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Noninducible Tet Repressor Mutations Map from the Operator Binding Motif to the C Terminus

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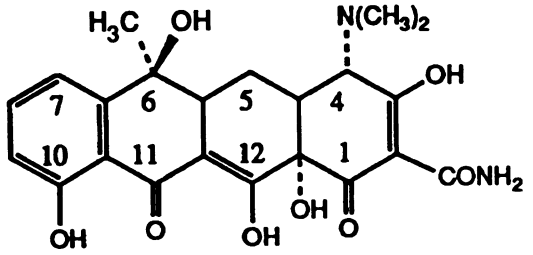
We have developed a new genetic selection system for Tet repressor mutations with a noninducible phenotype for tetracycline (TetR^s). Extensive chemical mutagenesis of *tetR* yielded 93 single-site Tet repressor mutations. They map from residue 23 preceding the α-helix-turn-α-helix operator binding motif to residue 196 close to the C terminus of the repressor. Thirty-three of the mutations are clustered between residues 95 and 117, and another 27 are clustered between residues 131 to 158. Several of the mutants were characterized quantitatively in vivo for induction by tetracycline and anhydrotetracycline. While all of these are severely reduced in tetracycline-mediated induction, only some of them are affected for anhydrotetracycline-mediated induction.

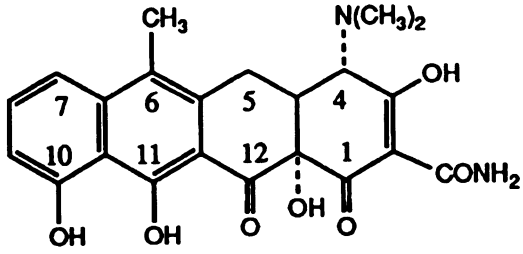
Resistance to tetracycline (TC) in gram-negative bacteria is mostly mediated by determinants encoding active efflux of the drug from the resistant cell. These determinants have been grouped into five different classes which share extensive sequence homology in their structural genes. In addition, their genetic organization is conserved, and a strict regulation of expression of the resistance gene is found in nearly all natural isolates (13). Regulation is mediated by the Tet repressor protein (4), which binds to two operators in the absence of TC (9). The inducer is the antibiotic TC itself, which binds to the Tet repressor and eliminates operator recognition. The induction of resistance must be very efficient to ensure expression of the export protein prior to inhibition of translation by TC. This is reflected by an unusually high association constant of about 10^9 M^{-1} for the Tet repressor-TC complex (6, 8, 21, 22). While these results have been obtained for the Tn*10*-encoded Tet repressor, the extensive homology of primary structures of repressors from the five determinants suggests that this unusually efficient inducibility may be shared by all of them (23). Therefore, it is interesting to study the molecular basis of Tet repressor-TC recognition.

A previous approach to obtain noninducible Tet repressor mutations made use of the facts that anhydro-TC is a potent inducer but a poor antibiotic and that expression of the Tn*10*-encoded TC resistance is toxic for *Escherichia coli* (19). A screen for survivors in the presence of anhydro-TC yielded a number of TetR mutations which were clustered between positions 64 and 107 and which caused a noninducible phenotype for anhydro-TC (19). A preliminary in vitro analysis of some of these mutants indicated that they were impaired in TC binding (19). We have designed a new genetic selection system for noninducible TetR mutations in the presence of TC and noticed that most of the mutants with a noninducible phenotype for TC are still inducible by anhydro-TC. Therefore, we have isolated and characterized an extensive set of TetR mutants with a noninducible phenotype for TC in vivo and describe in this report that they extend over nearly the entire primary structure of TetR and that many of them retain inducibility by anhydro-TC. The chemical structures of TC and anhydro-TC are depicted in Fig. 1.

The experimental scheme used for saturating mutagenesis of *tetR* from Tn*10* is depicted in Fig. 2. Random mutagenesis was performed on the coding and noncoding single strands of *tetR*. Since this procedure yielded multiple mutations in *tetR*, three fragments of the gene of roughly 200 bp each were subcloned into wild-type *tetR* on pWH1919. This resulted in mostly single mutations in the respective section



tetracycline



anhydrotetracycline

FIG. 1. Chemical structures of tetracycline and anhydrotetracycline.

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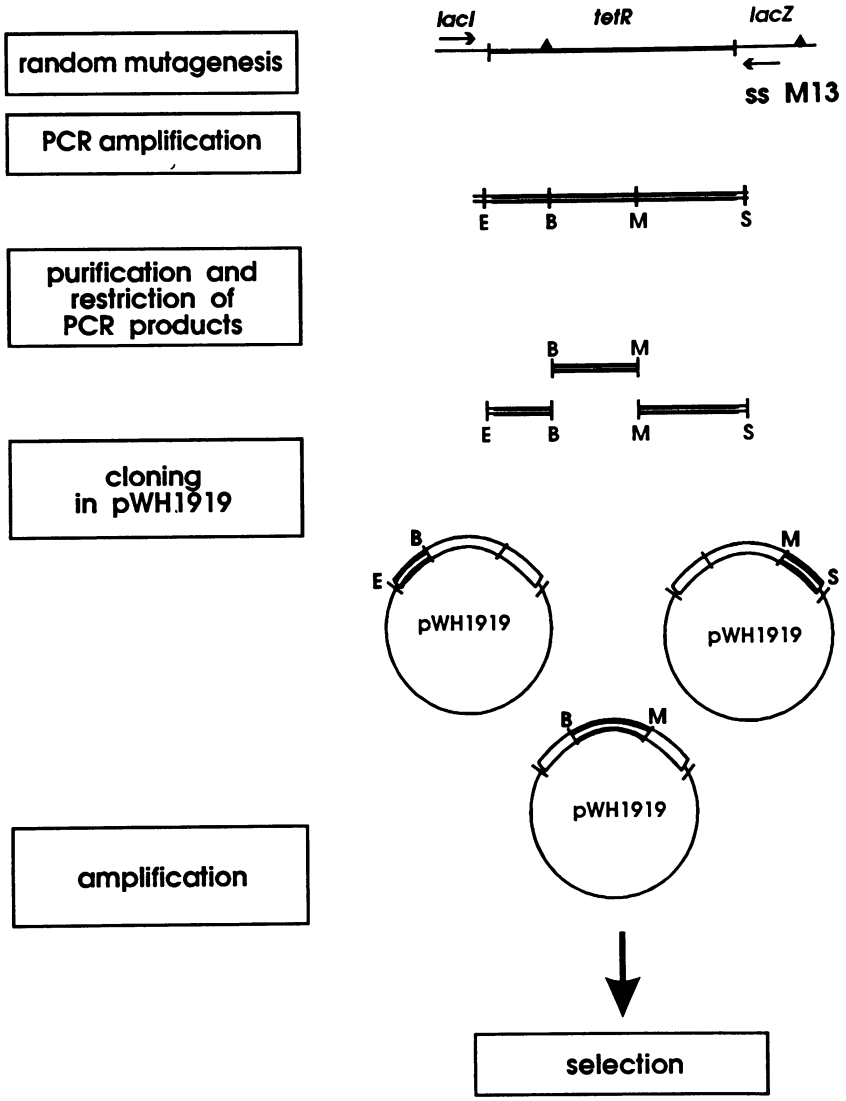

FIG. 2. Scheme of saturation mutagenesis of *tetR*. mWH819 (24) was used to introduce unique restriction sites in *tetR* by oligonucleotide-directed mutagenesis (12, 20). The L 34 codon CTA in *tetR* was changed to CTT, creating a recognition site for *Bst*XI. The N 129, A 130, and L 131 codons were changed to the sequence AACGCGTTA containing an *Mlu*I site. This mWH819 derivative was called mWH892. The same construction in M13mp18 (16) was designated mWH893. The 808-bp *Eco*RI-*Sph*I fragment was cloned into *Eco*RI-*Sph*I-digested pWH1201 (1). The resulting plasmid, pWH1919, is a pWH520 derivative (5). A region-specific mutagenesis was performed as described before (2). Single-stranded (ss) DNAs (40 μg in 40 μl of Tris-EDTA buffer) of mWH892 and -893 were mixed with 10 μl of 2.5 M sodium acetate (pH 4.3) and 50 μl of sodium nitrite ranging between 0.25 M and 2 M and incubated for 45 to 60 min at room temperature. Furthermore, the same amount of DNA was incubated with 60 μl of 18 M formic acid for 6 to 10 min at room temperature and, in a separate reaction, for the same time with 60 μl of 12 M hydrazine. Each of the DNA samples was treated as described previously (2), and the complementary strand was synthesized by using reverse transcriptase (not shown) starting with primer 5′-CCTTGGTGATCAAATAATTC (coding strand of *tetR*) or 5′-CGCCGTACTGCCCGCTTGG (noncoding strand of *tetR*). Alternatively, the *tetR* sequence was amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase as shown. The products were digested with *Eco*RI (E), *Bst*XI (B), *Mlu*I (M), and *Sph*I (S), and each of the three fragments was gel eluted and cloned (7, 14) into the respective sites of pWH1919. The resulting pool of *tetR* mutations was first amplified in *E. coli* DH5α and then transformed into the selection system (25).

of the gene. After amplification in *E. coli* DH5α, this approach yielded an extensively mutagenized *tetR* pool which was transformed into *E. coli* WH207(λWH25)/pWH414. The basic construction of this genetic system has been described previously in detail (25). It allows growth on minimal medium with galactose as the sole carbon source only when the Tet repressor interacts productively with the *tet* operator and blocks *lacI* expression, which, in turn, allows expression of *galK*. In addition, β-galactosidase (β-Gal) expression is repressed under these conditions, serving as a second marker.

In the presence of TC, these phenotypes occur with *tetR* mutations deficient in induction. Mutants that are only partially deficient in induction will nevertheless fail to grow on galactose minimal medium, because they synthesize small amounts of Lac repressor. To isolate these types of mutants, the stringency of selection was reduced by adding IPTG (isopropyl-β-D-thiogalactoside). The concentration of

TABLE 1. Induction efficiencies of wild-type and mutant Tet repressors by TC and anhydro-TC[a]

Inducer	β-Gal activities obtained with *tetR* allele (%)										
	Wild type	EK23	EA37	DG53	LS60	HR64	HQ64	NS82	SN85	SG85	FS86
None	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
TC	81.0 ± 0.9	16.3 ± 0.1	5.3 ± 0.1	4.0 ± 1.0	23.3 ± 0.7	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.6 ± 0.2	7.3 ± 0.3	0.1 ± 0.0
Anhydro-TC	96.8 ± 2.2	63.9 ± 1.5	40.0 ± 4.7	32.4 ± 0.5	95.9 ± 1.7	64.6 ± 3.0	66.3 ± 0.6	39.8 ± 6.6	82.8 ± 3.9	87.7 ± 5.6	28.3 ± 6.1

[a] The induction experiments were performed with *E. coli* WH207/pWH1012 (18, 25) transformed with pWH1919 or its mutants. Cells were grown in LB medium supplemented with the appropriate antibiotics. TC and anhydro-TC were freshly suspended in methanol or water for each experiment. The final concentration was 0.2 mg/liter for both the overnight and the log cultures. β-Gal assays were done as described previously (15) and repeated at least twice. The β-Gal expression in the absence of a *tetR* allele was set to 100% and corresponds to about 270 U, as defined by Miller (15).

IPTG was adjusted to induce only a small amount of Lac repressor. The upper limit was set by using the TetR mutant AD89, which shows a fourfold-reduced inducibility by TC (25). *E. coli* WH207(λWH25)/pWH414 (see reference 25 for details) was transformed with pWH1919 or pRT240 AD89, which express wild-type TetR and TetR AD89, respectively. The strains were selected for growth on galactose in the absence of IPTG and with IPTG concentrations varying between 10^{-6} and 10^{-2} M. Both strains grew alike at 10^{-3} M IPTG and did not grow at 10^{-2} M IPTG, which may be a toxic concentration for *E. coli*. In the presence of 0.5 mg of TC per liter, only the strain with pRT240 AD89 grew at 10^{-3} M IPTG, whereas the strain with pWH1919 did not. Thus, mutants which are at least as impaired in induction as TetR AD89 can be selected by growth on galactose in the presence of 0.5 mg of TC per liter and 10^{-3} M IPTG. Colonies with a white or light blue phenotype were restreaked, and the mutagenized *tetR* portion was sequenced. This approach yielded 93 *tetR* mutations with single amino acid replacements as shown in Fig. 3. The TetR mutant EA37 from a previous study (24) was also included in the further analysis.

The mutations obtained by Smith and Bertrand (19) map within a distinct region of the primary structure of TetR between residues 64 and 107 (Fig. 3). The mutations obtained here span a considerably larger region of the Tet repressor primary structure, ranging from residues 23 to 196 (Fig. 3). The first, TetR EK23, is located N terminal from the proposed α-helix-turn-α-helix operator binding domain and C terminal of a proposed α-helix preceding that motif (5, 10, 11). TetR EK37 and EA37 are in the turn of that motif, and

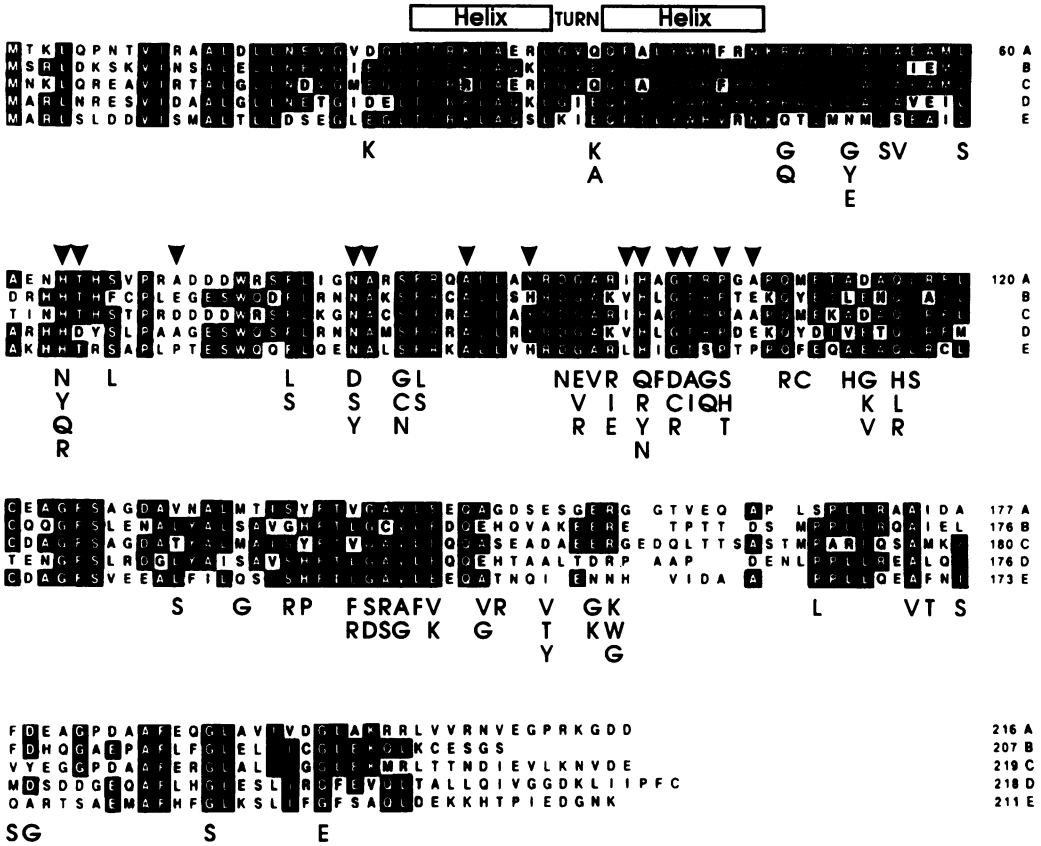

FIG. 3. Location of TetR[s] mutations. The primary structures of five naturally occurring *tetR* genes (23) are compared. Amino acids are given in one-letter abbreviations in inverted print when at least three of five residues are identical. Previously identified mutants with a TetR[s] phenotype for anhydro-TC are indicated by triangles (19). TetR[s] mutations for TC isolated in this work are indicated by amino acids in the one-letter code below the comparison of the five Tet repressor classes.

TABLE 1—*Continued.*

β-Gal activities obtained with *tetR* allele (%)												
DN95	RQ104	PT105	QR109	QR116	LS131	CR144	VA145	EG150	HR151	PL167	DG178	GE196
0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	9.0 ± 0.2
26.0 ± 0.2	18.0 ± 4.9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	35.2 ± 1.7	9.2 ± 0.8	30.5 ± 0.5	10.1 ± 1.5	5.9 ± 0.9	15.2 ± 1.2	0.2 ± 0.0	41.0 ± 2.4
81.2 ± 1.8	67.6 ± 2.9	0.7 ± 0.1	39.3 ± 9.3	1.3 ± 1.1	102.5 ± 0.8	92.5 ± 1.6	91.4 ± 0.8	84.3 ± 4.1	72.1 ± 0.6	100.7 ± 2.9	61.0 ± 4.0	101.6 ± 0.7

TetR RG49 and RQ49 are just C terminal of it. Since induction requires a conformational change of the operator binding motif, it makes sense to find noninducible mutations in that region of the repressor.

Two regions of Tet repressor with clustered mutations are found, namely, between residues 95 and 117 and residues 131 to 158 (Fig. 3). Whereas the first cluster of mutations overlaps to some extent with the previously described region (19), the C-terminal region has not been recognized as being important for induction before. A high frequency of mutations scattered over the entire sequence is found in the region between the α-helix-turn-α-helix motif and the first cluster. For four positions, D53, H64, N82, and S85, three or more mutations were found. No mutation was found in the region between the two blocks from residues 118 to 130. A few mutations are located between residues 167 and 196 at the C terminus of the repressor. Taken together, these results indicate that TetR^s mutations are spread over the entire TetR structure with two regions of increased frequency.

To quantitate the inducibility of TetR^s mutations, 23 of the 93 pWH1919 derivatives with single mutations were transformed into *E. coli* WH207/pWH1012 containing a *tetA-lacZ* fusion (18). The β-Gal expression was determined at 37°C in the absence of TC and with 0.2 mg of TC and 0.2 mg of anhydro-TC per liter. The results are presented in Table 1. They confirm that inducibility by TC is severely reduced in all mutants, albeit to different extents. TetR GE196 is inducible to about 40% of the β-Gal activity obtained with fully induced wild-type TetR and seems to represent the highest inducibility obtained in this selection. However, the inducibility of most of the mutants is much lower, with <10% of that obtained with the wild type. Only TetR DN95, LS131, VA145, and GE196 show a higher inducibility. Western blots (immunoblots) (24) with extracts from some of these mutants confirmed their roughly identical steady-state protein levels compared with the wild type (data not shown). This indicates that the lack of inducibility is an intrinsic property of the respective TetR mutant. Only a few possibilities could lead to this phenotype. The first would be impaired binding of TC, and the second would involve an impaired allosteric structural change of the mutant proteins so that they could bind TC but would not release the *tet* operator. These phenotypes can only be distinguished by in vitro binding studies. Formally, a superrepressor phenotype could also lead to these expression properties. Increased operator binding is very unlikely for the majority of the mutations, because they do not map in the α-helix-turn-α-helix *tet* operator binding motif (1, 3, 11, 24). TetR EA37 is not a superrepressor, as determined previously (24). The genetic system employed here is not suited to reveal a superrepressor phenotype. However, the results obtained with these 23 TetR^s mutations characterized in vivo indicate that the genetic selection yields the desired phenotype.

Induction studies with anhydro-TC reveal increased inducibility of most TetR^s mutations (Table 1). This cannot simply be due to a possible increased uptake of anhydro-TC or the previously reported better binding of that analog to the Tet repressor (6) because the induction efficiencies of TC and anhydro-TC are not correlated for various TetR^s mutations. For example, the induction efficiency of TetR LS60 is 23% with TC and 96% with anhydro-TC compared with TetR DN95, which is 26% for TC and only 81% for anhydro-TC. In contrast to TetR DN95, the mutants TetR SG85 and CR144 are 88 and 92% inducible with anhydro-TC, but only 7 and 9% inducible by TC, respectively. This suggests different induction or binding mechanisms for TC and anhydro-TC which would resemble their different mechanisms of antibiotic activities reported recently (17). Only some of the TetR^s mutations show a clearly reduced inducibility for anhydro-TC. They fall into two separate groups. One contains mutations inducible to about 30 to 40% with TetR EA37, DG53, NS82, FS86, and QR109. The other contains two mutants, namely, PT105 and QR116, which are completely noninducible by anhydro-TC. The mutations located in the region where previously reported TetR^s mutations with a noninducible phenotype for anhydro-TC occurred are about 60% inducible by anhydro-TC, except TetR PT105. This indicates that the selection used here yields a broader class of TetR^s mutations which can be differentiated by their various inducibilities with anhydro-TC.

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