

## R Factor Tetracycline and Chloramphenicol Resistance in *Escherichia coli* K12 *cmlB* Mutants

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### SUMMARY

The isolation of *Escherichia coli* chromosomal mutants that increased the level of resistance of a partially tetracycline-sensitive mutant of R100-1 is described. Plasmid-less derivatives of these moderately resistant mutants were phenotypically similar to the *cmlB* mutants described by Reeve (1966, 1968), and also mapped in the same region. The level of intrinsic resistance to both chloramphenicol and tetracycline was increased about twofold. Also, the levels of R factor-determined resistance to these drugs were increased by this host mutation and tetracycline resistance was expressed constitutively. A *cmlB* mutant accumulated tetracycline at a threefold lower rate than the wild-type strain, and it is proposed that the mutants have an altered permeability to the drugs and that this acts synergistically with the products of the R factor chloramphenicol and tetracycline resistance genes.

### INTRODUCTION

R factors may confer high-level resistance to chloramphenicol in *Escherichia coli* by determining chloramphenicol acetyltransferase, a constitutively synthesized intracellular enzyme that inactivates the drug by 3-*O*-acetylation (Shaw & Brodsky, 1968). R factor-mediated tetracycline resistance, however, is an inducible property which prevents the active accumulation of the drug in the bacterial cell (Franklin, 1967; Robertson & Reeve, 1972).

Colony formation by sensitive strains of *E. coli* is prevented by the incorporation of chloramphenicol or tetracycline into agar at 1 to 2 µg/ml. Single-step mutants which are more resistant to these drugs have been described (Reeve & Bishop, 1965; Reeve, 1968). One such mutant (*cmlB*) also increases the level of R factor-specified resistance to both drugs (Reeve, 1966).

This paper describes the isolation and characterization of *E. coli* chromosomal mutants that increase the tetracycline-resistance level of strains harbouring a partially tetracycline-sensitive mutant of R100-1. They were phenotypically similar to the *cmlB* mutants described by Reeve (1966, 1968) and also mapped in the *cmlB* region of the chromosome. Experiments to elucidate the synergism between the products of the *cmlB* locus and the R factor tetracycline and chloramphenicol resistance genes are described.

Table 1. *Escherichia coli* strains and plasmids

Strain no.	Genotype and plasmid carried	Source
DU1004	<i>pro-1 met-2 rif</i>	A spontaneous <i>Rif-r</i> mutant of J5-3. This paper
DU2500	DU1004 carrying pDU2*	
DU3081	DU1004 carrying R100-1	
DU2503	<i>pro-1 met-2 rif cmlB</i>	This paper
DU2529	DU2503 carrying R100-1	
RE1	<i>proB trp his lacY str</i>	E. C. R. Reeve. Also called J6-2
RE107	<i>proB trp his lacY str cmlB</i>	E. C. R. Reeve. A substrain of J6-2
X'-121	<i>thi tyrA his trp pyrD galK malA mtl xyl str</i>	B. Bachmann

\* pDU2 is a Tet-s mutant of R100-1 which has a single-site lesion in the *tet* locus. The plasmid confers resistance to about 7 µg Tet/ml (see Table 2 and Foster, 1975). R100-1 confers resistance to tetracycline (Tet-r), chloramphenicol (Chl-r), streptomycin (Str-r) and sulphonamides (Sul-r). It was first described by Egawa & Hirota (1962) and was obtained from Dr N. Datta in the host strain J5-3.

#### METHODS

**Media.** The composition of the nutrient broth and agar used in these experiments has been described elsewhere (Foster & Walsh, 1975). Minimal salts agar contained (g/l): K<sub>2</sub>HPO<sub>4</sub>, 7; KH<sub>2</sub>PO<sub>4</sub>, 3; MgSO<sub>4</sub>, 0.2; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1; Difco Bacto agar, 20.

**Antibiotics.** Antibiotics were used routinely at the following concentrations (µg/ml): tetracycline (Tet) and chloramphenicol (Chl), 20; rifampicin (Rif), 40; streptomycin (Str), 200 for chromosomal resistance and 10 for plasmid-determined resistance. Sulphonamide resistance was tested on agar containing 3% (v/v) lysed horse blood and 500 µg sulphathiazole/ml.

Tetracycline, chloramphenicol, rifampicin and streptomycin were gifts from Lederle Laboratories, Parke Davis, Ciba and Glaxo respectively. Sulphathiazole was obtained from Abbott Laboratories.

**Bacterial strains and plasmids.** These are listed in Table 1.

**Plasmid transfer by conjugation.** Equal volumes of exponential broth cultures of the plasmid-bearing donor and a suitably marked recipient were mixed and incubated for 60 min before streaking on media selective for transconjugants. Plasmids R100-1 and pDU2 were transferred to DU1004 and DU2503 by selecting for Chl-r [Rif-r] progeny and to RE1 and RE107 by selecting for Chl-r [His<sup>+</sup> Trp<sup>+</sup>] progeny.

**Transduction.** The procedure for transduction with phage P1<sub>Kc</sub> was that described by Lennox (1955) and was applied to R factor transduction as in Watanabe & Fukasawa (1961).

**Resistance level determinations.** The methods for the determination of resistance to chloramphenicol and tetracycline by colony formation on agar, and by growth and challenge tests in broth, have been described previously (Foster & Walsh, 1975). Drug resistance was also estimated by measuring whole cell protein synthesis as follows: Starter cultures were grown overnight in supplemented minimal salts solution and diluted into fresh medium next day. The induced cultures were grown in the presence of 1 µg Tet/ml. Exponential cultures were harvested and resuspended in fresh growth medium at an E<sub>500</sub> of 0.8, and incubation continued. The challenging dose of Tet (100 µg/ml) was added, followed shortly afterwards by 0.2 µCi L-[<sup>14</sup>C]leucine (The Radiochemical Centre, Amersham, Buckinghamshire)/ml in 20 µg cold L-leucine carrier/ml. Samples (0.2 ml) were withdrawn at intervals and chilled immediately in 0.2 ml ice-cold 10% trichloroacetic acid (TCA).

After at least 30 min in the cold TCA, the samples were filtered on to Whatman glass-fibre (GF/C) filters, washed with two 3 ml volumes of cold 5 % TCA and the filters placed in scintillation vials. After drying, 5 ml of scintillant (PPO, 6 g/l in toluene) was added and the radioactivity estimated in a Packard Tricarb liquid scintillation spectrometer.

*Plasmid curing.* Broth containing 30  $\mu\text{g}$  ethidium bromide (BDH)/ml was inoculated with approximately  $10^8$  cells of the R<sup>+</sup> strain. After overnight incubation, dilutions were plated on drug-free agar for single colonies which were replica-plated on to Chl agar. Those colonies which failed to grow were picked from the master plate, purified and tested for the absence of Str-r and Sul-r, the other pDU<sub>2</sub> resistance determinants.

*Frequency of mutation to increased tetracycline resistance.* Ten different cultures of DU2500 (DU1004 harbouring pDU<sub>2</sub>) were grown overnight in broth, diluted and spread on agar as follows: samples of  $10^{-1}$  and  $10^{-2}$  dilutions were plated on agar containing 20  $\mu\text{g}$  Tet/ml to estimate the total number of Tet-r derivatives. The overnight cultures were also concentrated ten-fold and spread on agar containing 50  $\mu\text{g}$  Tet/ml, a concentration which allowed only the high-level Tet-r revertants of pDU<sub>2</sub> to grow. Resistant colonies of each type were counted and expressed as a fraction of the total viable cells.

*Preparation and assay of chloramphenicol acetyltransferase.* The chloramphenicol acetyltransferase (CAT) from crude culture sonicated supernatants was prepared and measured as follows: Broth (100 ml portions in 250 ml flasks) was inoculated with 5 ml starter cultures and shaken overnight at 37 °C. Cultures were harvested by centrifuging at 10000 g for 10 min and the pellet resuspended in 10 ml ice-cold tris buffer (0.01 M, pH 7.8). All subsequent manipulations were performed at 4 °C. Cell breakage was achieved by sonication using an MSE ultrasonic disintegrator operating at maximum output. Three 60 s periods of sonication with 60 s cooling intervals were used. The cell debris was removed by centrifuging at 35000 g for 45 min. The supernatants were dialysed overnight against 0.01 M-tris pH 7.8 and stored without further purification at -20 °C. CAT was measured by the method of Shaw & Brodsky (1968). Reactions were performed in a Varian Techtron recording spectrophotometer at 37 °C. Specific enzyme activities were calculated as  $\mu\text{mol}$  acetyl coenzyme A utilized/min/mg protein. Protein was assayed by the method of Lowry *et al.* (1951).

*Tetracycline uptake experiments.* Exponential broth cultures were resuspended in fresh broth at an  $E_{500}$  of 0.8. After incubation at 37 °C for 15 min, 0.5  $\mu\text{Ci}$  7- $^3\text{H}$ (N)tetracycline-HCl (The Radiochemical Centre)/ml, in either 10 or 100  $\mu\text{g}$  cold carrier tetracycline/ml, was added and incubation continued. At intervals, 0.5 ml samples were withdrawn and immediately deposited on Whatman glass-fibre filters, washed with two 4 ml volumes of warm broth and placed in scintillation vials. Radioactivity was counted as described above. Control experiments for non-specific binding of  $^3\text{H}$ tetracycline to the filters were performed. This figure (usually less than 1000 c.p.m.) was subtracted from the experimental values.

## RESULTS

### *Tetracycline-resistant derivatives of Escherichia coli harbouring pDU<sub>2</sub>*

pDU<sub>2</sub> is a tetracycline-sensitive (Tet-s) mutant of R100-1 that retains resistance to about 7  $\mu\text{g}$  Tet/ml, threefold greater than the R<sup>-</sup> parent strain. It is presumed to be a missense mutant (Foster, 1975). When  $10^8$  cells of DU2500 were spread on agar containing 20  $\mu\text{g}$  Tet/ml, many small colonies grew after overnight incubation. Six derivatives isolated from independent cultures were purified on Tet agar. Plating tests showed that these strains

inherited resistance to 30  $\mu\text{g}$  Tet/ml. This resistance was about fourfold greater than the original pDU2-bearing strain, but less than would be expected for reversions of the lesion in the R factor *tet* locus. Only when  $10^9$  cells were spread on 50  $\mu\text{g}$  Tet/ml, a concentration which prevented growth of the low-level (30  $\mu\text{g}/\text{ml}$ ) resistant strains, were revertants with high-level resistance isolated. The average number of these two types of Tet-r derivative of pDU2-bearing strains was estimated as outlined in Methods. The partially resistant mutants occurred at a frequency of  $7 \times 10^{-6}$ , while the high-level resistant revertants occurred at  $7 \times 10^{-9}$ .

If the mutations causing increased tetracycline resistance are located on the plasmid, then the Tet-r phenotypes should be co-transducible with other R factor markers by phage  $\text{P}\lambda\text{kc}$ . The Tet-r and Chl-r markers of the high-level resistant derivatives were transduced to DU1004 at similar frequencies. All Chl-r transductants (100/100) from one such experiment were Tet-r, and vice versa. This demonstrated that the mutations restoring high-level resistance were located on the plasmid, and were presumably reversions of the Tet-s mutation carried by pDU2.

The low-level Tet-r phenotype could not be transduced by selection on agar containing 20  $\mu\text{g}$  Tet/ml. In addition, Chl-r R factor transductants were resistant to only 7  $\mu\text{g}$  Tet/ml, the same level as the original pDU2-bearing strain. Thus the mutation causing low-level resistance is not located on the plasmid, and is presumably chromosomal.

To test this further, R<sup>-</sup> derivatives of five independently-isolated low-level Tet-r strains were obtained after growth in ethidium bromide broth (see Methods). The cured strains had a 2- to 3-fold higher level of resistance to both tetracycline and chloramphenicol than the parental R<sup>-</sup> strain DU1004 (Table 2), a phenotypic characteristic of *cmlB* mutants of *E. coli* described by Reeve (1968). One such derivative is DU2503 (Table 1). *cmlB* is closely linked to *pyrD* on the *E. coli* chromosome with a co-transduction frequency of 54% (Reeve & Dougherty, 1968).  $\text{P}\lambda\text{kc}$  was propagated on DU2503, one of the putative *cmlB* mutants of DU1004, and *pyrD*<sup>+</sup> transductions of  $\chi'121$  selected; 57/103 (53%) of such transductants grew when streaked on agar containing 4  $\mu\text{g}$  chloramphenicol/ml, indicating that DU2503 was indeed a mutant in the *cmlB* locus.

*Effects of the chromosomal cmlB mutation on the level of R factor-determined resistance to tetracycline and chloramphenicol*

R100-1 and the Tet-s mutant pDU2 were introduced into RE1, RE107 (*cmlB*), DU1004 and DU2503 (*cmlB*) by conjugation. The plate resistance levels to tetracycline of both induced and uninduced cultures were determined (Table 2). Chloramphenicol resistance determined by R100-1 was also tested. The level of R factor-determined resistance to these drugs was always higher in the *cmlB* hosts, confirming the observation of Reeve (1966). Also, the Tet-r level conferred by R100-1 was higher in *cmlB* strains whether the cultures were induced or not. With wild-type hosts, uninduced cultures always have a lower resistance level in plating tests than those induced by growth in 1  $\mu\text{g}$  Tet/ml (Foster & Walsh, 1975). The level of resistance conferred by pDU2 in the *cmlB* strains was 30  $\mu\text{g}/\text{ml}$ , the same as the original low-level resistant mutants of DU1004 harbouring pDU2. It is interesting to note that R factor Chl-r levels in RE1 and RE107 were lower than in the corresponding J5-3 strains DU1004 and DU2503. This is probably a strain difference, since the R<sup>-</sup> strains RE1 and RE107 are slightly more sensitive to both Tet and Chl than the J5-3 strains (Table 2).

The level of CAT determined by R100-1 in wild-type and *cmlB* strains was compared. Ten independent cultures of DU3081 and DU2529 were grown and the CAT prepared and measured as described in Methods. The mean specific activities of the DU3081 and the

Table 2. Chloramphenicol and tetracycline resistance levels in Escherichia coli strains

Strain	Tetracycline resistance level ( $\mu\text{g/ml}$ )				Chloramphenicol resistance level ( $\mu\text{g/ml}$ )	
	R <sup>-</sup>	pDU2	R100-1		R <sup>-</sup>	R100-1
			Uninduced	Induced*		
RE1	0.8	7	91	200	0.9	200
RE107	1.25	30	250	250	2.5	350
DU1004	1.25	7	100	200	1.25	275
DU2503	3.75	30	250	250	5.0	400

\* Induced cultures were grown overnight in 1  $\mu\text{g}$  tetracycline/ml.

DU2529 (*cmlB*) enzymes were  $9.94 \pm 0.94$  and  $9.77 \pm 0.82$ , respectively. The two values were not significantly different ( $P = 0.1$ ; Student's *t* test) indicating that the increased chloramphenicol resistance of DU2529 cannot be due to an increase in CAT activity. In addition, the R<sup>-</sup> strain DU2503 (*cmlB*) did not contain detectable CAT ( $< 0.007$ ).

*Effects of the chromosomal cmlB mutation on the expression of R factor-determined tetracycline resistance*

The ability of an exponentially-growing culture of wild-type *E. coli* carrying a Tet-r R factor to continue protein synthesis and growth after challenge with a high concentration of tetracycline is dependent on its induction by prior exposure to a low concentration of the drug (see Fig. 1 and Franklin, 1967; Robertson & Reeve, 1972; Foster & Walsh, 1975). In growth and challenge tests with R100-1 bearing derivatives of RE107 and DU2503 (*cmlB* mutants), exponential growth continued after challenge of uninduced cultures. Fig. 1(a) shows one such experiment with DU2529 and DU3081. The rate of growth of the induced culture of DU2529 (*cmlB*) after challenge with 100  $\mu\text{g}$  Tet/ml was similar to the uninduced unchallenged control culture, whereas the uninduced culture continued growth at a slightly faster rate than DU3081 induced. Thus, although Tet-r was seemingly expressed constitutively in *cmlB* strains, an even greater level of resistance was obtained after exposure to 1  $\mu\text{g}$  Tet/ml. Similar results were obtained in experiments with R100-1 in RE1 and RE107 and also derivatives of these strains harbouring N3, RP1, R46 and R62 (unpublished data). In addition, a similar pattern of expression of tetracycline resistance was obtained by directly measuring protein synthesis over a shorter time period, by following the incorporation of [<sup>14</sup>C]leucine into whole cell protein (Fig. 1 b).

*Tetracycline uptake experiments.* The rate of uptake of [<sup>3</sup>H]tetracycline by DU1004 and DU2503 (*cmlB*) was compared as described in Methods. When challenged with 10  $\mu\text{g}$  Tet/ml, exponential broth cultures of DU2503 accumulated the drug at a threefold lower rate than DU1004 (Fig. 2a). This could be explained by the *cmlB* mutant being less permeable to the drug because of an altered envelope structure.

A similar experiment was performed with uninduced and induced cultures of DU2529 and DU3081 challenged with 100  $\mu\text{g}$  Tet/ml (Fig. 2 b). The amount of drug accumulated by these strains was consistent with the level of resistance expressed in growth and challenge tests described previously (Fig. 1). The uninduced culture of DU3081, which is phenotypically sensitive in growth and challenge tests, accumulated 3- to 4-fold more tetracycline than the induced culture of DU2529; DU3081 induced and DU2529 uninduced accumulated intermediate levels of the drug (Fig. 2 b).

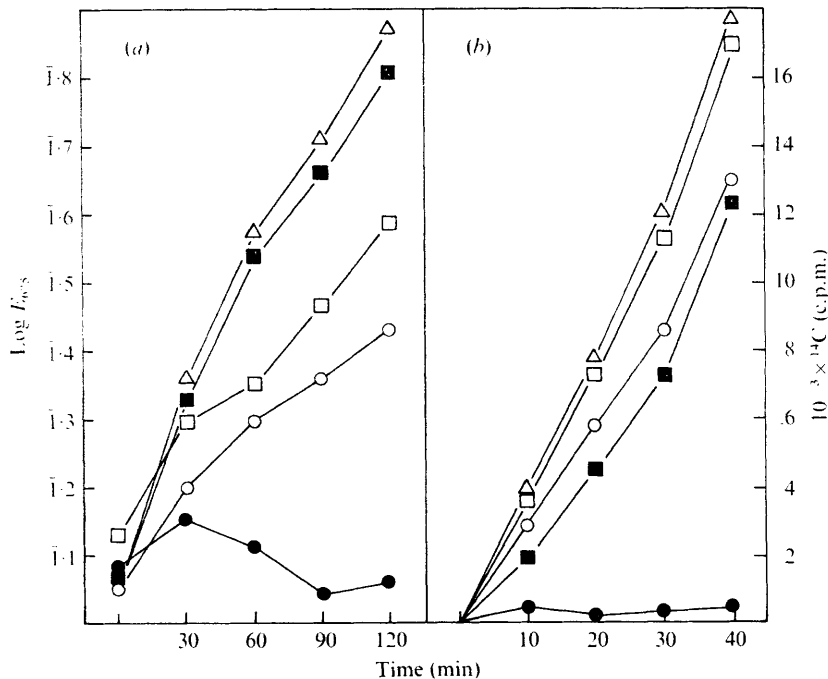


Fig. 1. Growth and challenge tests with wild-type and *cmlB* mutant *E. coli* harbouring R100-1. Exponentially-growing cultures in broth (a) or supplemented minimal salts (b), either uninduced or induced in 1  $\mu\text{g}$  tetracycline/ml, were challenged with 100  $\mu\text{g}$  Tet/ml. In (a), the growth was monitored by measuring the change in  $E_{675}$ . In (b), 0.2  $\mu\text{Ci/ml}$  L-[ $^{14}\text{C}$ ]leucine was added and protein synthesis followed by measuring the radioactivity incorporated into whole cell protein as described in Methods.  $\Delta$ , DU2529 (-, -);  $\blacksquare$ , DU2529 (+, +);  $\square$ , DU2529 (-, +);  $\circ$ , DU3081 (+, +);  $\bullet$ , DU3081 (-, +). The symbols in parentheses indicate whether the culture was induced or challenged, respectively.

#### DISCUSSION

Two types of tetracycline-resistant derivatives of *E. coli* strains harbouring pDU2 (a Tet-s mutant of R100-1) were distinguished during reversion tests. Apart from the expected reversions of the plasmid Tet-s lesion, mutations located in the chromosomal *cmlB* locus, which determined an intermediate level of resistance in pDU2-bearing strains, were isolated. The latter type of mutation occurred at a high frequency and could easily be confused with true revertants unless a higher concentration of tetracycline was used.

The *cmlB* mutation, which was first described by Reeve (1966, 1968), increases by about two-fold the intrinsic resistance of R<sup>-</sup> *E. coli* strains to Tet and Chl. This pleiotropic effect could be explained by the mutants having an altered envelope structure which impairs the uptake of the drugs. In support of this hypothesis, DU2503 (*cmlB*) accumulated [ $^3\text{H}$ ]tetracycline at a threefold lower rate than the wild-type strain DU1004 (Fig. 2a). However, the *cmlB* mutants did not exhibit any phenotypic properties associated with envelope mutants, such as slower growth rate and increased sensitivity to detergents and dyes (unpublished experiments). These negative findings do not rule out the possibility of altered membrane permeability being involved.

The increase in R factor-determined resistance to Tet and Chl in *cmlB* strains could also be explained by this hypothesis. In the case of Chl resistance, a simple synergism between the intracellular CAT and the *cmlB* mutant envelope impermeability is envisaged. The CAT

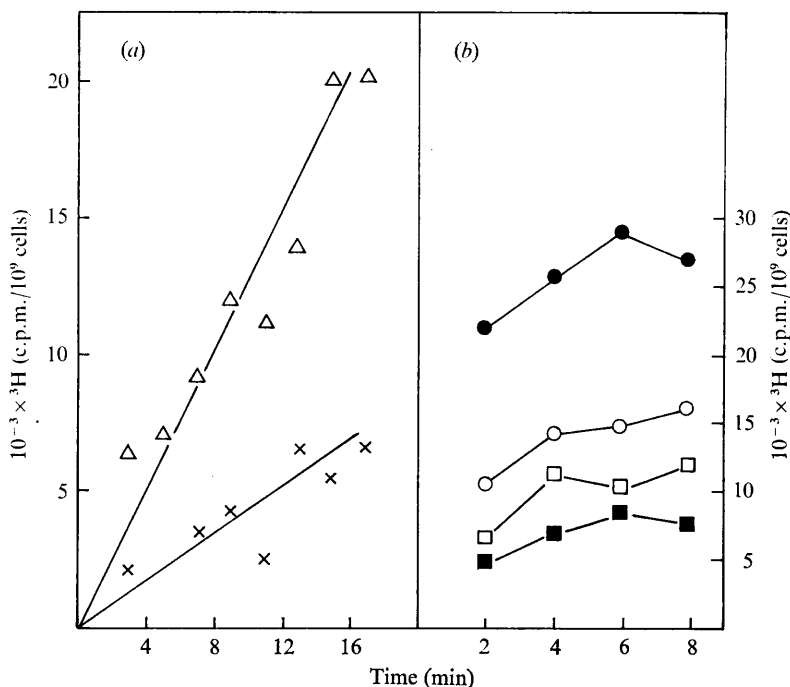


Fig. 2. Uptake of tetracycline by (a) DU1004 and DU2503, and (b) induced and uninduced cultures of DU3081 and DU2529. Exponential broth cultures of DU1004 ( $\Delta$ ) and DU2503 ( $\times$ ) were challenged with tetracycline ( $10 \mu\text{g/ml}$ ) containing  $0.5 \mu\text{Ci}$  [ $^3\text{H}$ ]tetracycline/ml. Samples ( $0.5 \text{ ml}$ ) were withdrawn and cell-bound radioactivity measured as described in Methods. (b) Exponential broth cultures either uninduced or induced by growth in  $1 \mu\text{g}$  tetracycline/ml were challenged with tetracycline ( $100 \mu\text{g/ml}$ ) containing  $0.5 \mu\text{Ci}$  [ $^3\text{H}$ ]tetracycline/ml. Samples ( $0.5 \text{ ml}$ ) were withdrawn and the cell-bound radioactivity measured as described in Methods. For symbols, see Fig. 1.

activities of R100-1 bearing derivatives of DU1004 and DU2503 were indistinguishable, ruling out the possibility of altered CAT levels being involved.

The seemingly constitutive expression of R factor-mediated tetracycline resistance in *cmlB* strains is more difficult to explain. The product of the R factor *tet* gene(s) is a polypeptide of 50000 mol. wt, which is incorporated into the *E. coli* minicell membrane (Levy & McMurray, 1974), where it presumably prevents Tet from being accumulated by blocking the active transport site. The *cmlB* mutation might result in a reduction in the initial uptake of Tet into uninduced  $\text{R}^+$  Tet-r cells challenged with  $100 \mu\text{g}$  of the drug/ml, allowing partial induction of high-level resistance before an inhibitory intracellular concentration can be reached.  $\text{R}^+$  *cmlB* cultures grown in  $1 \mu\text{g}$  Tet/ml exhibit a rate of protein synthesis and growth when challenged which is considerably greater than that of the uninduced culture, and also that of the induced wild-type  $\text{R}^+$  culture. This suggests that the R factor *tet* gene product acts synergistically with the *cmlB* mutant product to produce greater resistance. In this case the interaction occurs at the level of the cell envelope.

The high frequency at which *cmlB* mutants arise in populations of *E. coli* has made it difficult to isolate R factor mutants constitutive for the expression of tetracycline resistance. Most survivors of uninduced  $\text{R}^+$  Tet-r cultures challenged with  $100 \mu\text{g}$  Tet/ml which express Tet-r constitutively are mutant in chromosomal genes (unpublished experiments).

In addition, it is likely that the constitutive mutant described by Franklin & Cook (1971) is of this type.

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