Reduced Expression of Tn10-mediated Tetracycline Resistance in *Escherichia coli* Containing More Than One Copy of the Transposon

By IAN CHOPRA,* STUART W. SHALES, JOHN M. WARD† AND LINDA J. WALLACE†

Department of Bacteriology, University of Bristol, Bristol BS8 1TD, U.K.

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In *Escherichia coli* a single copy of Tn10 confers high-level resistance to tetracycline. Resistance itself results from expression of three distinct mechanisms which normally act together (Shales *et al.*, 1980). In cells containing two copies of Tn10, the level of resistance to tetracycline was reduced. This was not due to overproduction of the repressor which controls the resistance genes, because strains diploid for an operator-constitutive allele of Tn10 also exhibited reduced expression of resistance. The negative gene dosage effect resulted from decreased expression of two mechanisms (1 and 2) consequent on enhanced expression of the third mechanism. The net result of increasing the copy number was a decrease in resistance because mechanism 3 was less efficient than mechanisms 1 and 2 in protecting the cell against tetracycline. The DNA sequence responsible for the reduced expression of resistance was contained in the internal *Bgl*III fragment of Tn10. This sequence, which is probably unique to Tn10, may encode the protein which mediates mechanism 3. Elevated levels of this protein probably cause decreased expression of mechanisms 1 and 2.

INTRODUCTION

Gene amplification of plasmid-located antibiotic resistance determinants frequently leads to increased levels of antibiotic resistance. For instance, an increase in gene dosage leads to greater resistance to ampicillin, chloramphenicol and streptomycin (Nordstrom *et al.*, 1972; Uhlin & Nordstrom, 1975, 1977). There is a similar relationship for several tetracycline resistance determinants. When the plasmid pSC101, present at 5 copies per chromosome, is linked to ColE1 to produce a hybrid plasmid with a copy number of 16 there is a concomitant increase in tetracycline resistance (Cabello *et al.*, 1976). The tetracycline resistance determinant on plasmid pRSD1 is located in a transposon designated Tn1721 (Schmitt *et al.*, 1979). Growth of *Escherichia coli* harbouring pRSD1 in the presence of tetracycline leads to an increase in the copy number of the transposon and increased expression of resistance (Mattes *et al.*, 1979; Schmitt *et al.*, 1979). Cloning of the tetracycline resistance determinant of RP1 (2 copies per chromosome) into a derivative of RSF2124 (30 copies per chromosome) produces a 3-fold increase in the level of resistance to tetracycline (P. M. Bennett, I. Chopra & S. W. Shales, unpublished data).

In some cases, plasmid-determined tetracycline resistance is encoded by a transposon termed Tn10 that is 9300 base pairs long (Jorgensen & Reznikoff, 1979). In contrast to the above findings, Taylor *et al.* (1977) found that multiple copies of Tn10 per cell, obtained by generating a copy number mutant of NR1 (pRR12), led to a decrease in resistance of *E. coli* carrier strains compared with cells harbouring wild-type NR1. Since expression of

† Present address: Department of Biochemistry, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester M60 1QD, U.K.

Tn10-mediated tetracycline resistance is subject to negative regulatory control (Yang *et al.*, 1976), resistance conferred in *E. coli* by pRR12 ($3 \mu\text{g ml}^{-1}$) relative to NR1 ($100 \mu\text{g ml}^{-1}$) might result from a higher level of tetracycline gene repressor owing to a repressor gene dosage effect (see discussion in Jorgensen & Reznikoff, 1979). However, this is an unlikely explanation because expression of resistance coded for by the determinants in pSC101, pRSD1 and RP1, all of which show positive gene dosage effects, is also probably subject to negative regulatory control (Chopra & Howe, 1978; Mattes *et al.*, 1979; Shales *et al.*, 1980; P. M. Bennett, unpublished data).

Whether a tetracycline resistance determinant shows a positive or a negative gene dosage response may be related to the nature of the structural genes it carries. Thus, the resistance region of Tn10, which shows a negative response, is genetically distinct from the resistance determinants in pSC101, pRSD1 and RP1 (Mendez *et al.*, 1980), each of which shows a positive response. The lack of nucleotide sequence homology between the resistance region of Tn10 and the other determinants clearly implies that the products of Tn10 differ from those encoded by the other plasmids. Recent studies on expression of Tn10-mediated resistance (Beck, 1979; Shales *et al.*, 1980) imply that resistance results from the interplay of at least three separate resistance mechanisms each of which may be mediated by separate proteins. To understand fully the negative gene dosage response associated with Tn10 it seems necessary to examine expression of each of the three resistance mechanisms as a function of Tn10 copy number. Unfortunately the products that mediate the three mechanisms have not yet been unequivocally identified and so cannot be quantified. However, it is possible to determine expression of the resistance mechanisms from phenotypic studies (Shales *et al.*, 1980). The phenotypes associated with these mechanisms are as follows (Ball *et al.*, 1980; Shales *et al.*, 1980):

(a) A 10- to 20-fold increase in resistance to tetracycline that is not associated with decreased antibiotic accumulation – mechanism 1.

(b) A 4- to 7-fold increase in resistance to tetracycline that is associated with decreased uptake of the antibiotic – mechanism 2. This mechanism results at least partially from accelerated efflux of the drug from resistant bacteria.

(c) A 2- to 3-fold increase in resistance to both tetracycline and its derivative minocycline, that is not associated with decreased accumulation of either antibiotic – mechanism 3.

With wild-type Tn10 derived from R100-1, mechanisms 2 and 3 are inducible by tetracycline whereas mechanism 1 is expressed constitutively (Shales *et al.*, 1980). We have now examined expression of these phenotypes in strains having different numbers of Tn10 per cell in the hope that we might clarify the basis of the negative gene dosage response exhibited by Tn10.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains and plasmids used are listed in Table 1. Some of the plasmids were specifically constructed for this study and their genealogy is described in Figs 1 and 2. Plasmids were constructed according to the methods of Ward & Grinsted (1978), except that buffers were changed after precipitation of DNA by ethanol instead of being removed by dialysis.

General methods. Determination of concentrations of antibiotic causing a 50% reduction in growth rate (IC_{50}), estimation of covalently closed circular plasmid DNA content and determination of the accumulation of tetracycline by spectrofluorimetry were performed as described previously (Ball *et al.*, 1980; Shales *et al.*, 1980).

RESULTS

Possible role of repressor in reduced expression of Tn10-mediated tetracycline resistance

The availability of mutants of Tn10 which express resistance constitutively (Foster, 1977) provides a means to determine whether the tetracycline gene repressor has a role in reducing resistance in the multicopy state. The plasmids pDU300 and pDU301 are reported to contain

Table 1. *Escherichia coli* K12 strains and plasmids

Strain	Strain description	Plasmid markers*	Reference/source
JC3272	<i>his trp lys strA</i>		Achtman <i>et al.</i> (1971)
DU1200	Transposition of Tn10 from pDU300 to the chromosome of JC3272		Foster (1977)
DU1203	Transposition of Tn10 from pDU301 to the chromosome of JC3272		Foster (1977)
DU3091	Transposition of Tn10 from R100-1 to the chromosome of JC3272		Foster <i>et al.</i> (1975)
UB1528	JC3272(pIP7)	Tc Km Sm Su	Shales <i>et al.</i> (1980)
UB1529	JC3272(pIP69)	Tc Km Cb	Shales <i>et al.</i> (1980)
UB1533	JC3272(R100-1)	Tc Cm Sm Hg	Foster <i>et al.</i> (1975)
UB1553	JC3272(pDU301)	Tc Cm Sm Hg	Shales <i>et al.</i> (1980)
UB5793	DU3091(R100-1)	Tc Cm Sm Hg	This paper
UB6012	JC3272(pUB5554)	Tc Tp Su	This paper
UB6014	JC3272(pUB5555)	Tc Tp	This paper
UB6121	DU1203(pDU301)	Tc Cm Sm Hg	This paper
UB6122	DU1203(pIP7)	Tc Km Sm Su	This paper
UB6123	JC3272(pUB1640)	Tc Tp	This paper
UB6124	JC3272(pUB1641)	Tc Tp	This paper
UB6125	DU1203(pUB1640)	Tc Tp	This paper
UB6126	DU1203(pUB1641)	Tc Tp	This paper
UB6127	DU1203(pUB5556)	Tp	This paper
UB6128	DU1203(pUB5572)	Tp	This paper
UB6129	DU1203(pUB5502)	Tp	This paper

* Resistance respectively to carbenicillin (Cb), chloramphenicol (Cm), kanamycin (Km), mercuric ions (Hg), tetracycline (Tc), trimethoprim (Tp), streptomycin (Sm) and sulphonamide (Su).

operator-constitutive (O^c) mutants of Tn10 (Foster, 1977). We confirmed the constitutive phenotypes conferred by these plasmids since exposure of strain UB1553, which carries pDU301, to sub-inhibitory concentrations of tetracycline failed to increase resistance (Table 2). Furthermore, expression of resistance in a strain (DU1200) in which the constitutive (O^c) derivative of Tn10 had transposed from pDU300 to the chromosome of JC3272 (Foster, 1977) was also constitutive (Table 2). In contrast, both strains UB1533 and DU3091, each of which contains wild-type Tn10, exhibited inducible resistance to tetracycline (Table 2).

Plasmid pDU301 was transferred to strain DU1203 to produce strain UB6121, which carries constitutive alleles of Tn10 in both chromosomal and plasmid locations (Table 1). Its level of resistance to tetracycline was less than that exhibited by cells carrying a determinant in one location only (Table 2). The reduction in resistance cannot be attributed to accumulation of repressor because exposure to a sub-inhibitory level of tetracycline ($0.5 \mu\text{g ml}^{-1}$) did not enhance resistance (Table 2).

Further evidence that repressor is not involved in lowering resistance was provided by experiments in which the plasmid R100-1 was introduced into strain DU3091 to produce strain UB5793, which therefore carried wild-type Tn10 in both chromosomal and plasmid locations. Its level of resistance to tetracycline following exposure to $0.5 \mu\text{g}$ tetracycline ml^{-1} was less than that exhibited by DU3091 which carries the determinant in one location only (Table 2). Prior exposure of strain UB5793 to higher concentrations of tetracycline (1.0 and $5.0 \mu\text{g ml}^{-1}$) failed to increase further its level of resistance to tetracycline (data not shown), which was therefore still lower than that of induced cultures of DU3091. Again, these results showed that the reduction in resistance cannot be attributed to accumulation of functional repressor molecules.

Fig. 1

Fig. 2

Fig. 1. Construction of plasmids pUB5502, pUB5554, pUB5555 and pUB5556. *Step 1*: formation of pUB5502 from R388 as described previously (Ward & Grinsted, 1978). *Step 2*: transposition of Tn10 from the chromosome of DU1200 into the small (1 kb) *Pst*I fragment of R388 to form pUB5554 (R388::Tn10). *Step 3*: ligation of the *Pst*I fragment of pUB5554, containing Tn10, into the single *Pst*I site of pUB5502 to form pUB5555. *Step 4*: deletion in vitro, using *Bgl*III, of DNA between the *Bgl*III sites of Tn10 in pUB5555, resulting in the formation of pUB5556 which has lost the 2.7 kb *Bgl*III fragment of Tn10. Plasmid pUB5554 has a copy number of 3 per chromosome and pUB5555, 8 per chromosome. Abbreviations for restriction endonucleases: B, *Bam*HI; B_{II}, *Bgl*III; E, *Eco*RI; P, *Pst*I. (kb denotes 10³ base pairs.)

Fig. 2. Construction of plasmids pUB5572, pUB1640 and pUB1641. *Step 1*: digestion of R388 with *Bgl*III to form pUB5572. *Step 2*: cloning of the 2.7 kb *Bgl*III fragment from pUB5555 (Fig. 1) into the *Bgl*III site of pUB5572 in both orientations to give pUB1640 and pUB1641. Abbreviations for restriction endonucleases: B, *Bam*HI; B_{II}, *Bgl*III; E, *Eco*RI; P, *Pst*I.

Reduced expression of tetracycline resistance in Tn10 diploids occurs after induction

Tetracycline resistance in uninduced cultures of strain UB5793 (containing 2 copies per chromosome of wild-type Tn10) was the sum of the basal levels expressed in the hosts (UB1533 and DU3091) containing only a single copy of Tn10 (Table 2). We attribute this to a positive gene dosage effect for mechanism 1. Induction of UB1533 and DU3091 led, respectively, to 10- and 5-fold increases in tetracycline resistance (Table 2). Similar treatment of UB5793 led only to a 2-fold increase (Table 2). Therefore, the mechanism which reduced resistance when 2 copies of Tn10 were present was inducible by tetracycline.

Table 2. Expression of tetracycline resistance in strains of *E. coli* K12 containing wild-type and constitutive derivatives of Tn10

Strain	Tetracycline resistance genes†		Mean no. of Tn10 copies per chromosome‡	Resistance level (IC ₅₀) to tetracycline (µg ml ⁻¹)§	
	Plasmid	Chromosomal		Growth without Tc	Growth with Tc
JC3272*	—	—	—	0.29 ± 0.02	—
UB1553	pDU301 (O ^c)	—	1	61.0 ± 1.0	63.0 ± 0.9
DU1200	—	From pDU300 (O ^c)	1	71.5 ± 3.5	73.0 ± 2.0
UB6121	pDU301 (O ^c)	From pDU301 (O ^c)	2	51.8 ± 0.8	53.0 ± 1.4
UB1533	R100-1 (WT)	—	1	3.05 ± 0.05	30.8 ± 0.3
DU3091	—	From R100-1 (WT)	1	11.8 ± 0.4	58.0 ± 8.5
UB5793	R100-1 (WT)	From R100-1 (WT)	2	15.3 ± 1.3	31.0 ± 0.5

* JC3272 is the tetracycline-sensitive, plasmid-free, parent of all other strains.

† WT and O^c refer to wild-type (WT) Tn10, in which expression of resistance is inducible, and operator-constitutive (O^c) Tn10 mutants.

‡ Plasmid copy numbers determined as described by Shales *et al.* (1980).

§ Mean values ± 1 standard deviation.

|| Strains pre-grown for at least 10 generations in the presence of 0.5 µg tetracycline ml⁻¹.

Nature of the product mediating reduced expression in strains carrying more than one copy of Tn10

Reduced expression of resistance in the Tn10 diploid UB5793 occurred after induction thereby implicating a product of the Tn10 structural genes in the effect. The response could therefore be mediated either by the product conferring mechanism 2, or that necessary for mechanism 3, because both mechanisms of resistance are inducible in Tn10 derived from wild-type R100-1 (Shales *et al.*, 1980).

The plasmid pIP7 was used to investigate the possible role of a mechanism 2 product in the negative gene dosage effect of Tn10. Plasmid pIP7 (with a copy number of 2 per chromosome) mediates resistance to tetracycline but not minocycline (Table 3), and induction of resistance leads only to decreased accumulation of tetracycline (Shales *et al.*, 1980). The pIP7-encoded product that confers decreased accumulation is functionally, but probably not structurally, analogous to that determined by Tn10 (Ball *et al.*, 1980; Mendez *et al.*, 1980; Shales *et al.*, 1980). Inducible products of pIP7 did not reduce the expression of Tn10-mediated resistance to tetracycline or minocycline (Table 3), thereby implying that the pIP7 product mediating mechanism 2 did not decrease the expression of Tn10-determined resistance.

Because the reduction in resistance in the Tn10 diploids was probably caused by the synthesis of the product required for joint resistance to tetracycline and minocycline (i.e. mechanism 3), we attempted to produce a Tn10 derivative in which the structural gene for the joint resistance mechanism was deleted, but which still encoded a functional mechanism 2. Jorgensen & Reznikoff (1979) concluded that the structural gene for a 36 kilodalton (36K) envelope protein (probably mediating mechanism 2) resides mainly within an internal 2025 base pair (bp) fragment of Tn10 produced by cleavage with *Hpa*I (see Fig. 4). The Tn10 fragment generated by digestion with *Bgl*II should therefore contain the structural gene for the 36K protein (Jorgensen & Reznikoff, 1979), but might lack the gene required for expression of mechanism 3. Plasmids pUB1640 and pUB1641 were therefore constructed which contain the internal *Bgl*II fragment of Tn10 cloned in opposite orientations (Fig. 2).

Both plasmids conferred decreased accumulation of tetracycline (Table 4) consistent with a role for the 36K protein in mechanism 2. However, the recombinant plasmids were not deleted for mechanism 3 because they encoded minocycline resistance (Table 4). The extent to which mechanism 2 was expressed by these plasmids was inversely related to the level of

Table 3. Expression of tetracycline and minocycline resistance in strains of *E. coli* K12 carrying plasmid pIP7

Strain	Tetracycline resistance genes†		Mean no. of copies per chromosome		Resistance level (IC ₅₀) to tetracycline (µg ml ⁻¹)§		Resistance level (IC ₅₀) to minocycline (µg ml ⁻¹)§	
	Plasmid	Chromosomal	pIP7‡	Tn10	Growth without Tc	Growth with Tc	Growth without Tc	Growth with Tc
JC3272*	—	—	—	—	0.29 ± 0.02	—	0.42 ± 0.08	—
UB1528	pIP7 (WT)	—	2	—	3.1 ± 0.1	21.0 ± 2.0	0.37 ± 0.04	0.45 ± 0.01
DU1203	—	From pDU301 (O ^c)	—	1	54.0 ± 0.0	ND	2.87 ± 0.06	ND
UB6122	pIP7 (WT)	From pDU301 (O ^c)	2	1	48.0 ± 0.5	46.8 ± 0.8	2.13 ± 0.13	2.13 ± 0.13

ND, Not determined.

* JC3272 is the tetracycline-sensitive, plasmid-free, parent of all other strains.

† WT and O^c refer to the wild-type (WT) tetracycline resistance determinant of pIP7, in which expression of resistance is inducible, and the operator-constitutive (O^c) Tn10 mutant found in pDU301.‡ See Shales *et al.* (1980).

§ Mean values ± 1 standard deviation.

|| Strains pre-grown for at least 10 generations in the presence of 0.5 µg tetracycline ml⁻¹.Table 4. Expression of tetracycline (Tc) and minocycline (Mn) resistance in strains of *E. coli* K12: actual and predicted resistance levels

Strain	Plasmid	Tetracycline accumulated (arbitrary fluorescence units)†	Expression of mechanism 2‡	Actual resistance levels (IC ₅₀) to Tc and Mn (µg ml ⁻¹)†		Expression of mechanism 3§	Predicted resistance level (IC ₅₀) to Tc (µg ml ⁻¹) assuming expression of mechanisms 2, 3		Calculated expression of mechanism 1¶
				Tc	Mn				
JC3272*	—	70 ± 10	—	0.29 ± 0.02	0.42 ± 0.08	—	—	—	—
UB6012	pUB5554	13.5 ± 0.5	5.2-fold	54.7 ± 1.4	1.58 ± 0.18	3.8-fold	5.7	9.6-fold	9.6-fold
UB6123	pUB1640	1.8 ± 0.3	38.9-fold	33.5 ± 2.5	1.05 ± 0.05	2.5-fold	28.3	1-fold	1-fold
UB6124	pUB1641	3.0 ± 0.4	23.3-fold	22.5 ± 2.5	1.56 ± 0.02	3.7-fold	24.7	1-fold	1-fold

* JC3272 is the tetracycline sensitive, plasmid-free, parent of the other strains.

† Mean values ± 1 standard deviation: determinations performed on strains not previously exposed to tetracycline.

‡ From fluorescence data in column 3; also see text.

§ From minocycline resistance levels in column 6; also see text.

|| Based on the IC₅₀ of tetracycline for JC3272 (0.29 µg ml⁻¹) and the values for expression of mechanisms 2 and 3 (columns 4 and 7); also see text.

¶ Calculated after allowing for resistance contributed by mechanisms 2 and 3; see text.

Fig. 3. Relationship between the predicted expression of the Tn10-encoded resistance mechanisms 1 and 2, and the level of minocycline resistance (mechanism 3). Strains containing Tn10 or genes derived from Tn10 were examined for expression of three proposed resistance mechanisms (see text and Shales *et al.*, 1980). Expression of mechanism 2 was determined from fluorescence data and mechanism 3 from the level of resistance (IC_{50}) to minocycline. Any residual resistance to tetracycline after accounting for mechanisms 2 and 3 was attributed to expression of mechanism 1 (see text and Table 4).

Key to strains: (1) UB6012, (2) DU1200, (3) UB6123, (4) UB6014, (5) DU3091, (6) UB1553, (7) UB6127, (8) DU1203, (9) UB6128, (10) UB6124, (11) UB5793, (12) UB6129, (13) UB6125, (14) UB1529, (15) UB1533, (16) UB6126.

resistance to minocycline (Table 4). Thus, the ratio of minocycline resistance levels for UB6124:UB6123 was 1.5 and the ratio of tetracycline accumulation for UB6124:UB6123 was 1.7. The data therefore support the notion that the product involved in resistance to minocycline (mechanism 3) can affect the expression of mechanism 2.

Expression of mechanisms 1 and 2 is inversely related to that of mechanism 3

Data presented in the previous sections imply that elevated levels of the mechanism 3 product conferring joint resistance to tetracycline and minocycline can lead to reduced expression of mechanism 2. However, some simple calculations (see below) predicted that expression of mechanism 1 might also be affected by the mechanism 3 product.

If expression of both mechanisms 1 and 2 is inversely related to that of mechanism 3 we would predict a negative correlation between expression of mechanisms 1 and 2 together and mechanism 3. To test this possible relationship, additional data were collected for strains containing Tn10 and were combined with results already presented. In each case expression of mechanisms 1, 2 and 3 was determined. Expression of mechanism 3 was obtained directly from the IC_{50} data for minocycline. Expression of mechanism 2 was calculated using data from fluorimeter traces. For example, expression of mechanism 2 in UB6012 was estimated as follows: strain UB6012 accumulated 13.5 units of tetracycline whereas strain JC3272 (the plasmid-free host) accumulated 70 units, and so expression of mechanism 2 in UB6012 was $70/13.5$, or 5.2-fold (Table 4). If mechanism 2 only was expressed it would result in cells with an IC_{50} of $1.5 \mu\text{g tetracycline ml}^{-1}$ [i.e. $5.2 \times (IC_{50} \text{ of tetracycline for JC3272}) = 5.2 \times 0.29 \mu\text{g ml}^{-1}$]. Expression of mechanism 1 was calculated from the total level of resistance to tetracycline after accounting for expression of mechanisms 2 and 3. For strain UB6012, for example, mechanism 2 contributed a 5.2-fold increase in resistance and mechanism 3 a 3.8-fold increase [i.e. $(IC_{50} \text{ of minocycline for UB6012})/(IC_{50} \text{ of minocycline for JC3272}) =$

1.58/0.42], and so we predict that these mechanisms alone would produce cells with an IC_{50} of $5.7 \mu\text{g tetracycline ml}^{-1}$ [i.e. $5.2 \times 3.8 \times (IC_{50} \text{ of tetracycline for JC3272}) = 5.2 \times 3.8 \times 0.29 \mu\text{g ml}^{-1}$] (Table 4). Since the actual IC_{50} of tetracycline for UB6012 was $54.7 \mu\text{g ml}^{-1}$ (Table 4), we predict that expression of mechanism 1 in UB6012 contributed a 9.6-fold increase in resistance to tetracycline (i.e. $54.7/5.7$). Calculated values for expression of mechanism 1 in other strains varied between 10-fold and 1-fold (i.e. not expressed). Strains UB6123 and UB6124 are examples of bacteria which did not appear to express mechanism 1 (Table 4).

In Fig. 3, data for a variety of strains containing Tn10 are plotted as the theoretical resistance levels conferred by mechanisms 1 and 2 alone versus the logarithm of the minocycline IC_{50} (i.e. resistance conferred by mechanism 3). Two parallel lines were obtained which imply a negative correlation between expression of mechanisms 1 and 2 together and mechanism 3. One group of strains (dashed line) was more sensitive to minocycline. The basis of this difference between the two groups is not known.

DISCUSSION

While the work described here was in progress, Jorgensen & Reznikoff (1979) provided data from which it can also be concluded that the repressor is not involved in reducing the level of resistance in strains containing more than one copy of Tn10. The repressor is also unlikely to be involved because the ratio between its content and the number of operator regions with which it interacts should not change with alterations in Tn10 copy number.

As noted in the Introduction, Tn10-mediated resistance results from the interplay of at least three separate resistance mechanisms, each of which may be mediated by separate Tn10-encoded proteins. Mechanism 2, involving energy-dependent efflux of tetracycline, is likely to be mediated by a 36K protein located in the cytoplasmic membrane (Chopra *et al.*, 1981), whereas mechanism 3 may involve protection of ribosomes by a 15K protein (Levy *et al.*, 1977; Levy & McMurry, 1978). The nature of the product mediating mechanism 1 is unknown. From the data presented here we conclude that increased expression of resistance to minocycline (mechanism 3) leads to decreased expression of the other mechanisms (mechanisms 1 and 2). Although enhanced expression of mechanism 3 should lead *per se* to an increase in resistance to tetracycline, the overall level of resistance falls because mechanisms 1 and 2, which are more efficient than mechanism 3, are suppressed.

If the Tn10 protein which mediates minocycline resistance caused lowered resistance in the multicopy state, then those tetracycline resistance determinants which show a positive gene dosage effect should not encode resistance to minocycline. This is true for pSC101, RP1 (Mendez *et al.*, 1980; Shales *et al.*, 1980) and pRSD1 (I. Chopra, unpublished data). Extrapolation of the continuous line in Fig. 3 to the point where the theoretical expression of mechanism 3 is 1-fold [i.e. $\log_{10}(\text{minocycline } IC_{50}) = -0.38$] gives an IC_{50} value of $29 \mu\text{g tetracycline ml}^{-1}$ for the combined expression of mechanisms 1 and 2. This corresponds closely to the levels of tetracycline resistance mediated by the determinants in pIP7 and RP1, neither of which confers minocycline resistance (Shales *et al.*, 1980).

Decreased expression of mechanisms 1 and 2 when Tn10 is present in several copies might be the consequence of reduced transcription of the respective resistance genes. This model therefore suggests that, in addition to its role in mediating resistance, the mechanism 3 product may bind to DNA and regulate transcription. As yet, there is no evidence to support this contention.

Data presented here, in conjunction with reports by Jorgensen *et al.* (1978), Jorgensen & Reznikoff (1979) and Beck (1979), permit conclusions to be drawn on the organization of genes within the resistance region of Tn10 (Fig. 4). The gene for the 36K protein (probably mediating mechanism 2) resides within the *HincII* fragment of 1275 bp and the structural gene for the repressor lies to the left of this within the *HincII* fragment of 695 bp (Jorgensen &

Fig. 4. Transcriptional and restriction site map of the resistance region of Tn10. The vertical lines above the linear map show some of the restriction endonuclease cleavage sites located in Tn10: the lengths of various regions are given in base pairs. Below the linear map the regions which probably code for the 36K and 15K resistance proteins are indicated, together with their directions of transcription. Horizontal lines designate the general location of RNA polymerase binding sites. The model is compiled from data presented in this paper and by Jorgensen *et al.* (1978), Jorgensen *et al.* (1979), Jorgensen & Reznikoff (1979), Beck (1979) and Shales *et al.* (1980). Abbreviations for restriction endonucleases: B, *Bam*HI; B_{II}, *Bgl*II; H_{II}, *Hinc*II; H_PI, *Hpa*I.

Reznikoff, 1979). Plasmids pUB1640 and pUB1641 contain both these fragments and additional sequences at either end. The gene conferring joint resistance to tetracycline and minocycline (mechanism 3) and probably coding for the 15K protein (see above) may not be transcribed from a promoter within the *Bgl*II fragment because the expression of minocycline resistance conferred by plasmids pUB1640 and pUB1641 is affected by the orientation of the *Bgl*II fragment. In plasmids pUB1640 and pUB1641 transcription of the 15K protein may therefore be mediated by R388 promoters that are weaker than the normal Tn10 promoter. The structural gene for the 15K protein may therefore reside to the right of the *Hinc*II fragment of 1275 bp and its promoter may be located within or to the right of the *Bgl*II cut site on the right of the transposon. Jorgensen & Reznikoff (1979) concluded that the sequence coding for the carboxy-terminus of the 36K protein probably lies within 100 bp of the *Hinc*II/*Hpa*I site that defines the right hand side of the 2025 bp fragment (Fig. 4). The gene encoding minocycline resistance may therefore be located in a 540 bp sequence, lying within the 640 bp region defined by the *Hinc*II/*Hpa*I and *Bgl*II cut sites. Assuming that the average molecular mass of an amino acid is 115, then a 15K protein will require a coding sequence of about 400 bp (Chopra & Howe, 1978), a length consistent with the size of the region thought to contain the structural gene. Jorgensen & Reznikoff (1979) concluded that the product effecting the multicopy response is coded by a region of Tn10 lying to the right of the 640 bp region defined by the *Hinc*II/*Hpa*I and *Bgl*II cut sites shown in Fig. 4. We believe that this region contains the promoter for the 15K protein rather than the structural gene. However, the data of Jorgensen & Reznikoff (1979) is still consistent with our model because deletions to the right of the 640 bp region in their recombinant plasmids (unrelated to R388) could have eliminated the promoter and abolished synthesis of the 15K protein. Our contention that the region to the right of the 640 bp fragment contains a promoter is also consistent with data showing an RNA polymerase binding site close to the insertion sequence at the right hand side of Tn10 (see Fig. 4).

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