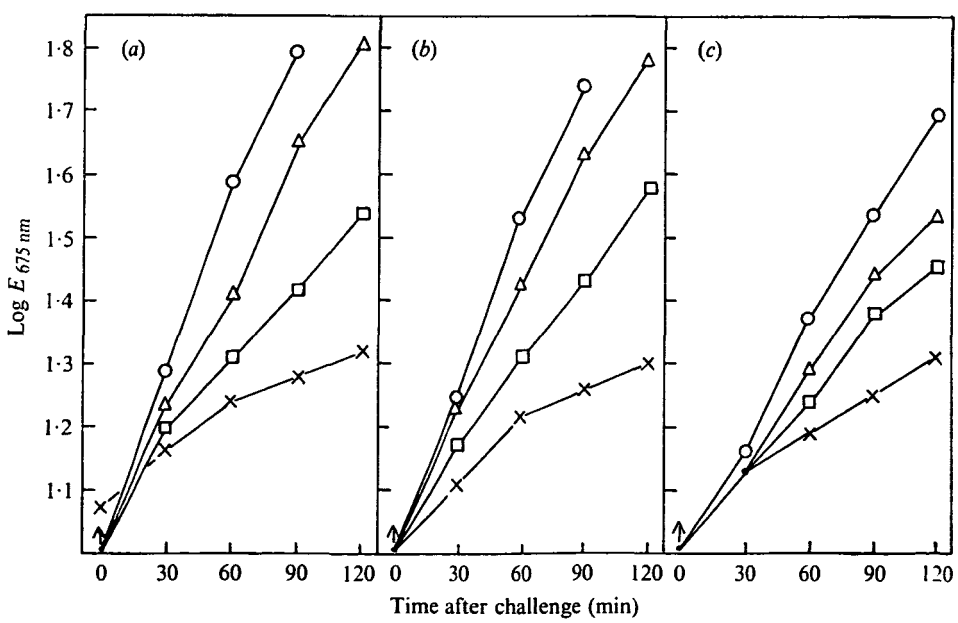

Fig. 5. Growth and challenge tests on *E. coli* J5–3 carrying (*a*) (R6–S), (*b*) (R69) or (*c*) R6–S and R69. Cultures were induced for 16 h with 1 μg/ml Otc and then were challenged with the following concentrations of Otc; 50 μg/ml (△), 100 μg/ml (□), 150 μg/ml (×), unchallenged (○).

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Table 3. *Summary of phenotypic classes of R-factor tetracycline-resistance determinants*

Phenotypic class	R-factors	Resistance levels (μg/ml)* of fully induced cultures		Induction profile
		Otc	Mc	
Ia	R6–S, R100–1 R64, R69, RM2, RM4, RM16, RM35	>200	15–25	Otc better than Mc in short exposures, Mc = Otc in longer exposures.
Ib i	N3, R245	>200	5–6	Mc better than Otc in long exposures.
ii	RP1, RM22†, RM34	118–150	2·9–4·3	Neither Otc nor Mc induced in short exposures, Mc = Otc in longer exposures.
IIa	R46, R48, R205	50–70	2·8–3·4	Otc and Mc induced only after long exposures.
IIb	R62, R390	50–75	0·9–1·25	Otc and Mc induced only after long exposures.

* Induced cultures were grown overnight in Otc (1 μg/ml) for both Otc and Mc resistance-level determinations. † RM22 is an exception in having an induction profile similar to N3 and R245, but conferring lower Otc and Mc resistance.

Table 4. *Oxytetracycline and minocycline resistance levels of strains carrying two R-factors*

R-factors	Resistance level (μg/ml)*				% growth-rate of fully induced† cultures challenged with Otc. Otc (μg/ml)					
	Otc		Mc							
	Uninduced	Induced	Uninduced	Induced	12·5	25	50	100	150	200
R6–S	106	200	7·5	19·7	—	—	84	69	41	14
R69	141	200	8·7	13·3	—	—	85	58	35	—
R64	87	166	8·7	13·3	—	—	—	65	36	—
RP1	33	116	2·5	3·7	—	—	55	20	—	—
RM22	37	108	2·3	3·3	—	—	—	—	—	—
R46	16	41	1·8	2·3	89	34	—	—	—	—
R6–S/R69	50	183	5·4	13·3	—	—	83	50	43	—
R6–S/R64	87	183	9·1	19	—	—	—	66	46	21
R6–S/RP1	54	183	6·2	14	—	—	89	68	43	—
R6–S/R46	61	200	6·6	15	—	—	75	50	21	—
RP1/RM22	40	112	3·3	3·3						

* Each value is the mean of three experiments. Induced cultures were grown overnight in the presence of Otc (1 μg/ml) for both Otc and Mc resistance determinations. Ampicillin (40 μg/ml) and chloramphenicol (20 μg/ml) were used to select for all strains carrying two R-factors except R6–S/R64 where chloramphenicol and streptomycin (10 μg/ml) were employed.

† Fig. 5 shows graphically results of a typical experiment involving R6–S, R69 and R6–S/R69.

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growth in 0·2 μg/ml Mc selected for faster-growing mutants was ruled out by including 60 min inductions in some experiments; these cultures gave the same response to challenge as those induced overnight with Mc. Also, clones purified after overnight growth in 0·2 μg/ml Mc did not inherit this phenotype. The phenotypic heterogeneity in R-factor-borne tetracycline resistance could reflect differences in the control systems or plasmid-specific differences in expression.

Minocycline is a more effective inhibitor of *E. coli* carrying an R-factor than tetracycline. Some plasmids (class Ia), however, confer resistance to 15–25 μg/ml Mc. These were isolated from clinical material before the introduction of the antibiotic. If this level of resistance is enough for the organism to withstand the antibiotic in the body, then its clinical use will be restricted. Furthermore, mutations in the tetracycline-resistance locus can result in a higher level of minocycline resistance (Scavizzi, 1972). In our experience, however, it is difficult to isolate such mutants from class Ia Tet^r R-factors when cultures are plated on agar containing high concentrations of Otc or Mc because a chromosomal mutation is selected which confers a low level of tetracycline or minocycline resistance in an R$^-$ host, but together with a wild-type Tet^r R-factor, an Otc or Mc resistance level higher than that characteristic of the plasmid in a wild-type host. This resistance is often phenotypically constitutive in growth and challenge tests (E. C. R. Reeve, personal communication; T. J. F., unpublished experiments).

When multiple copies of R-factor genes are present in the same cell, a concomitant increase in β-lactamase and chloramphenicol acetyltransferase has been reported (Nordstrom, Ingram & Lundback, 1972). Stable strains carrying two compatible R-factors were constructed and no increase in Tet^r of induced cultures was demonstrated, suggesting that one gene copy of the effector is enough to saturate all the hypothesized sites of action in the cytoplasmic membrane. Moreover, the absence of synergism suggests that class I and class II Tet^r determinants block Otc uptake by similar mechanisms. The uninduced resistance levels of strains carrying two R-factors were lower than those of cells carrying a class Ia R-factor alone, which may indicate that induction on the surface of the plate is reduced in these strains. This phenomenon might account for high resistance levels of uninduced cultures of cells carrying a single R-factor when tested for resistance on agar, and possibly for the variation in experimental results obtained for uninduced cultures by this method. The resistance levels of fully induced cultures were more reproducible.

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