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Mutations in Multicopy Tn*10 tet* Plasmids That Confer Resistance to Inhibitory Effects of Inducers of *tet* Gene Expression

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Escherichia coli K-12 strains that carry the Tn*10* tetracycline resistance determinant (*tet*) on multicopy plasmids are hypersensitive to 5a,6-anhydrotetracycline and heated chlortetracycline, two tetracycline derivatives that are relatively more effective as inducers of *tet* gene expression than as inhibitors of bacterial growth. Twenty spontaneous mutations that confer resistance to anhydrotetracycline (At^r) and resistance to heated chlortetracycline (Ct^r) were isolated and characterized. All of these At^r mutations are located in the Tn*10 tet* region; the majority (18 of 20) have no effect on *tetR* repressor function. At^r mutations can increase, reduce, or eliminate the phenotypic expression of plasmid tetracycline resistance (Tc^r). IS insertions that result in an At^r Tc^s phenotype are clustered in a 150-base-pair promoter-proximal region of the *tetA* resistance gene. Some At^r mutations reduce expression of the *tetA* gene by altering either the *tetR* repressor or the *tetA* promoter. In addition, it appears that *E. coli* cannot tolerate constitutive expression of the wild-type *tetA* gene from a multicopy plasmid containing a *tetR* deletion. These observations support the proposal that high level expression of the 36-kilodalton *tetA* gene product inhibits the growth of *E. coli*. We speculate that this inhibition is related to the interaction of the *tetA* gene product with the cytoplasmic membrane.

The Tn*10* tetracycline resistance determinant (*tet*) exhibits a negative gene dosage effect in *Escherichia coli* K-12; strains that contain multicopy *tet* plasmids are generally less resistant to tetracycline than are strains that contain *tet* in a low-copy-number state (3, 5, 11, 17, 19). Furthermore, multicopy *tet* strains are hypersensitive to certain tetracycline derivatives; concentrations of 5a,6-anhydrotetracycline and heated chlortetracycline that have little or no effect on the growth of tetracycline-sensitive (Tc^s) control strains inhibit the growth of multicopy stains (17). The hypersensitivity of multicopy strains to anhydrotetracycline and heated chlortetracycline appears to be related to the action of anhydrotetracycline and heated chlortetracycline as inducers of *tet* gene expression, as opposed to their action as inhibitors of protein synthesis. Previous explanations of the reduced Tc^r of multicopy strains have, in general, assumed regulatory mechanisms that reduce expression of the *tet* genes in these strains (3, 5, 19). We propose, instead, that high level expression of the 36-kilodalton *tetA* gene product inhibits the growth of *E. coli* and accounts for both the reduced tetracycline resistance and anhydrotetracycline hypersensitivity of multicopy Tn*10 tet* strains.

Here we describe the isolation and characterization of mutations that confer resistance to the inhibitory effects of anhydrotetracycline and heated chlortetracycline. These studies reinforce our proposal regarding the basis of the Tn*10* negative gene dosage effect and provide a preliminary characterization of several interesting classes of Tn*10 tet* regulatory mutations.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. With the exception of plasmid pBT1071, bacterial strains, plasmids, and phages are described in the accompanying paper (17). pBT1071 is a *tetR* deletion derivative of pBT107. The 44-base-pair (bp) *BclI* and 266-bp *BclI* fragments that span the carboxy-terminal end of the *tetR* gene (Fig. 1b) were deleted by digestion of pBT107 with *BclI*: the digested DNA was religated, and ampicillin-resistant (Ap^r) transformants of JA221 were selected.

Selection of mutants. Independent spontaneous mutations were selected by plating saturated LB broth cultures of JA221(pBT107) on either ATZ medium or CTZ medium. ATZ medium is TYE agar (10 g of tryptone [Difco Laboratories, Detroit, Mich.] per liter,

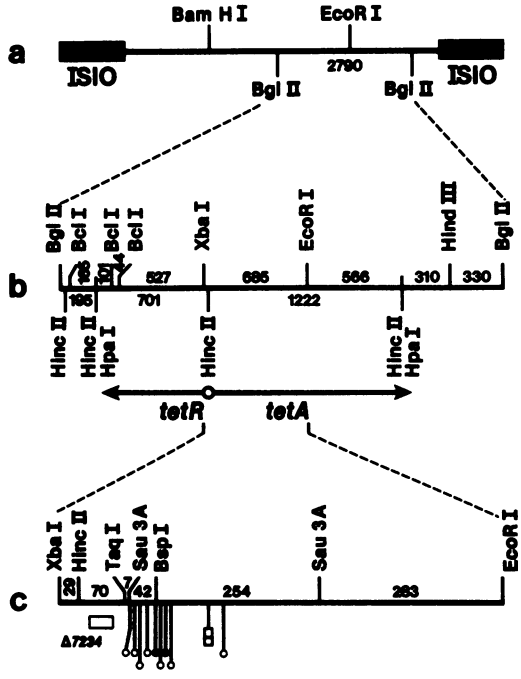

FIG. 1. (a) Restriction map of Tn*10* showing the relative position and orientation of the *Bgl*II 2,790-bp fragment that encodes Tc^r (11). Tn*10* consists of a central region (6,400 bp) flanked by two copies of IS*10* (1,450 bp) in inverted orientation. (b) Restriction map of the *Bgl*II 2,790-bp fragment of Tn*10*. The lengths of DNA segments within the central *Hpa*I fragment are based on DNA sequence data. The extent and direction of transcription of the repressor (*tetR*) and the resistance (*tetA*) genes are indicated by the arrows below the restriction map; the open circle indicates the regulatory region spanning the *Hin*cII site. (c) Restriction map of the *Xba*I-*Eco*RI 685-bp fragment of Tn*10*, showing the positions of At^r mutations. Δ7234 (pBT7234) is a 30 to 35-bp deletion within the 70-bp *Hin*cII-*Taq*I segment. Symbols: (○) IS*1* insertions oriented such that the order of restriction sites is *Xba*I (Tn*10*), *Pst*I (IS*1*), *Bal*I(IS*1*); (●) IS*1* insertions in the opposite orientation; (□) two independent IS*5* insertions. Only the *Taq*I and *Bsp*I sites nearest the *Xba*I site are shown.

8 g of NaCl per liter, 5 g of Difco yeast extract per liter, and 15 g of Difco agar per liter) containing 100 μg of ampicillin per ml, 10 μg of 5a,6-anhydrotetracycline (lot no. 4967-261-3, Pfizer) per ml, and 0.6 mM $ZnCl_2$. CTZ medium contains 23 g of Difco nutrient agar, 10 g of NaCl per liter, 10 g of NaH_2PO_4 per liter, 100 μg of ampicillin per ml, 50 μg of chlortetracycline hydrochloride (Sigma Chemical Co., St. Louis, Mo.) per ml, 8 μg of fusaric acid (Sigma) per ml, and 0.1 mM $ZnCl_2$; the nutrient agar, NaCl, NaH_2PO_4, and chlortetracycline are mixed and then sterilized by autoclaving (30 min, 15 lb/in^2). Sterile stocks of ampicillin (1 mg/ml), anhydrotetracycline (0.5 mg/ml in methanol), fusaric acid (2 mg/ml), and $ZnCl_2$ (20 mM) were prepared separately. CTZ medium is essentially the medium described by Maloy and Nunn (14) for the enrichment of Tc^s derivatives of Tn*10*-containing *E. coli* strains; it is a modification of a similar medium described by Bochner et al. (1a).

Restriction analysis of mutant plasmids. The small deletion in the anhydrotetracycline-resistant (At^r) Tc^s plasmid pBT7234 is within the 70-bp *Hin*cII-*Taq*I fragment that spans the *tetA* and *tetR* promoters (Fig. 1c) (1, 22). The 660-bp *Hin*cII-*Eco*RI fragment in pBT107 is replaced by a 625-bp fragment in pBT7234. Similarly, the 158-bp *Taq*I fragment in pBT107 is replaced by a 128-bp *Taq*I fragment in pBT7234, providing an estimate of 30 to 35 bp for the length of the deletion in pBT7234.

Nine of the At^r Tc^s plasmids have an insertion of approximately 750 bp within the *Hin*cII 1,275-bp fragment of pBT107 (Fig. 2). These insertions were identified as IS*1* elements and localized by analyzing *Xba*I-*Pst*I and *Xba*I-*Bal*I digests (Fig. 1c). IS*1* has unique *Pst*I and *Bal*I restriction sites situated as follows: left end, 180 bp, *Pst*I, 53 bp, *Bal*I, 535 bp, right end (18). The positions of the IS*1* insertions were confirmed by examining the *Sau*3A and *Bsp*I digests of the At^r plasmids. The arrangement of sites in this region of pBT107 is as follows: *Xba*I, 106 bp, *Sau*3A, 42 bp, *Bsp*I, 254 bp, *Sau*3A. All of the insertions are in the 296-bp *Sau*3A fragment; they lie either to the left or right of the *Bsp*I site as indicated in Fig. 1c.

Two of the At^r Tc^s plasmids (pBT7312 and pBT768) have insertions of approximately 1,180 bp in the *Hin*cII 1,275-bp fragment of pBT107 (Fig. 2). These insertions were identified as IS*5* elements and localized by analyzing *Xba*I-*Bgl*II, *Xba*I-*Bal*I, *Xba*I-*Pvu*II, and *Xba*I-*Eco*RI digests of the plasmids (Fig. 1c). The DNA sequence of IS*5* predicts the following sequence

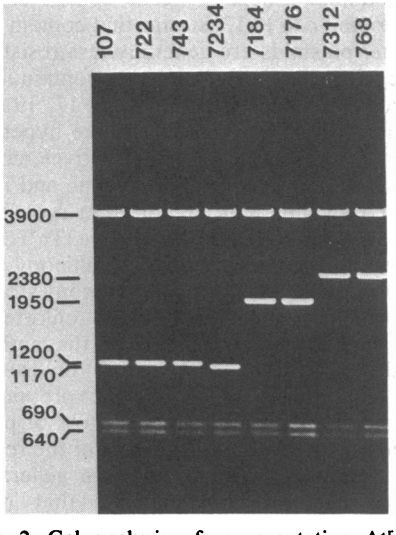

FIG. 2. Gel analysis of representative At^r plasmids. *Hin*cII digests of (left to right) pBT107, pBT722, pBT743, pBT7234, pBT7184, pBT7176, pBT7312, and pBT768 electrophoresed in a 1% agarose gel. Fragment lengths are given in base pairs.

of restriction sites: left end, 152 bp, *Bgl*II, 48 bp, *Bal*I, 310 bp, *Pvu*II, 585 bp, *Eco*RI, 94 bp, right end (7). The IS5 insertions are in the same region of pBT107 as the IS1 insertions (Fig. 1c).

Antibiotic resistance and β-galactosidase activity. Minimum inhibitory concentrations of tetracycline and anhydrotetracycline and levels of β-galactosidase in *tet-lac* fusion strains were determined as described in the accompanying paper (17).

Plasmid DNA preparation, restriction enzymes, and gel electrophoresis. Plasmid-containing strains were grown to saturation in M9 minimal medium (16) supplemented with 0.4% glucose, 0.2% Casamino Acids, 40 μg of L-tryptophan per ml, 4 μg of thiamine per ml, 1 mM $MgSO_4$, and 100 μg of ampicillin per ml. Plasmid DNA was prepared by the method of Humphreys et al. (9).

Restriction enzymes were obtained from Bethesda Research Laboratories (Rockville, Md.), except for *Bsp*I and *Xba*I, which were prepared by K. Postle and K. Toth, respectively. Restriction digests were fractionated by electrophoresis in agarose or polyacrylamide slab gels. Agarose gels (1%) were prepared in TEA buffer (50 mM Tris, 20 mM sodium acetate, 2 mM disodium EDTA, pH 8.05 [8]). Polyacrylamide gels (5 and 8%) were prepared in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM disodium EDTA, pH 8.3) as described by Maniatis et al. (15), except that the gels contained 5% glycerol.

Construction of mosaic plasmids. The *Xba*I-*Eco*RI 5,800-bp and 685-bp restriction fragments of pBT107 and mutant plasmids were electrophoresed in 8% polyacrylamide gels, eluted, and religated in various combinations. Reactions containing 0.5 to 2.0 pmol of each DNA fragment were ligated and used to transform competent JA221; Ap^r transformants were selected and screened for their Tc^r and At^r phenotypes. In general, the purified *Xba*I-*Eco*RI 5,800-bp fragments gave rise to a few Ap^r transformants due to contamination of the 5,800-bp fragments with low levels of partial digestion products. In reactions containing *Xba*I-*Eco*RI 5,800-bp and 685-bp fragments, the majority (>98%) of the Ap^r transformants were Tc^r, indicating that the *Eco*RI site in *tetA* was restored.

RESULTS

Isolation of At^r plasmids. With the exception of pBT782, the mutant plasmids described in Table 1 were isolated by selecting derivatives of JA221(pBT107) that grow on CTZ medium. The majority (>90%) of spontaneous JA221(pBT107) mutants that grow on CTZ medium are Tc^r. The mutant phenotype, in every case examined, was due to a mutation in the plasmid, since it could be transferred to JA221 by plasmid DNA isolated from mutant bacteria. Subsequent studies showed that JA221(pBT107) is inhibited to the same extent by CTZ medium lacking fusaric acid. With regard to the mutants listed in Table 1, it appears that the principal selective agent in CTZ medium is the heated chlortetracycline. $ZnCl_2$ enhances the inhibitory effects of anhydrotetracycline and heated chlortetracycline on the growth of JA221(pBT107) (17).

TABLE 1. Characteristics of At^r plasmids derived from pBT107

Plasmid	MIC[a] of tetracycline (μg/ml)	Characteristics
pACYC177	3.0	Vector: At^r Tc^s Ap^r Nm^r
pBT107	28	Parent: At^s Tc^r Ap^r Nm^r
pBT7152	50	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT791	50	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT711	40	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT7111	40	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT7101	18	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT743	18	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT771	18	*tet Xba*I-*Eco*RI 685-bp point mutation[b]
pBT722	16	*tetR* point mutation[b]
pBT712	3.0	*tetA* IS1 insertion
pBT721	3.0	*tetA* IS1 insertion
pBT735	3.0	*tetA* IS1 insertion
pBT7512	3.0	*tetA* IS1 insertion
pBT782	3.0	*tetA* IS1 insertion
pBT7112	3.0	*tetA* IS1 insertion
pBT7161	3.0	*tetA* IS1 insertion
pBT7176	3.0	*tetA* IS1 insertion
pBT7184	3.0	*tetA* IS1 insertion
pBT7312	3.0	*tetA* IS5 insertion
pBT768	3.0	*tetA* IS5 insertion
pBT7234	3.0	*tet* promoter deletion

[a] Minimal inhibitory concentrations (MICs) were determined with plasmids in JA221 background.

[b] No change was detected in the sizes of *tet* restriction fragments.

The mutant plasmid pBT782 was isolated by selection for JA221(pBT107) derivatives that grow on ATZ medium. Since the mutants isolated on CTZ medium also grow on ATZ medium, we have chosen, for convenience, to refer to all of these mutants as At^r mutants.

Tc^r phenotypes of At^r plasmids. The At^r plasmids do not differ significantly from pACYC177 in their At^r phenotypes; however, there is considerable variation in the Tc^r phenotypes of these plasmids. We have characterized 12 At^r plasmids that confer no detectable Tc^r and 8 At^r plasmids that confer various degrees of Tc^r (Table 1). Four of the At^r Tc^r plasmids confer higher Tc^r than the pBT107 parent.

In addition to enhancing the sensitivity of JA221(pBT107) to anhydrotetracycline and heated chlortetracycline, $ZnCl_2$ further reduces the resistance of JA221(pBT107) to tetracycline (17). In contrast, $ZnCl_2$ has little effect on the Tc^r conferred by the At^r plasmids (data not shown). Consequently, At^r plasmids that confer

relatively high levels of Tc^r (pBT7152, pBT791, pBT711, and pBT7111) can also be distinguished from pBT107 on the basis of growth on TYE medium containing 20 μg of tetracycline per ml and 0.6 mM $ZnCl_2$.

Repressor function in At^r plasmids. Tn*10 tetR* repressor function can be assessed independently of the Tc^r phenotype of a plamid by introducing the plasmid into B2550(λ *tetA-lacZ43*), which contains a transcriptional gene fusion of the Tn*10 tetA* promoter to the *lacZ* gene (K. Bertrand, L. Wray, and W. Reznikoff, manuscript in preparation). In the absence of *tetR*, B2550(λ *tetA-lacZ43*) synthesizes high levels of β-galactosidase (Table 2). When pBT107 is introduced into this strain, β-galactosidase levels are reduced 20- to 50-fold; this repression can be overcome by the addition of appropriate concentrations of anhydrotetracycline (17). The majority of the At^r plasmids do not differ significantly from pBT107 either in their ability to repress β-galactosidase synthesis or in their response to inducing concentrations of anhydrotetracycline. However, two of the At^r plasmids tested do show a significant difference in repressor function. pBT7234 does not repress β-galactosidase synthesis in the *tet-lac* fusion strain as effectively as pBT107 (10-fold versus 25- to 50-fold). As discussed below, the At^r lesion in pBT7234 most likely impairs the *tetR* promoter. In contrast, pBT722 reduces β-galactosidase synthesis to the same extent as pBT107; however, the addition of anhydrotetracycline does not overcome repression by pBT722. pBT722-mediated repression is also insensitive to concentrations of tetracycline that lead to a 20- to 30-fold induction when pBT107 is the source of the repressor (data not shown). As discussed below, pBT722 appears to encode a mutant *tetR* repressor that fails to respond to inducing concentrations of anhydrotetracycline or tetracycline.

Effect of At^r plasmids on expression of chromosomally determined Tn*10* Tc^r. Multicopy Tn*10 tet* plasmids not only confer markedly lower levels of Tc^r than a chromosomal copy of Tn*10*, but also appear to suppress the expression of high-level chromosomally determined Tc^r (5). Thus, the Tc^r phenotype of pBT107 is dominant to the Tc^r phenotype of chromosomal Tn*10* (Table 3). Similarly, the At^s phenotype of pBT107 is dominant to the At^r phenotype of chromosomal Tn*10*. The effects of representative At^r plasmids on the expression of chromosomal Tn*10* Tc^r were also determined. In contrast to pBT107, the At^r Tc^r plasmids pBT743 and pBT7111, as well as the At^r Tc^s plasmids pBT7176, pBT7312, and pBT7234, have no significant effect on the expression of high-level chromosomal Tn*10* Tc^r. The At^r Tc^r plasmid pBT722 is an interesting exception; the reduced Tc^r phenotype of pBT722 is dominant to the high-level Tc^r phenotype of chromosomal Tn*10*.

Mapping the At^r mutations. *Hin*cII cleaves pBT107 into five fragments that are approximately 3,700, 1,275, 690, 640, and 195 bp in length (Fig. 2). The *Hin*cII cleavage patterns of the 8 At^r Tc^r plasmids appear identical to the pBT107 pattern, whereas the 12 At^r Tc^s plas-

TABLE 2. Repressor function in At^r plasmids[a]

Plasmid	β-galactosidase activity[b]	
	Uninduced	Induced[c]
pACYC177	5,120	5,100
pBT107	111	4,020
pBT7152	188	4,590
pBT791	160	5,270
pBT711	90	4,730
pBT7111	141	4,370
pBT7101	164	4,750
pBT743	155	4,625
pBT771	154	4,975
pBT722	125	108
pBT7176	118	4,250
pBT7184	101	4,240
pBT7312	85	4,280
pBT7234	673	5,400
pBT1071	4,895	4,700

[a] Capacity of At^r plasmids to repress β-galactosidase synthesis in B2550(λ *tetA-lacZ*43).

[b] Activity is expressed in units as defined by Miller (16).

[c] Induced cultures received 0.1 μg of anhydrotetracycline per ml, except for the pBT107 culture, which received 0.02 μg of anhydrotetracycline per ml.

TABLE 3. Effect of At^r plasmids on expression of high-level Tc^r conferred by chromosomal Tn*10*

Plasmid	Background	MIC[a] (μg/ml)	
		Tetracycline	Anhydrotetracycline
pACYC177	JA221	3.0	16
	JA221::Tn*10*	150	20
pBT107	JA221	24	0.8
	JA221::Tn*10*	36	0.8
pBT7111	JA221	40	18
	JA221::Tn*10*	175	22
pBT743	JA221	18	18
	JA221::Tn*10*	150	22
pBT722	JA221	14	18
	JA221::Tn*10*	16	18
pBT7176	JA221	3.0	18
	JA221::Tn*10*	150	22
pBT7312	JA221	3.0	18
	JA221::Tn*10*	175	22
pBT7234	JA221	3.0	18
	JA221::Tn*10*	175	22

[a] MIC, Minimal inhibitory concentration.

TABLE 4. Localization of At^r Tc^r mutations by analysis of mosaic plasmids[a]

Expt	*Xba*I-*Eco*RI fragment		Ap^r trans-formants	Mutant phenotype frequency[b]
	685 bp	5,800 bp		
1	Parent		0	
		Parent	7	0/1
	Parent	Parent	64	1/58
	pBT7111		0	
		pBT7111	9	7/7
	pBT7111	pBT7111	58	56/56
	Parent	pBT7111	69	5/65
	pBT7111	Parent	69	67/67
2	Parent		0	
		Parent	13	0/2
	pBT711		0	
	pBT711	Parent	87	77/78
	pBT743		0	
	pBT743	Parent	64	60/60
	pBT771		0	
	pBT771	Parent	58	56/56
	pBT791		0	
	pBT791	Parent	68	62/63
	pBT7101		0	
	pBT7101	Parent	64	59/59
	pBT7152		0	
	pBT7152	Parent	74	72/72
3	Parent		0	
		Parent	19	0/7
	pBT722	Parent	92	0/90
		pBT722	3	3/3
	Parent	pBT722	28	27/27

[a] Plasmids were reconstructed by ligating purified restriction fragments prepared from pBT107 (parent) and At^r mutant plasmids.

[b] The number of Ap^r JA221 transformants resistant to 20 μg of tetracycline per ml plus 0.6 mM $ZnCl_2$ relative to the number resistant to 20 μg of tetracycline per ml. For mosaic plasmids containing fragments from pBT743, pBT771, pBT7101, and pBT722, the number of transformants resistant to 4 μg of anhydrotetracycline per ml plus 0.6 mM $ZnCl_2$ relative to the number resistant to 5 μg of tetracycline per ml.

mids have IS*1* or IS*5* insertions or, in one case, a deletion in the 1,275-bp *Hin*cII fragment that spans the *tetA* region of pBT107 (Fig. 1b and 2). Restriction analysis of the At^r Tc^s plasmids is described above and summarized in Fig. 1c. A striking feature of the IS*1* and IS*5* insertions is that they are clustered in a region that is 105 to 250 bp downstream from the *Xba*I site, or 40 to 185 bp downstream from the initiation site for *tetA* transcription (1).

The mutations in the At^r Tc^r plasmids were localized by examining the phenotypes of mosaic plasmids constucted by ligating purified restriction fragments from pBT107 and complementary fragments from each mutant plasmid. Ap^r transformants were selected and screened for their At^r and Tc^r phenotypes. In experiment 1 (Table 4), *Xba*I-*Eco*RI fragments prepared from pBT107 (parent) and pBT7111 (At^r mutant) were tested individually and in all possible combinations. Whereas the combination 685 bp (parent) plus 5,800 bp (pBT7111) primarily yields plasmids with a parental phenotype, the combination 685 bp (pBT7111) plus 5,800 bp (parent) yields plasmids with the mutant phenotype of pBT7111, indicating that the At^r mutation is in the *Xba*I-*Eco*RI 685-bp fragment of pBT7111. Similar results were obtained for the At^r mutations in pBT7111, pBT743, pBT771, pBT791, pBT7101, and pBT7152 (Table 4, experiment 2). In contrast, the At^r mutation in pBT722 is in the *Xba*I-*Eco*RI 5,800-bp fragment of this plasmid (Table 4, experiment 3). Thus, with the exception of pBT722, the mutations in the At^r Tc^r plasmids are in the *Xba*I-*Eco*RI 685-bp fragment that spans the *tetR* and *tetA* promoters and the promoter-proximal half of *tetA* (Fig. 1b) (1, 21).

Analysis of *tetR* deletion plasmid pBT7101. Deletion of the *Bcl*I 266-bp and *Bcl*I 44-bp fragments of pBT107 (Fig. 1b) removed DNA sequences that encode the carboxy-terminal 18 amino acids to the *tetR* repressor (K. Postle, T. Nguyen, and K. Bertrand, manuscript in preparation), with the result that repressor function is completely eliminated in pBT1071 (Table 2). pBT1071 confers greater resistance to anhydrotetracycline (18 μg/ml) and tetracycline (90 μg/ml) than does pBT107. The unexpected At^r phenotype of pBT1071 is explained by the finding that this plasmid has acquired a second mutation in addition to the *tetR* deletion introduced in vitro. This second mutation eliminates

TABLE 5. Analysis of the At^r repressor deletion plasmid pBT1071[a]

*Xba*I-*Eco*RI fragment		Ap^r trans-formants	Mutant phenotype frequency[b]
685 bp	5,800 bp		
Parent		0	
	Parent	8	
pBT1071		0	
	pBT1071	18	3/3
pBT7111		0	
Parent	Parent	272	0/268
pBT1071	pBT1071	201	192/192
Parent	pBT1071	16	4/4
pBT1071	Parent	241	237/237
pBT7111	Parent	239	239/239
pBT7111	pBT1071	150	137/137

[a] Plasmids were reconstructed by ligating purified restriction fragments from pBT107 (parent), pBT7111, and pBT1071.

[b] The number of Ap^r JA221 transformants resistant to 20 μg of tetracycline per ml plus 0.6 mM $ZnCl_2$ relative to the number resistant to 20 μg of tetracycline per ml.

the *Hin*cII site in the region of the *tetA* and *tetR* promoters (Fig. 1b). Whereas the *Bsp*I 450-bp fragment that spans the *tet* promoter region appears to be the same size in both pBT1071 and pBT107, the pBT1071 *Bsp*I 450-bp fragment is not cleaved by *Hin*cII.

The mutations in pBT1071 were further analyzed by preparing a series of mosaic plasmids derived from the purified *Xba*I-*Eco*RI fragments of pBT1071 (Table 5). Whereas the *Bcl*I repressor deletion is in the 5,800-bp fragment (a 5,500-bp fragment in the case of pBT1071), the mutated *Hin*cII site is in the 658-bp fragment. Two important results emerge from this set of experiments. First, the mutated *Hin*cII site is sufficient to account for the At^r character of pBT1071. Thus, the combination 685 bp (pBT1071) plus 5,800 bp (parent) yields plasmids with an At^r Tc^r phenotype. Second, the *Bcl*I *tetR* deletion yields viable mosaic plasmids only when it is combined with 685-bp fragments that carry an At^r mutation. Thus the combinations 685 bp (pBT7111) plus 5,800 bp (pBT1071) and 685 bp (pBT1071) plus 5,800 bp (pBT1071) yield many Ap^r transformants, but the combination 685 bp (parent) plus 5,800 bp (pBT1071) yields only a very few Ap^r Tc^r transformants. The few Ap^r Tc^r transformants that arise with the combination 685 bp (parent) plus 5,800 bp (pBT1071), as well as the Ap^r Tc^r transformants that arise with 5,800 bp (pBT1071) alone, are presumably the result of full-length pBT1071 molecules that contaminate the 5,800-bp fragment preparation.

DISCUSSION

We proposed that high-level expression of the 36-kilodalton Tn*10* *tetA* gene product inhibits the growth of *E. coli* and accounts for the hypersensitivity of multicopy *tet* strains to the inducers anhydrotetracycline and heated chlortetracycline (17). We have explored this proposal by analyzing mutations that overcome the hypersensitivity of JA221(pBT107) to anhydrotetracycline and heated chlortetracycline. Twenty spontaneous At^r mutations were characterized (Table 1). All of these mutations are located in the *tet* region of pBT107; the majority (18 of 20) do not influence *tetR* repressor activity, as judged by the capacity of the mutant plasmids to repress β-galactosidase synthesis in *tetA-lacZ* fusion strains and the response to inducing concentrations of anhydrotetracycline (Table 2). Eleven IS insertion mutations and one deletion mutation were localized by restriction mapping (Fig. 1c). Eight At^r point mutations were localized by constructing mosaic plasmids derived from pBT107 and each of the At^r plasmids (Table 4). With one exception (pBT722), all of the At^r mutations are within the 685-bp *Xba*I-*Eco*RI restriction fragment that spans the extreme promoter-proximal end of the *tetR* gene, the *tetR* and *tetA* promoters, and the promoter-proximal half of the *tetA* gene (Fig. 1) (1, 21). At^r mutations can increase (pBT7152, pBT791, pBT711, pBT7111), reduce (pBT7101, pBT743, pBT771, pBT722), or eliminate (pBT7234, IS insertions) the phenotypic expression of pBT107 Tc^r (Table 1). Thus, the level of plasmid-determined Tc^r is not the basis of the At^r character.

The ease of isolating mutations in the pBT107 region that increase the level of plasmid-determined Tc^r raises an important point; multicopy Tn*10* *tet* plasmids that are constructed or propagated (or both) under tetracycline selection may, in fact, no longer carry the wild-type Tn*10* *tet* region.

We find, in agreement with others (3, 5, 11, 19), that multicopy Tn*10* *tet* strains are less resistant to tetracycline than are single-copy *tet* strains (17). Coleman and Foster (5) analyzed this phenomenon in detail, and their results provide a basis for discussing the data presented here. Specifically, Coleman and Foster showed the following. (i) The reduced Tc^r conferred by multicopy Tn*10* *tet* plasmids is phenotypic; the plasmid *tet* genes confer high-level Tc^r when reintroduced into the bacterial chromosome. (ii) The reduced Tc^r phenotype is not due to overproduction of the *tetR* repressor as previously suggested (19). (iii) Multicopy Tn*10* *tet* plasmids inhibit the expression of high-level Tc^r by chromosomal Tn*10*; that is, the reduced Tc^r phenotype of multicopy *tet* plasmids is *trans* dominant to the Tc^r phenotype of chromosomal Tn*10* (the *trans* effect). (iv) All Tn*5* insertions within the 1,275-bp *Hin*cII fragment that spans *tetA* eliminate expression of plasmid Tc^r; however, only those insertions within a 40-bp region adjacent to the *tetA* promoter eliminate the plasmids' *trans* effect on expression of chromosomal Tn*10* Tc^r.

The data presented here show that the At^s and reduced Tc^r phenotypes of JA221(pBT107) are related in a fundamental manner. (i) The At^s and reduced Tc^r phenotypes are dominant to the At^r and high-level Tc^r phenotypes of chromosomal Tn*10* (Table 3). (ii) With one exception (pBT722), the At^r mutations result in loss of this *trans* effect on expression of chromosomal Tn*10* Tc^r (Table 3). (iii) IS insertions that result in an At^r Tc^s phenotype are clustered within a 150-bp region that spans the location reported by Coleman and Foster (5) for Tc^s Tn*5* insertions that, like the IS insertions, eliminate the *trans* effect on expression of chromosomal Tn*10* Tc^r (Table 3). However, the phenotypes of the At^r mutants show that level of plasmid-determined Tc^r is not the basis of either the At^s character or the *trans* effect.

At least two of the At^r plasmids have regula-

tory mutations that reduce the level of transcription of the wild-type *tetA* gene. pBT722 appears to encode a mutant *tetR* repressor that has a much-reduced affinity for inducer or, alternatively, fails to respond to bound inducer in the usual manner (Table 2). The mutation in pBT722 maps outside the *Xba*I-*Eco*RI 685-bp fragment (Table 4), which is consistent with it being in the *tetR* structural gene (1, 21). The repressed level of *tetA* promoter function in pBT722 apparently accounts for the At^r phenotype and the low-level Tc^r phenotype of this plasmid (Table 1). The secondary mutation in the *Hin*cII site of pBT1071 changes the DNA sequence in the "−35 region" of the *tetA* promoter (1) and results in a 50- to 100-fold reduction in *tetA* transcription (unpublished observation). Mosaic plasmids that contain the pBT1071 *tetA* promoter mutation and the wild-type *tetR* repressor gene have an At^r Tc^r phenotype (Table 5), again suggesting that reduced *tetA* promoter function is sufficient to account for the At^r phenotype.

The finding that the *tetR* deletion plasmid pBT1071 contains a secondary At^r mutation in the *tetA* promoter resolved an apparent contradiction to the proposal that high-level expression of *tetA* is inhibitory. In fact, the *tetR* deletion in pBT1071 is not viable unless it is present in a plasmid background that also contains a compensating At^r mutation (Table 5). We conclude that the *E. coli* strain JA221 cannot tolerate the high-level constitutive expression of the wild-type *tetA* gene. As our experience with pBT1071 illustrates, efforts to isolate constitutive *tetR* derivatives of multicopy *tet* plasmids can be complicated by the ease of selecting compensating mutations in the *tet* regulatory region.

DNA sequencing studies indicate that the Tn*10* *Hin*cII 1,275-bp fragment contains a single extended open translation reading frame; deletions introduced at either end of this region alter the 36-kd *tetA* protein and therefore define the extent of the *tetA* structural gene (T. Nguyen, K. Postle, and K. Bertrand, in press). Curiale and Levy (6) and Coleman et al. (4) showed that Tc^s mutations within the *Hin*cII 1,275-bp fragment define two genetic complementation groups. We interpret these results to indicate that some *tetA* mutations are capable of intracistronic complementation. The At^r Tc^s *tetA*::IS insertions and the Tc^s *tetA*::Tn*5* insertions that eliminate the *trans* effect (5) are clustered in the promoter-proximal region of the *tetA* structural gene.

The genetic evidence presented here reinforces our proposal that high-level expression of the 36-kilodalton *tetA* protein inhibits *E. coli* growth and accounts for the reduced Tc^r phenotype and the At^s phenotype of multicopy Tn*10* *tet* strains. The 36-kilodalton *tetA* protein is associated with the cytoplasmic membrane (12, 13). It is tempting to speculate that the inhibition of growth associated with high-level *tetA* expression is a consequence of the interaction of high levels of the *tetA* protein with the cytoplasmic membrane. Other investigations have suggested that high-level expression of the *ompA* (2) and *phoE* (20) gene products, two outer membrane proteins, and high-level expression of alkaline phosphatase (*phoA*) (10), a periplasmic protein, also inhibit the growth of *E. coli*. The properties of promoter-distal *tetA*::Tn*5* insertions (5) may reflect the capacity of amino-terminal fragments of the *tetA* protein to interact with the cytoplasmic membrane in somewhat the same manner as the intact *tetA* protein. Further analysis of the At^r mutations described here should provide insight into the regulation of *tet* gene expression and, perhaps, the mechanism by which the *tetA* protein is inserted into the cytoplasmic membrane.

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