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Multicopy Tn*10* *tet* Plasmids Confer Sensitivity to Induction of *tet* Gene Expression

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We inserted the Tn*10* tetracycline resistance determinant (*tet*) into the multicopy plasmid pACYC177, and we examined the phenotype of *Escherichia coli* K-12 strains harboring these plasmids. In agreement with others, we find that Tn*10* *tet* exhibits a negative gene dosage effect. Strains carrying multicopy Tn*10* *tet* plasmids are 4- to 12-fold less resistant to tetracycline than are strains with a single copy of Tn*10* in the bacterial chromosome. In addition, we find that multicopy *tet* strains are 30- to 100-fold less resistant to the tetracycline derivative 5a,6-anhydrotetracycline than are single-copy *tet* strains. Multicopy *tet* strains are, in fact, 10- to 25-fold more sensitive to anhydrotetracycline than are strains that lack *tet* altogether. The hypersensitivity of multi-copy strains to anhydrotetracycline is correlated with the effectiveness of anhydrotetracycline as an inducer of *tet* gene expression, rather than its effectiveness as an inhibitor of protein synthesis. Anhydrotetracycline is 50- to 100-fold more effective than tetracycline as an inducer of tetracycline resistance and as an inducer of β-galactosidase in strains that harbor *tet-lac* gene fusions. In contrast, anhydrotetracycline appears to be two- to fourfold less effective than tetracycline as an inhibitor of protein synthesis. Both anhydrotetracycline and tetracycline induce synthesis of *tet* polypeptides in minicells harboring multicopy *tet* plasmids. Differences between *E. coli* K-12 backgrounds influence the tetracycline and anhydrotetracycline sensitivity of multicopy strains; $ZnCl_2$ enhances the tetracycline and anhydrotetracycline sensitivity of these strains two- to threefold. We propose that the overexpression of one or more Tn*10* *tet* gene products inhibits the growth of multicopy *tet* strains and accounts for their relative sensitivity to inducers of *tet* gene expression.

The transposable element Tn*10* determines high-level tetracycline resistance in *Escherichia coli* and other enteric bacteria (12, 17). The expression of resistance is regulated, in that exposure of resistant bacteria to subinhibitory levels of tetracycline increases the level of resistance (14). Although there is general agreement that resistance involves reduced accumulation of tetracycline (13, 22, 28), the mechanism of resistance is only partially understood. McMurry et al. (24) and Ball et al. (2) have reported that active efflux of tetracycline is a component of the resistance mechanism. Additional components have also been implicated (21, 22, 28).

The region of Tn*10* involved in the expression of tetracycline resistance lies within a central 2,790-base-pair *Bgl*II restriction fragment (9, 16). This region directs the synthesis of two tetracycline-inducible proteins; a 36-kilodalton (kd) protein that is associated with the cytoplasmic membrane and appears to be essential for resistance (16, 20, 22, 33) and a 25-kd repressor protein that, in the absence of tetracycline, functions to inhibit its own synthesis as well as synthesis of the 36-kd resistance protein (3, 32). The structural genes for these proteins, designated *tetA* (36 kd) and *tetR* (25 kd), have been localized within the physical map of Tn*10* (3, 9, 16, 32); *tetA* and *tetR* are transcribed in opposite directions from overlapping divergent promoters (3, 3a, 32).

In contrast to most plasmid-encoded antibiotic resistance determinants, the Tn*10* tetracycline resistance determinant appears to exhibit a negative gene dosage effect (7, 9, 30). Strains that carry the Tn*10* *tet* genes on multicopy plasmids are often significantly less resistant to tetracycline than are strains that carry the *tet* genes in a low copy state. Taylor et al. (30) described copy mutants of NR1 (also called R222 or R100) that increase plasmid copy number 3- to 6-fold and confer 15- to 30-fold lower tetracycline resistance than NR1 in *E. coli* K-12. More recently,

Coleman and Foster (9) characterized pBR322-Tn*10* *tet* derivatives that confer 4- to 18-fold lower tetracycline resistance than chromosomal Tn*10* in *E. coli* K-12. There is not, however, a consensus as to the magnitude of this effect. Jorgensen and Reznikoff (16) reported a less than twofold reduction in tetracycline resistance with derivatives of *E. coli* K-12 C600 carrying multicopy pVH51-Tn*10* *tet* plasmids, and several of the pVH51 *tet* plasmids described by these authors confer high-level tetracycline resistance comparable to that of single-copy Tn*10* strains. Interestingly, the negative gene dosage effect observed with Tn*10* is not characteristic of other tetracycline resistance determinants (5, 31).

We find that, in addition to conferring reduced levels of tetracycline resistance, multicopy Tn*10* *tet* plasmids confer hypersensitivity to some tetracycline derivatives. Concentrations of 5a,6-anhydrotetracycline or heated chlortetracycline that have little, if any, effect on the growth of tetracycline-sensitive bacteria significantly inhibit the growth of tetracycline-resistant bacteria containing multicopy *tet* plasmids. It has been suggested that the reduced tetracycline resistance of multicopy Tn*10* *tet* strains is due to reduced synthesis of *tet* gene products (7, 9, 30). Our studies, which have focused on the hypersensitivity of multicopy strains to anhydrotetracycline, indicate that hypersensitivity to anhydrotetracycline and heated chlortetracycline is related to the action of these tetracycline derivatives as inducers of *tet* gene expression. This finding leads us to conclude that the reduced tetracycline resistance conferred by multicopy *tet* plasmids is a consequence of induction of the *tet* genes and does not reflect increased inhibition of protein synthesis by tetracycline due to decreased expression of the *tet* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The *E. coli* K-12 strains, plasmids, and phages used in this study are described in Table 1. pACYC177 (6) is a small (3.67-kilobase) multicopy plasmid that confers resistance to both ampicillin and neomycin (or kanamycin). pBT1010 (Fig. 1) was constructed by transposition of Tn*10* from λ NK370 into pACYC177. pBT107 (Fig. 1) was constructed in vitro by ligation of the Tn*10* *Bgl*II 2,790-base-pair fragment from λ::Tn*10*$_1$ (15) into the *Bam*HI site of pACYC177. λ *tetA-lacZ43* carries a transcriptional fusion of the Tn*10* *tetA* promoter to the *lacZ* gene. This phage was constructed by inserting the *Taq* I 158-base-pair fragment that spans the *tetA* promoter-operator (3a, 32) into the promoter expression vector λ RS205 (K. Bertrand, L. Wray, and W. Reznikoff, manuscript in preparation). The expression of *lacZ* in B2550 (λ *tetA-lacZ43*) lysogens is regulated by the Tn*10* *tetR* repressor and tetracycline.

Media. LB broth contains (per liter) 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 10 g of NaCl, and 5 g of yeast extract (Difco). TYE agar contains (per liter) 10 g of tryptone, 8 g of NaCl, 5 g of yeast extract, and 15 g of agar (Difco). All media contained 100 μg of ampicillin (Polycillin N; Bristol Laboratories, Syracuse, N.Y.) per ml to assure plasmid retention. Tetracycline hydrochloride was purchased from Sigma Chemical Co., St. Louis, Mo. 5a,6-Anhydrotetracycline (lot no. 4967-261-3) was kindly provided by N. Belcher, Pfizer Inc., Central Research, Groton, Conn. Nonaqueous stocks of tetracycline (20 mg/ml in methanol) and anhydrotetracycline (0.5 mg/ml in ethanol) were stored at −20°C. $ZnCl_2$ solutions were sterilized by filtration and added to autoclaved TYE agar as indicated. All cultures were incubated at 37°C, except *tetA-lacZ* fusion strains, which were incubated at 32°C.

Determination of antibiotic resistance. Minimum inhibitory concentrations (MICs) of tetracycline and anhydrotetracycline were determined by the agar dilution method (11). Saturated LB broth cultures were diluted 1:25 in LB broth, grown until the optical density at 550 nm reached 0.8 to 1.0, diluted 1:100, and

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Genetic properties or derivation	Source or reference
E. coli K-12		
B2550	F′ *proAB* Δ*lacS20*/W3110 Δ*trpEA2 tna2* Δ*lacS20* Nal^r	K. Bertrand and W. Reznikoff
JA221	F$^-$ *lacY1 leuB6 thi-1 tonA2 supE44* Δ*trpE5 recA1 hsr*	Clarke and Carbon (8)
P678-54	F$^-$ *lacY1 leuB6 thi-1 tonA2 supE44 thr-1 minA1 minB2 ara-13 gal-6 malA1 xyl-7 mtl-2 azi-8 rpsl135* λ^r	Adler et al. (1) via B. Bachmann
B2550::Tn*10*	Transduction of B2550 to Tc^r by λNK370	This work
JA221::Tn*10*	Transduction of JA221 to Tc^r by λNK370	This work
Plasmids		
pACYC177	Ap^r Nm^r	Chang and Cohen (7)
pBT1010	Ap^r Nm^r Tc^r	K. Postle
pBT107	Ap^r Nm^r Tc^r	K. Bertrand
Phages		
λNK370	λ*b*221 cI857 cII171::Tn*10* *Ouga261*	N. Kleckner via R. Simons
λ *tetA-lacZ43*	*lacZ*$^+$ *bet gam* cI857 *nin5* *tetA-lacZ* transcriptional fusion	K. Bertrand and W. Reznikoff

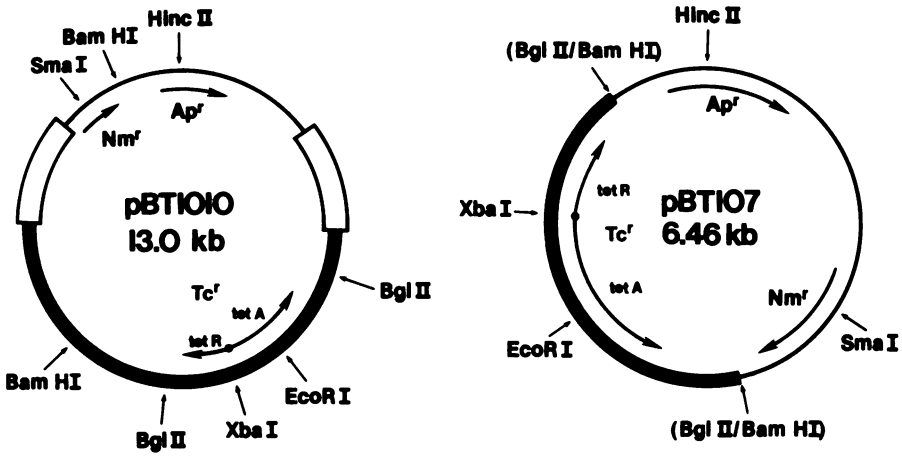

FIG. 1. Structures of the plasmids pBT1010 and pBT107. Arrows indicate the approximate extent and direction of transcription of the regions that encode resistance to ampicillin (Ap^r), neomycin (Nm^r), and tetracycline (Tc^r). Tn*10* sequences are shown as thick lines, except the IS*10* sequences of pBT1010, which are shown as open boxes (15).

spotted (0.003 ml) on TYE agar plates containing various concentrations of tetracycline or anhydrotetracycline. The LB broth and TYE agar contained 100 μg of ampicillin per ml; omission of ampicillin from the TYE agar had no effect on the MICs for tetracycline. Plates were incubated for 18 to 20 h at 37°C, and the least amount of drug that caused inhibition of confluent growth was taken as the MIC. A slight haze or a few individual colonies was ignored. The exposure of cultures to inducing levels of tetracycline before plating appeared to have little effect on the MICs determined by this method.

The low solubility of anhydrotetracycline (0.5 mg/ml in ethanol) poses a problem for MIC determinations with relatively resistant strains. Significant amounts of ethanol were added to the medium to obtain high anhydrotetracycline concentrations; however, the ethanol added to plates containing up to 22 μg of anhydrotetracycline per ml did not, by itself, inhibit growth.

β-Galactosidase assays. *tetA-lacZ* fusion strains were incubated at 32°C in TYE broth containing 100 μg of ampicillin per ml. Single colonies were inoculated into 5 ml of medium and incubated overnight. These cultures were diluted 1:50 into fresh medium and incubated until the optical density at 550 nm reached 0.3 to 0.6. Cultures to be induced contained the indicated amounts of tetracycline or anhydrotetracycline during both the pregrowth and subculturing steps. β-Galactosidase activities were determined as described by Miller (25).

Minicell analysis. Plasmids pBT1010 and pBT107 were introduced into the minicell-producing strain P678-54 (1), and minicells were prepared essentially as described by Roozen et al. (27). Washed minicells were suspended in labeling medium consisting of 25% (vol/vol) methionine assay medium (Difco) in M9 minimal salts (25) and then incubated at 37°C for 30 min. Cycloserine (Sigma) was added to 200 μg/ml, and the minicells were incubated for an additional 30 min. For labeling, [^{35}S]methionine (600 to 1,400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to 10 to 20 μCi/ml, and the minicell suspension was incubated for 15 min. Samples to be induced received either 1 μg of tetracycline per ml or 1 μg of anhydrotetracycline per ml immediately before the addition of the [^{35}S]methionine. Labeled minicells were washed in M9 minimal salts and stored at −20°C. Samples for electrophoresis were prepared as described by Lane (19); the minicell pellets were suspended in sample buffer consisting of 60 mM Tris-hydrochloride (pH 8.8), 10% glycerol, 2% sodium dodecyl sulfate, 2 mM disodium EDTA, 5 mM dithiothreitol, and 0.075% phenol red, incubated for 1 h at room temperature, made 12.5 mM in iodoacetamide, and incubated for an additional 1 h at room temperature. Approximately 100,000 cpm of radioactivity was loaded into each well of a 12.5% polyacrylamide-sodium dodecyl sulfate slab gel (18). The gels were impregnated with Autofluor (National Diagnostics), dried under vacuum, and exposed to Kodak XR-5 film for 1 to 3 days. The molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were as follows: lysozyme (14.4 kd), soybean trypsin inhibitor (21.5 kd), carbonic anhydrase (31.0 kd), ovalbumin (45.0 kd), bovine serum albumin (66.2 kd), and phosphorylase B (92.5 kd).

RESULTS

Inhibition by tetracycline and anhydrotetracycline. Tn*10 tet* was introduced into two different *E. coli* K-12 strains by transposition from a λ::Tn*10* phage into the bacterial chromosome or by transformation with pBT1010 or pBT107 plasmid DNA; MICs of tetracycline and anhydrotetracycline were determined (Table 2). A single chromosomal copy of Tn*10 tet* confers relatively high level tetracycline resistance in JA221 (160 μg/ml), whereas the multicopy *tet* plasmids pBT1010 and pBT107 confer four- to fivefold lower levels of tetracycline resistance in this strain. The negative gene dosage effect is

TABLE 2. Resistance of JA221 and B2550 derivatives to tetracycline and anhydrotetracycline

Strain	Plasmid	MIC (μg/ml)			
		$-ZnCl_2$		$+ZnCl_2$	
		Tetracycline	Anhydrotetracycline	Tetracycline	Anhydrotetracycline
JA221	pACYC177	2.5	10	3.0	8.0
JA221::Tn*10*	pACYC177	160	18	120	14
JA221	pBT1010	40	1.0	16	0.4
JA221	pBT107	30	1.0	12	0.4
B2550	pACYC177	2.0	4.0	2.5	4.0
B2550::Tn*10*	pACYC177	120	6.0	100	5.0
B2550	pBT1010	10	0.16	4.0	0.05
B2550	pBT107	10	0.16	4.0	0.05

more pronounced in the B2550 background, where pBT1010 and pBT107 confer 12-fold lower levels of tetracycline resistance than a single copy of Tn*10* in the chromosome.

Anhydrotetracycline is about fourfold less effective than tetracycline as an inhibitor of JA221(pACYC177). We assume that this difference reflects the relative efficacies of tetracycline and anhydrotetracycline as inhibitors of protein synthesis, although differences in the extent to which JA221(pACYC177) accumulates tetracycline, as compared with anhydrotetracycline, could also effect the MICs. The overall pattern of anhydrotetracycline resistance differs from the pattern of tetracycline resistance in two ways. First, single-copy Tn*10* confers only marginal resistance to anhydrotetracycline. Second, strains harboring multicopy *tet* plasmids are not only less resistant to anhydrotetracycline than single-copy Tn*10* strains, they are less resistant to anhydrotetracycline than are strains that lack Tn*10* *tet* altogether. In the JA221 background, pBT1010 and pBT107 confer 18-fold lower levels of anhydrotetracycline resistance than single-copy Tn*10*, and 10-fold lower anhydrotetracycline resistance than pACYC177. As with tetracycline resistance, the difference is even greater in the B2550 background, where pBT1010 and pBT107 confer 40-fold lower anhydrotetracycline resistance than single-copy Tn*10* and 25-fold lower anhydrotetracycline resistance than pACYC177. Thus multicopy *tet* strains are hypersensitive to anhydrotetracycline and, as with the reduced tetracycline resistance of these strains, the anhydrotetracycline hypersensitivity is significantly influenced by differences between *E. coli* K-12 backgrounds.

Effect of $ZnCl_2$. A survey of the effects of divalent metals revealed that the resistance of multicopy *tet* strains to tetracycline and anhydrotetracycline is further reduced by $ZnCl_2$ (Table 2). Whereas 0.6 mM $ZnCl_2$ has only a modest effect on the resistance of either JA221(pACYC177) or JA221::Tn*10*(pACYC177) to tetracycline and anhydrotetracycline, it reduces the resistance of JA221(pBT1010) and JA221(pBT107) to tetracycline and anhydrotetracycline by two- to threefold. $ZnCl_2$ has a comparable effect on B2550 derivatives.

Induction of tetracycline resistance. Maximal expression of the Tn*10* tetracycline resistance phenotype is induced by exposure to subinhibitory tetracycline. Anhydrotetracycline is about 100-fold more effective than tetracycline as an inducer of tetracycline resistance in single-copy Tn*10* strains (Fig. 2).

Induction of β-galactosidase in *tet-lac* fusion strains. The effectiveness of tetracycline and anhydrotetracycline as inducers can also be demonstrated at the level of *tet* gene expression.

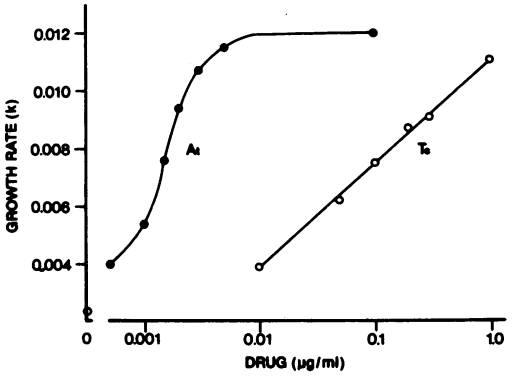

FIG. 2. Comparison of tetracycline and anhydrotetracycline as inducers of tetracycline resistance. B2550::Tn*10*(pACYC177) was inoculated into LB broth containing the indicated amounts of tetracycline (○) or anhydrotetracycline (●). Cultures were incubated overnight, diluted 1:50 into the same media, and, after log phase growth had resumed, challenged by the addition of 50 μg of tetracycline per ml. Absorbance at 550 nm (A) was monitored at 30-min intervals over a period of 120 min. The specific growth rate (K) was calculated using the equation $K = (\ln A_t - \ln A_{t_0})/(t - t_0)$ in which t is expressed in minutes.

Levels of β-galactosidase activity in strains carrying *tetA-lacZ* gene fusions provide an indirect measure of transcription initiated at the *tetA* promoter. Since λ *tetA-lacZ43* does not carry the *tetR* repressor gene, B2550(λ *tetA-lacZ43*) lysogens synthesize high levels of β-galactosidase; the presence of functional *tetR* on a plasmid or on a second lysogenic phage results in a 15- to 100-fold repression of β-galactosidase activity, depending on the source of the *tet* repressor. The ability of tetracycline and anhydrotetracycline to induce β-galactosidase synthesis was determined in a single-copy *tet* strain, B2550(λ *tetA-lacZ43*)(λ::Tn*10*$_1$)(pACYC177) (Fig. 3a), and in a multicopy *tet* strain, B2550(λ *tetA-lacZ43*)(pBT107) (Fig. 3b). Anhydrotetracycline is 50- to 100-fold more effective than tetracycline as an inducer in both strains; however, half-maximal induction in the multicopy strain requires approximately 10-fold higher levels of each inducer. Whereas it is possible to approach maximal induction in the single-copy strain, growth of the multicopy strain is significantly inhibited by concentrations of inducers that result in greater than 40 to 50% maximal induction of β-galactosidase levels.

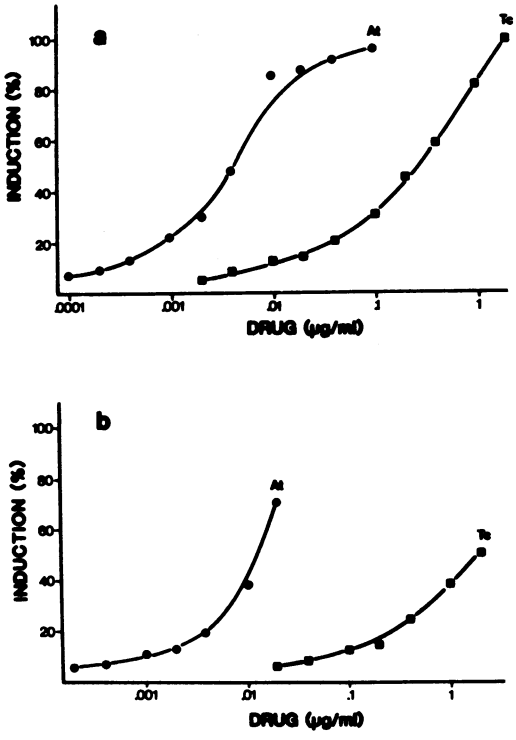

FIG. 3. Comparison of tetracycline and anhydrotetracycline as inducers of β-galactosidase in *tetA-lacZ* fusion strains. (a) B2550(λ *tetA-lacZ43*)(λ::Tn*10*$_1$)(pACYC177) was inoculated into LB broth containing the indicated amounts of tetracycline (■) and anhydrotetracycline (●). Cultures were incubated overnight, subcultured, and, after several generations of log phase growth, assayed for β-galactosidase activity. In the absence of inducers, 265 U of β-galactosidase was synthesized; in the absence of *tetR* repressor B2550(λ *tetA-lacZ43*)(pACYC177) 5,550 U of β-galactosidase was synthesized. (b) B2550(λ *tetA-lacZ43*)(pBT107) was treated as in (a). In the absence of inducers, 115 U of β-galactosidase was synthesized.

Induction of *tet* proteins in minicells. The ability of the multicopy plasmids pBT1010 and pBT107 to direct the synthesis of *tet* proteins in minicells was examined. Two *tet*-specific proteins can be detected in minicells containing these plasmids, the *tetA* gene product, which has an apparent molecular mass of 36 kd (16, 20), and the *tetR* gene product, which has an apparent molecular mass of 25 kd (3, 33). Both tetracycline and anhydrotetracycline induce significant synthesis of the 36-kd protein and, to a lesser extent, the 25-kd protein in minicells containing either pBT1010 or pBT107 (Fig. 4). The minicell strain P678-54 exhibits a relatively severe negative gene dosage effect (data not shown); the concentration of anhydrotetracycline used here (1 μg/ml) is sufficient to com-

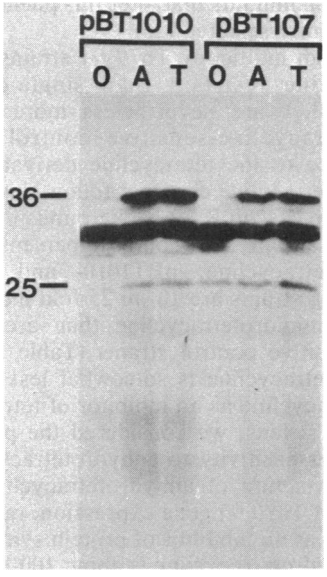

FIG. 4. Induction of *tet* protein synthesis in minicells. Minicells harboring either pBT1010 or pBT107 were labeled with [^{35}S]methionine in the absence of anhydrotetracycline or tetracycline (0) or in the presence of either 1 μg of anhydrotetracycline per ml (A) or 1 μg of tetracycline per ml (T). The figure shows a fluorogram of labeled proteins fractionated on a 12.5% acrylamide-sodium dodecyl sulfate gel. The positions of the 36-kd *tetA* protein and the 25-kd *tetR* protein are indicated.

pletely inhibit the growth of P678-54 when it contains either pBT1010 or pBT107.

DISCUSSION

We find, in agreement with others (7, 9, 30), that the Tn*10* tetracycline resistance determinant shows a negative gene dosage effect. In the two *E. coli* K-12 backgrounds examined, the multicopy Tn*10 tet* plasmids pBT1010 and pBT107 confer 4- to 12-fold lower levels of tetracycline resistance than a chromosomal copy of Tn*10* (Table 2). The near identity of pBT107 and pBT1010, in this regard, strongly suggests that the region of Tn*10* responsible for the negative gene dosage effect is within the central 2,790-bp *Bgl*II fragment that encodes the *tetA* and *tetR* genes (3, 9, 16, 32). Whereas pBT107 and pBT1010 show a significant negative gene dosage effect for tetracycline resistance, other pACYC177-Tn*10 tet* plasmids that we have examined do not (data not shown). Differences between the tetracycline resistance phenotypes of multicopy Tn*10 tet* strains, as reported in the literature (7, 9, 10, 16, 30, 32), undoubtedly reflect several factors, including differences in plasmid copy number, the extent of the Tn*10 tet* DNA present, the influence of vector promoters on *tet* expression, strain differences, and, perhaps most importantly, the ease with which mutants that alter this phenotype are inadvertently selected (26).

Although multicopy Tn*10 tet* strains are less tetracycline resistant than single-copy *tet* strains, they are nevertheless more resistant than tetracycline-sensitive control strains. Resistance to the tetracycline derivative 5a,6-anhydrotetracyline does not follow this pattern. In the two *E. coli* K-12 backgrounds examined, pBT1010 and pBT107 confer hypersensitivity to anhydrotetracycline; pBT1010- and pBT107-containing strains are 10- to 25-fold more sensitive to anhydrotetracycline than are tetracycline-sensitive control strains (Table 2). Since anhydrotetracycline is somewhat less effective than tetracycline as an inhibitor of tetracycline-sensitive strains, we considered the possibility that hypersensitivity to anhydrotetracycline reflects the action of anhydrotetracycline as an inducer of Tn*10 tet* gene expression, rather than its action as an inhibitor of protein synthesis. In fact, anhydrotetracycline is about 100-fold more effective than tetracycline as an inducer of tetracycline resistance in single-copy Tn*10 tet* strains (Fig. 2), and it is 50- to 100-fold more effective than tetracycline as an inducer of β-galactosidase synthesis in both single-copy and multicopy *tet* strains carrying *tetA-lacZ* gene fusions (Fig. 3). We interpret the latter result to mean that anhydrotetracycline is about 50- to 100-fold more effective than tetracycline as an inducer of transcription initiation at the *tetA* promoter. In contrast to what we have observed for Tn*10 tet* in *E. coli* K-12, anhydrotetracycline is apparently no more effective than tetracycline as an inducer of tetracycline resistance in *Staphylococcus aureus* (29).

We also find that multicopy Tn*10 tet* strains are hypersensitive to heated chlortetracycline, although the effect is less pronounced for heated chlortetracycline than for anhydrotetracycline (26). Heated chlortetracycline is an ingredient of media that favor the growth of tetracycline-sensitive derivatives of Tn*10*-containing *Salmonella typhimurium* and *E. coli* strains (4, 23), and it is reported to act as a gratuitous inducer of Tn*10 tet* gene expression under these conditions. Thus hypersensitivity of multicopy strains to anhydrotetracycline and heated chlortetracycline appears to reflect the greater activity of anhydrotetracycline and heated chlortetracycline as inducers of *tet* gene expression compared with their activity as inhibitors of protein synthesis. The relationship between induction and inhibition of multicopy strains is also evident in the experiment shown in Fig. 3b. Growth of B2550(λ *tetA-lacZ43*) containing the multicopy plasmid pBT107 is significantly inhibited by concentrations of either tetracycline or anhydrotetracycline that result in greater than about 40 to 50% maximal induction of β-galactosidase activity. Transcription of the pBT107 *tetA* region in this strain is presumably induced to the same extent as β-galactosidase under these conditions. In addition, the introduction of either pBT107 or pBT1010 into the minicell-producing strain P678-54 confers sensitivity to 1 μg of anhydrotetracycline per ml, although minicells isolated from these strains synthesize significant levels of *tet* proteins when induced with this concentration of anhydrotetracycline (Fig. 4).

The sensitivity of multicopy *tet* strains to tetracycline and anhydrotetracycline is enhanced by $ZnCl_2$. Bochner et al. (4) noted a similar effect of $ZnCl_2$ on the sensitivity of single-copy Tn*10* strains to media containing heated chlortetracycline and the lipophilic chelating agent fusaric acid. Although the role of $ZnCl_2$ in these phenomena is uncertain, our observations eliminate several possible mechanisms of action. $ZnCl_2$ does not enhance the intrinsic sensitivity of *E. coli* K-12 to either tetracycline or anhydrotetracycline (Table 2), and it does not enhance the effectiveness of anhydrotetracycline as an inducer of β-galactosidase synthesis in *tetA-lacZ* fusion strains (data not shown).

Previous explanations of the reduced tetracycline resistance phenotype of multicopy *tet* plasmids have assumed regulatory mechanisms that diminish expression of the *tet* genes in the

multicopy state and thereby reduce protection against the inhibitory action of tetracycline on ribosome function. Taylor et al. (30) proposed that increased synthesis of *tet* repressor in multicopy *tet* strains could account for reduced synthesis of tetracycline resistance functions. Shales et al. (28) and Chopra et al. (7) proposed that Tn*10* tetracycline resistance reflects the sum of three distinct resistance mechanisms, and that the negative gene dosage effect is the result of decreased expression of two of these mechanisms due to increased expression of the third mechanism. Coleman and Foster (9) demonstrated that increased synthesis of *tet* repressor is not the basis of the negative gene dosage effect; instead, they suggested a possible mechanism involving feedback inhibition at the level of translation resulting in reduced synthesis of the 36-kd *tetA* protein and a corresponding decrease in tetracycline resistance.

Although reduced *tet* gene expression can, in principle, provide an explanation for the reduced tetracycline resistance of multicopy *tet* strains, it cannot readily account for the hypersensitivity of these strains to anhydrotetracycline and heated chlortetracycline. Regardless of the mechanism involved, we propose that the reduced tetracycline resistance of multicopy *tet* strains and their hypersensitivity to anhydrotetracycline and heated chlortetracycline have a common physiological basis. Two observations lend further support to this proposal. First, the relative sensitivity of these strains to tetracycline, like their sensitivity to anhydrotetracycline, is enhanced by $ZnCl_2$. Second, strain differences (B2550 versus JA221) that influence the sensitivity of multicopy *tet* strains to tetracycline have a similar effect on their sensitivity to anhydrotetracycline. Taken together, our observations lead us to propose that the relative sensitivity of multicopy *tet* strains to tetracycline and their hypersensitivity to anhydrotetracycline and heated chlortetracycline are caused by the induction of multiple copies of the *tet* genes and the consequently higher levels of *tet* gene products, one or more of which inhibits the growth of *E. coli* K-12. Direct quantitation of *tet* proteins in whole cells has not, as yet, been possible. However, mutations in the *tet* region of JA221(pBT107) that overcome the hypersensitivity of this strain to anhydrotetracycline and heated chlortetracycline appear to either reduce expression of the *tetA* gene or alter the *tetA* structural gene per se, suggesting that it is overexpression of the 36-kd *tetA* gene product that is inhibitory (26).

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LITERATURE CITED

1. **Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree.** 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. U.S.A. **57**:321–326.
2. **Ball, P. R., S. W. Shales, and I. Chopra.** 1980. Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun. **93**:74–81.
3. **Beck, C. F., R. Mutzel, J. Barbe, and W. Muller.** 1982. A multifunctional gene (*tetR*) controls Tn*10*-encoded tetracycline resistance. J. Bacteriol. **150**:633–642.

3a. **Bertrand, K. P., K. Postle, L. V. Wray, Jr., and W. S. Reznikoff.** 1983. Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. Gene **23**:149–156.

4. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
5. **Cabello, F., K. Timmis, and S. N. Cohen.** 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. Nature (London) **259**:285–290.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
7. **Chopra, I., S. W. Shales, J. M. Ward, and L. J. Wallace.** 1981. Reduced expression of Tn*10*-mediated tetracycline resistance in *Escherichia coli* containing more than one copy of the transposon. J. Gen. Microbiol. **126**:45–54.
8. **Clarke, L., and J. Carbon.** 1978. Functional expression of cloned yeast DNA in *Escherichia coli*: Specific complementation of argininosuccinate lyase (*argH*) mutations. J. Mol. Biol. **120**:517–532.
9. **Coleman, D. C., and T. J. Foster.** 1981. Analysis of the reduction in expression of tetracycline resistance determined by transposon Tn*10* in the multicopy state. Mol. Gen. Genet. **182**:171–177.
10. **Curiale, M. S., and S. B. Levy.** 1982. Two complementation groups mediate tetracycline resistance determined by Tn*10*. J. Bacteriol. **151**:209–215.
11. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B. Suppl. **217**:1–90.
12. **Foster, T. J., T. G. B. Howe, and K. M. V. Richmond.** 1975. Translocation of the tetracycline resistance determinant from R100-1 to the *Escherichia coli* K-12 chromosome. J. Bacteriol. **124**:1153–1158.
13. **Franklin, T. J.** 1967. Resistance of *Escherichia coli* to tetracyclines. Changes in permeability to tetracyclines in *Escherichia coli* bearing transferable resistance factors. Biochem. J. **105**:371–378.
14. **Izaki, K., K. Kiuchi, and K. Arima.** 1966. Specificity and mechanism of tetracycline resistance in a multiple drug-resistant strain of *Escherichia coli*. J. Bacteriol. **91**:628–633.
15. **Jorgensen, R. A., D. E. Berg, B. Allet, and W. S. Reznikoff.** 1979. Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. J. Bacteriol. **137**:681–685.
16. **Jorgensen, R. A., and W. S. Reznikoff.** 1979. Organization of structural and regulatory genes that mediate tetracycline resistance in transposon Tn*10*. J. Bacteriol. **138**:705–714.
17. **Kleckner, N., R. K. Chan, B.-K. Tye, and D. Botstein.**

1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. J. Mol. Biol. **97**:561–575.

18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lane, L. C.** 1978. A simple method for stabilizing protein-sulfhydryl groups during SDS-gel electrophoresis. Anal. Biochem. **86**:655–664.
20. **Levy, S. B., and L. McMurry.** 1974. Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. Biochem. Biophys. Res. Commun. **56**:1060–1068.
21. **Levy, S. B., and L. McMurry.** 1978. Plasmid-determined tetracycline resistance involves new transport systems for tetracycline. Nature (London) **276**:90–92.
22. **Levy, S. B., L. McMurry, P. Onigman, and R. M. Saunders.** 1977. Plasmid-mediated tetracycline resistance in *E. coli*. Top. Infect. Dis. **2**:181–206.
23. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**:1110–1112.
24. **McMurry, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:3974–3977.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Moyed, H. S., and K. P. Bertrand.** 1983. Mutations in multicopy Tn*10 tet* plasmids that confer resistance to inhibitory effects of inducers of *tet* gene expression. J. Bacteriol. **155**:557–564.
27. **Roozen, K. J., R. G. Fenwick, Jr., and R. Curtiss III.** 1971. Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. J. Bacteriol. **107**:21–33.
28. **Shales, S. W., I. Chopra, and P. R. Ball.** 1980. Evidence for more than one mechanism of plasmid-determined tetracycline resistance in *Escherichia coli*. J. Gen. Microbiol. **121**:221–229.
29. **Sompolinsky, D., and J. Krausz.** 1973. Action of 12 tetracyclines on susceptible and resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **4**:237–247.
30. **Taylor, D. P., J. Greenberg, and R. H. Rownd.** 1977. Generation of miniplasmids from copy number mutants of the R plasmid NR1. J. Bacteriol. **132**:986–995.
31. **Wiebauer, K., S. Schraml, S. W. Shales, and R. Schmitt.** 1981. Tetracycline resistance transposon Tn*1721*:*recA*-dependent gene amplification and expression of tetracycline resistance. J. Bacteriol. **147**:851–859.
32. **Wray, L. V., Jr., R. A. Jorgensen, and W. S. Reznikoff.** 1981. Identification of the tetracycline resistance promoter and repressor in transposon Tn*10*. J. Bacteriol. **147**:297–304.
33. **Yang, H.-L., G. Zubay, and S. B. Levy.** 1976. Synthesis of an R plasmid protein associated with tetracycline resistance is negatively regulated. Proc. Natl. Acad. Sci. U.S.A. **73**:1509–1512.