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Sequence of a Class E Tetracycline Resistance Gene from *Escherichia coli* and Comparison of Related Tetracycline Efflux Proteins

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We determined the nucleotide sequence of the class E *tetA* gene on plasmid pSL1456 from *Escherichia coli* SLH1456A. The deduced amino acid sequence of the class E TetA protein shows 50 to 56% identity with the sequences of five related TetA proteins (classes A through D and G). Hydrophobicity profiles identify 12 putative transmembrane segments with similar boundaries in all six TetA sequences. The N-terminal α domain of the six sequences is more highly conserved than the C-terminal β domain; the central hydrophilic loop connecting the α and β domains is the least conserved region. Amino acid residues that have been shown to be important for class B (Tn*10*) TetA function are conserved in all six TetA sequences. Unlike the class B *tetA* gene, the class D and E *tetA* genes do not exhibit a negative gene dosage effect when present on multicopy plasmids derived from pACYC177.

Tetracycline resistance genes have been grouped into more than 12 classes on the basis of their lack of cross-hybridization under stringent conditions (for reviews, see references 5, 22, and 40). In addition, three biochemically distinct mechanisms of resistance have been identified: (i) energy-dependent tetracycline efflux, (ii) ribosome protection, and (iii) tetracycline modification. In members of the family *Enterobacteriaceae* and related genera, resistance is most often due to a family of related *tet* genes (classes A through E and G), all of which mediate tetracycline efflux (26, 27, 29, 62). The plasmids and transposons that define these six classes are RP1 and Tn*1721* (class A), Tn*10* (class B), pSC101 and pBR322 (class C), RA1 and pIP173 (class D), pSL1456 (class E), and pJA8122 (class G). In each case, the resistance determinant consists of two genes: *tetA*, which encodes an inner membrane resistance protein, and *tetR*, which encodes a repressor protein (24). Tetracycline induces transcription of both genes (9, 20). Recent studies of the Tn*10* TetA protein have shown that it functions as a tetracycline/proton antiporter and that the species transported out of the cell is a divalent metal-tetracycline complex (57, 60). Sequences for both *tetA* and *tetR* have been reported for class A (52), class B (*tetA* [19, 34]; *tetR* [37]), class C (*tetA* [36]; *tetR* [46]), class D (*tetA* [2]; *tetR* [47]), and class G (62). For class E, the sequence of the *tetR* gene but not the *tetA* gene has been reported (45).

Class E tetracycline resistance genes were initially identified on the plasmid pSL1456, a large transferable plasmid isolated from *Escherichia coli* SLH1456A (26). Although class E genes are rare among tetracycline-resistant *E. coli* strains, they are prevalent among *Aeromonas* species (26) and have recently been identified in *Vibrio salmonicida* (43). Marshall et al. (26) localized the pSL1456 *tetA* and *tetR* genes to a 3.5-kb *Bam*HI-*Pvu*I fragment on pSL1503, which they constructed by cloning the pSL1456 *tet* region into pACYC177.

Class E *tetA* sequence. Tovar et al. (45) identified a *Cla*I site in the *tetA* promoter-operator region of pSL1503. We determined the nucleotide sequence of the 1,268-bp region extending from this *Cla*I site downstream through the *tetA* structural gene (Fig. 1). The sequencing was done by the dideoxy method with [α-^{35}S]dATP and Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio). The 1.1-kb *Cla*I-*Pvu*II fragment from pSL1503 (Fig. 1, bp 1 to 1057) was cloned between the *Acc*I and *Sma*I sites of both M13mp18 and M13mp19 and sequenced with a universal primer. An internal primer (JAE2; CAGCCCGAACACAGCCAATG) was used to extend the sequence across the middle of this fragment. To sequence the 3′ end of *tetA*, another internal primer (JAE1; TTCGTGTCGGTCAGGTAGCA) was used on double-stranded pSL1503 DNA. Finally, to obtain a complementary strand sequence, the 464- and ~1,300-bp *Hin*cII fragments from pSL1503 (bp 14 to 478 and 478 to 1268+) were cloned into the *Sma*I site of M13mp18 and the *tetA* regions were sequenced with a universal primer. If translation initiates at the first AUG codon in the open reading frame, the pSL1503 *tetA* gene encodes a 405-amino-acid protein (calculated M_r = 43,469). In contrast to those of the class A through D and G *tetA* genes, the probable Shine-Dalgarno sequence for the pLS1503 *tetA* gene (GAGAA; bp 8 to 12) overlaps the adjacent *tet* operator sequence (CCCTATCATCGATAGAG; bp −7 to +10). The sequence reported by Tovar et al. (45) (EMBL accession number X14035) overlaps bp 1 to 26 of the sequence shown in Fig. 1.

Base composition. The class A through E and G *tet* genes differ significantly from each other in terms of their base composition. The G+C contents for *tetA* and *tetR*, respectively, are as follows: class B, 43 and 39%; class E, 47 and 47%; class D, 58 and 55%; class G, 58 and 60%; class C, 62 and 59%; and class A, 64 and 64%. These differences are even more striking in the third position of codons, where the G+C contents are as follows: class B, 39 and 31%; class E, 43 and 46%; class D, 64 and 59%; class G, 60 and 69%; class C, 73 and 68%; and class A, 75 and 75%. By comparison, the G+C contents for *E. coli* genes that encode proteins are 52% overall and 56% in the third position of codons (51). Not

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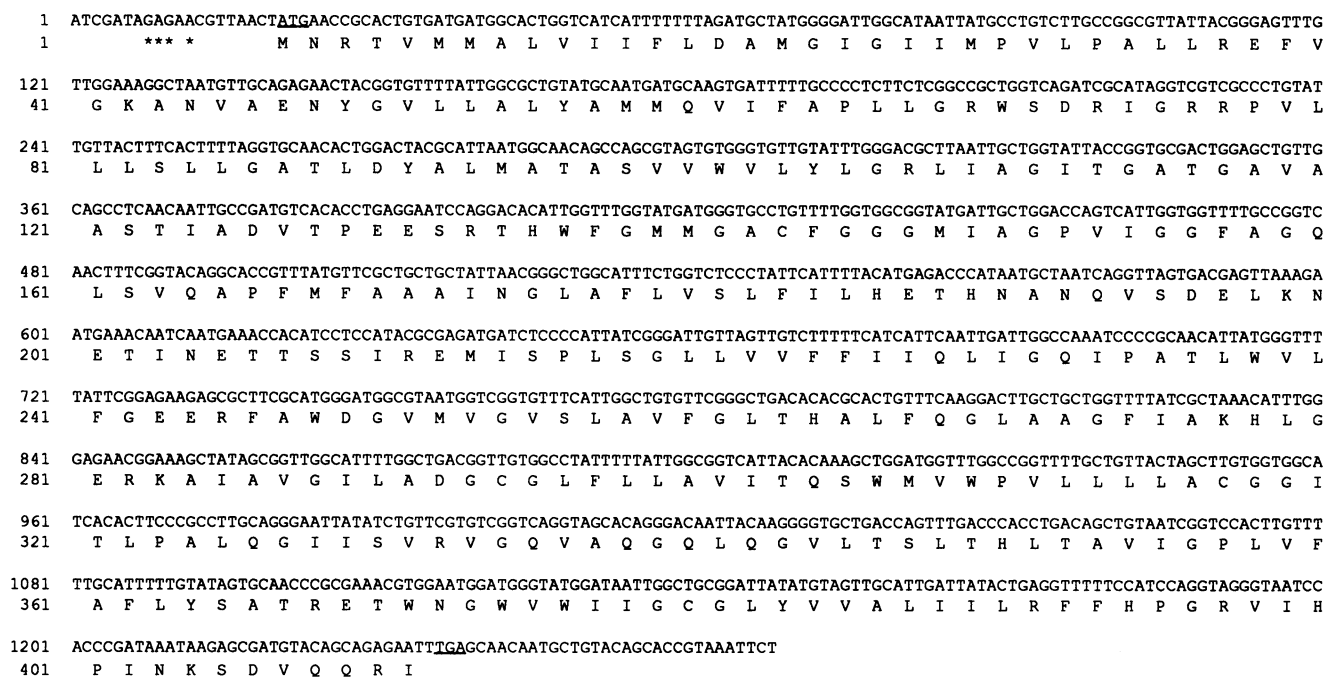

FIG. 1. Nucleotide sequence of the class E *tetA* gene from pSL1503. The deduced amino acid sequence of the TetA protein is shown below the nucleotide sequence. The initiation and termination codons for TetA are underlined, and the probable Shine-Dalgarno sequence is marked by asterisks.

surprisingly, the *tet* genes that differ most from *E. coli* genes in their G+C content show a corresponding difference in codon usage. Thus, the class B and A genes show a strong bias toward codons with A or U and with G or C, respectively, in the third position. These differences in G+C content presumably reflect differences in the base composition of the organisms in which the *tet* genes diverged from their common ancestral sequences (35).

Comparison of class A through E and G TetA sequences. Figure 2 shows an alignment of the deduced amino acid sequences of the class A through E and G TetA proteins. Sequence comparisons were performed by using version 7.0 of the University of Wisconsin Genetics Computer Group (UWGCG) software package (10). The six TetA sequences are identical at 129 of 405 positions (32% six-way identity) and similar at an additional 76 positions (51% six-way similarity). As noted for class A through D TetA (1, 2, 12, 17), hydrophobicity profiles of the class E TetA sequence suggest a 12-transmembrane-segment model for the topology of TetA in the inner membrane (Fig. 2 and 3). In fact, parallel analyses of the class A through E and G TetA sequences, with several different algorithms for identifying potential transmembrane segments (13, 14, 50), all identify 12 potential transmembrane segments with boundaries very similar to those shown in Fig. 2 for class E TetA (data not shown). Protease accessibility and antibody binding studies of class B TetA (12, 55) and *tetA-phoA* gene fusion studies of class C TetA (1) support many features of this model.

Figure 3 summarizes the sequence data in relation to the predicted membrane topology of class E TetA. In the following discussion, transmembrane segments are designated TM1 through TM12, and periplasmic and cytoplasmic segments are designated P1 through P6 and C1 through C7, respectively (1). As noted for the class A through D sequences (2, 8, 34), the central hydrophilic loop (C4) of the class E sequence shows little similarity to the other sequences. Additionally, the class E sequence has a 1-amino-acid insertion in this region relative to the class A through C and G sequences and a 3-amino-acid insertion relative to the class D sequence. Genetic studies of class B TetA suggest that the central region divides the TetA protein into two functional domains, an N-terminal α domain and a C-terminal β domain (8, 38). Overall, the α domain is more highly conserved than the β domain; the region from amino acid 1 to amino acid 200 shows 38% six-way identity, whereas the region from amino acid 201 to the C terminus shows 26% six-way identity. On the basis of the low, but statistically significant, degree of sequence similarity between the α and β domains of class A through C TetA, Rubin et al. (39) proposed that a gene duplication may have given rise to the common ancestor of the class A through C *tetA* genes. Comparisons of the α and β domains of class D, E, and G TetA also support this idea. Overall, the transmembrane regions are more highly conserved than the connecting loops (39 versus 20% six-way identity). However, a few of the connecting loops are also highly conserved (e.g., P1, P2, and C2). The R/KXGRR motif, which is present in a number of bacterial transport proteins (15, 17), is conserved with minor differences in the C2 loops of all six TetA sequences, and a variant of this motif, R/KXGER, is conserved with minor differences in the C5 loops.

Cytoplasmic membrane proteins generally have more positively charged amino acids flanking the cytoplasmic ends than the periplasmic ends of transmembrane segments (49). In class A, B, D, and G TetA, all 12 predicted transmembrane segments follow this positive-inside rule (Fig. 2). Class C has one exception (TM10), and class E has three exceptions (TM1, TM4, and TM11); in each of these cases, there

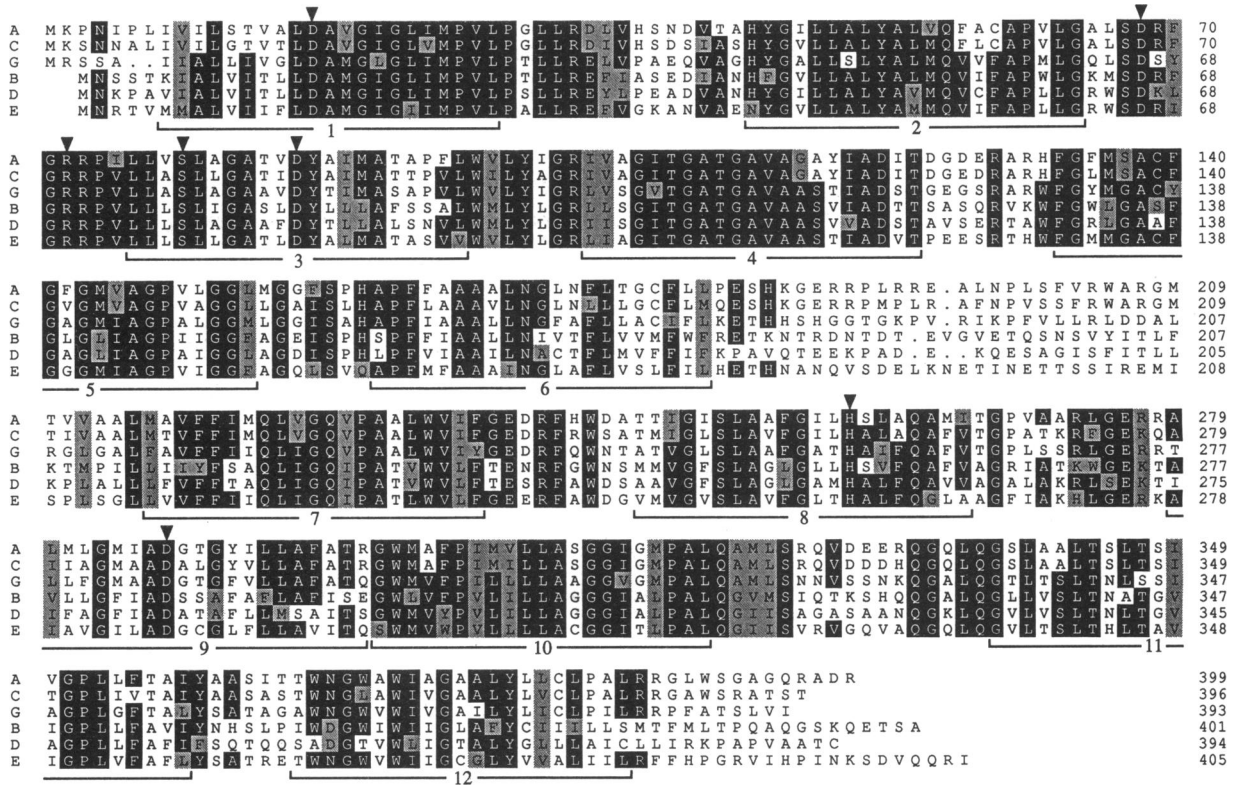

FIG. 2. Alignment of class A through E and G TetA amino acid sequences. The figure was generated by using the UWGCG programs PILEUP and PRETTYBOX. Black and grey backgrounds indicate identity and similarity, respectively. Numbered brackets indicate predicted transmembrane segments in class E TetA, determined by using the hydrophobicity scale of Engelman et al. (14), with a window size of 21. Arrowheads indicate amino acids that have been implicated in TetA function. Classes: A, Tn*1721* (NBRF accession number YTECR1) (52); B, Tn*10* (NBRF accession number YTECT0) (34); C, pBR322 (NBRF accession number YTEC32) (36); D, pIP173 (EMBL accession number X65876) (2); E, pSL1503 (GenBank accession number L06940) (this work); and G, pJA8122 (GenBank accession number S52437) (62).

are equal numbers of positive charges on both sides of the transmembrane segment.

Site-directed mutagenesis studies of the Tn*10* TetA protein (class B) have identified a number of amino acid residues that are important for TetA function. These include Asp-15 in TM1 and Asp-84 in TM3, which may be involved in substrate binding (28, 56); His-257 in TM8 and Asp-285 in TM9, which may be involved in proton translocation (54, 56); Asp-66 and Arg-70 in the C2 loop, which have been proposed to play a gating role in tetracycline transport (57, 59); and Ser-77 in TM3, which may be in the substrate translocation pathway (58). Site-directed mutagenesis studies of the Asp residues in TM1, TM3, and TM9 of the pBR322 TetA protein (class C) also confirm the importance of these residues in TetA function (1). As shown in Fig. 2 and 3, all of these residues are conserved in all six TetA sequences.

In a survey of the photosynthetic reaction centers, Schiffer et al. (41) noted that Trp residues tend to be clustered on the periplasmic face of the complex. They suggested that the hydrophobic character and hydrogen-bonding capability of Trp help to orient and anchor the transmembrane and periplasmic segments. The distribution of Trp residues in TetA follows a similar pattern. In particular, all five of the conserved Trp residues (Trp-95, Trp-232, Trp-242, Trp-300, and Trp-370 in class E TetA) are located either in a predicted periplasmic segment or near the periplasmic end of a transmembrane segment. Genetic studies of class B TetA suggested that TetA functions as an oligomer (18). In this regard, it is unlikely that either interchain or intrachain disulfide bonds play a role in the structure of TetA. None of the Cys residues in class A, C, D, E, and G TetA is conserved, and the single Cys residue in class B TetA (Cys-377) can be mutated to Ser without loss of function (our unpublished data).

We used the UWGCG program GAP to generate pairwise sequence alignments between the class A through E and G TetA sequences. The A-C and B-D sequence pairs are the most closely related (78 and 60% identity, respectively). In contrast, the A-C, A-D, C-B, and C-D sequence pairs are the least similar (45 to 46% identity). The class G sequence is more closely related to the A-C sequence pair (60 to 61% identity) than to the B-D sequence pair (50 to 52% identity), and the class E sequence is intermediate to the other five sequences (50 to 56% identity). Although the percent identity provides an intuitively straightforward measure of sequence similarity, the GAP alignment quality ratio provides a more precise measure since it takes into account all aspects of the alignment. These sequence relationships are summarized graphically in the upper portion of Fig. 4. Similar relationships exist for the corresponding class A through E and G TetR sequences (45, 62).

Comparison of class A through E and G TetA with other drug efflux proteins. Many bacterial transport proteins, including a number of drug efflux proteins, show sequence similarities with class A through C TetA (15, 23, 25). Among

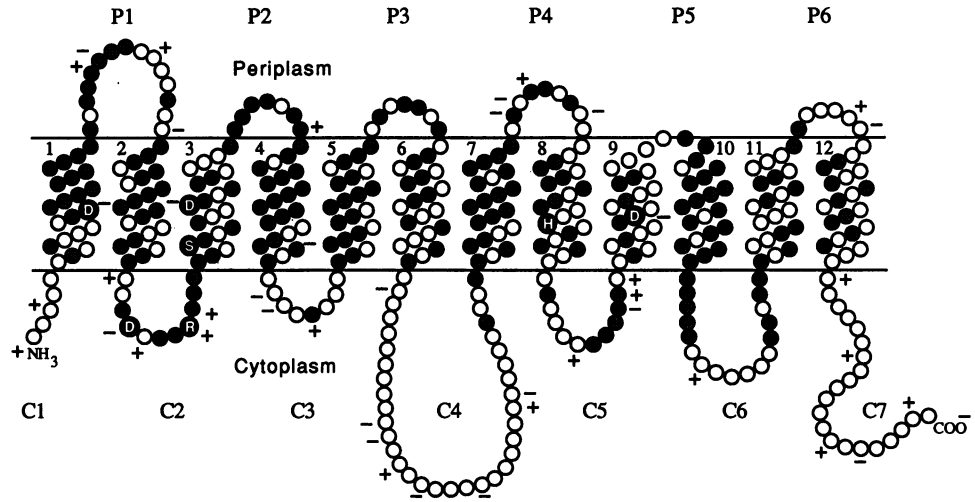

FIG. 3. Model of class E TetA membrane topology. The boundaries of the 12 predicted transmembrane segments are the same as those shown in Fig. 2. Periplasmic and cytoplasmic segments are labeled P1 through P6 and C1 through C7, respectively. Charged amino acids are indicated by + (Arg or Lys) and − (Asp or Glu). Black and grey circles, positions that are identical and similar in all six TetA sequences, respectively. Conserved amino acids that have been implicated in TetA function are indicated by white letters D, S, R, and H in black circles.

the sequences reported to date, the multidrug resistance protein (Bmr) from *Bacillus subtilis* (33) and the quinolone resistance protein (NorA) from *Staphylococcus aureus* (61) are the most closely related to class A through C TetA (25 to 26% identity). In addition, there are a number of proteins that show lower levels of sequence similarity with class A through C TetA. These include the Tet(K) and Tet(L) tetracycline efflux proteins from gram-positive bacteria (21, 30). We used GAP to generate pairwise alignments between sequences of class A through E and G TetA, Bmr, NorA, Tet(K), and Tet(L) (Fig. 4). To assess the statistical significance of the alignment scores, we compared them with the means of 100 alignment scores generated by randomizing one of the two sequences. For the alignments between either Bmr or NorA and class A through E and G TetA, the alignment scores were 16 to 19 standard deviations above the randomized means. For the alignments between either Tet(K) or Tet(L) and class A through E and G TetA, the alignment scores were 6 to 9 standard deviations above the randomized means. Finally, the class A through E and G TetA proteins also belong to a very large family of prokaryotic and eukaryotic transport proteins that appear to share a similar 12-transmembrane-segment structure (15, 23, 25).

Effect of gene dosage on tetracycline resistance. Multicopy plasmids carrying the Tn*10* *tetR* and *tetA* genes confer a substantially lower level of tetracycline resistance than low-copy-number plasmids or Tn*10* in the chromosome (7, 44). This negative gene dosage effect was initially attributed to reduced expression of *tetA*; however, subsequent work suggested that it was due to the toxicity associated with increased expression of *tetA* (9, 31, 32, 42). More recently, Eckert and Beck (11) showed that high-level expression of Tn*10* *tetA* results in loss of cytoplasmic membrane potential. In contrast to the class B *tetA* gene, the class A and C *tetA* genes exhibit a positive gene dosage effect (3, 53). To compare the effect of gene dosage on tetracycline resistance for the class B, D, and E *tetA* genes, we constructed a set of isogenic single-copy strains and a parallel set of multicopy strains.

The single-copy strains contain derivatives of pUC8 (48) integrated into the chromosome of the *polA* strain NO3434 via *lac* homology. This strategy exploits the fact that ColE1-like replicons cannot replicate in *polA* strains (16). Derivatives of pUC8 containing the class B, D, and E *tetR* and *tetA* genes were constructed as follows: (i) pBA1 (class B) contains the 2.8-kb *Bgl*II fragment from pBT1010 (32) inserted into the *Bam*HI site of pUC8, (ii) pBV1 (class D) contains the 3.5-kb *Sal*I-*Hin*dIII fragment from ColE1ΩG (2) inserted between the *Sal*I and *Hin*dIII sites of pUC8, and (iii) pBA2 (class E) contains the 3.6-kb *Bam*HI-*Pst*I fragment from pSL1503 inserted between the *Bam*HI and *Pst*I sites of

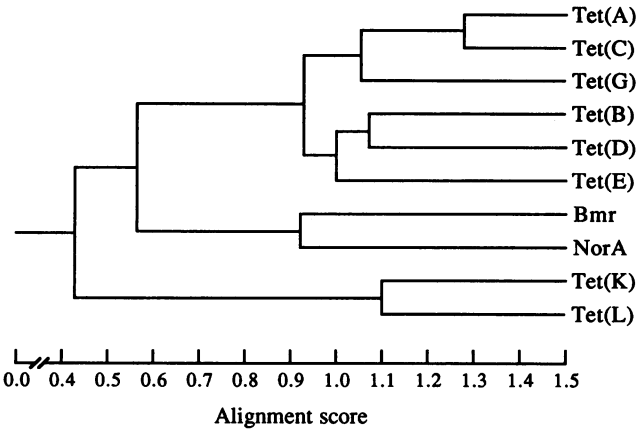

FIG. 4. Sequence relationships between class A through E and G TetA and other drug efflux proteins. The figure was generated by using the UWGCG program PILEUP and is based on the alignment quality ratios from the program GAP. Branch points were calculated by averaging scores of all pairwise alignments between the members of two branches. The TetA proteins of classes A through E and G are designated Tet(A) through Tet(E) and Tet(G), respectively. The sources of the sequences of Tet(A) through Tet(E) and Tet(G) are as described in the legend to Fig. 2. The sources of the other sequences are as follows: Bmr, GenBank accession number BACBMRX (33); NorA, GenBank accession number STANORA (61); Tet(K), SWISS-PROT accession number TCR_STAAU (30); and Tet(L), NBRF accession number YTBSRT (21).

TABLE 1. Effect of gene dosage on tetracycline resistance

Plasmid copy no. and *tet* class	Plasmid	MIC (μg/ml)[a]
Single copy[b]		
B	pBA1	240
D	pBV1	280
E	pBA2	160
Multicopy[c]		
B	pBT107	40
D	pBA3	300
E	pSL1503	240

[a] MICs of tetracycline determined by the agar plate dilution method (32). Values are means for duplicate experiments with two separate isolates. The MIC in the absence of any resistance gene was 3 μg/ml.

[b] *tetR*$^+$ *tetA*$^+$ derivatives of pUC8 integrated into the chromosome of *polA* strain NO3434 via *lac* homology.

[c] *tetR*$^+$ *tetA*$^+$ derivatives of pACYC177 in the *polA*$^+$ strain JA221.

pUC8. The *tetA* and *lacZ* promoters are in the same orientation in all three pUC8-*tet* plasmids.

Derivatives of NO3434 (*polA1 lysA rpsL*) (received from M. Nomura, University of California, Irvine) with the pUC8-*tet* plasmids integrated into the chromosome were constructed in three steps. First, the temperature-sensitive *polA* strain CP826 [*polA12*(Ts) *thr leu his rpsL*, Δ*cheA-Z* Km^r] (received from G. Hazelbauer, Washington State University) was transformed with monomeric plasmid, and Ap^r Lac$^+$ transformants were selected at 30°C on lactose-MacConkey agar containing 100 μg of ampicillin per ml. Second, purified transformants were grown at 42°C for 24 to 48 h, and Ap^r Tc^r Lac$^-$ clones were identified on lactose-MacConkey agar containing 25 μg of ampicillin and 5 μg of tetracycline per ml. Homologous recombination between the *lacZ*$^\alpha$ sequences in the plasmids and the *lacZ* gene in the CP826 chromosome yielded Ap^r Tc^r Lac$^-$ bacteria with plasmids integrated into the chromosome. Third, integrated plasmids were transferred from CP826 to NO3434 by P1 transduction, with selection for Ap^r Tc^r Lac$^-$ transductants at 37°C on lactose-MacConkey agar containing 25 μg of ampicillin and 5 μg of tetracycline per ml. The frequency of chromosomal integration was sufficiently low that it was difficult to obtain integrates by direct transformation of NO3434.

As shown in Table 1, MICs of tetracycline for the single-copy strains follow the order class D > class B > class E. This result differs somewhat from the class B > class D > class E order reported previously (26, 29). It may be significant that the class D *tetA* gene in our work was derived from pIP173 rather than RA1; however, the *tetR* genes from these two plasmids are essentially identical (2). In principle, the pUC-*polA* strategy for determining single-copy resistance phenotypes has two advantages: (i) gene copy number can be controlled more precisely than is possible with different naturally occurring low-copy-number plasmids, and (ii) the influence of other plasmid-encoded proteins on tetracycline resistance can be minimized. In a control experiment, integration of the class B plasmid (pBA1) into the chromosome of NO3434 conferred the same level of tetracycline resistance as insertion of Tn*10* into the NO3434 chromosome by transposition.

To determine the effect of gene dosage on tetracycline resistance, we also constructed a set of isogenic multicopy strains. Derivatives of pACYC177 (4) containing the class B, D, and E *tetR* and *tetA* genes were introduced into the *polA*$^+$ strain JA221 (*lacY1 leuB6 thi-1 tonA2 supE44* Δ*trpE5 recA1 hsdR*) (6). pBT107 (class B) (32) contains the 2.8-kb *Bgl*II fragment from Tn*10* inserted into the *Bam*HI site of pACYC177. pBA3 (class D) contains the 3.5-kb *Bam*HI-*Hin*dIII fragment from pBV1 inserted between the *Bam*HI and *Hin*dIII sites of pACYC177. pLS1503 (class E) (26) contains the 3.5-kb *Bam*HI-*Pvu*I fragment from pSL1456 inserted between the *Bam*HI and *Pvu*I sites of pACYC177. As seen previously, the class B genes exhibit a negative gene dosage effect; they confer substantially lower resistance when present on the multicopy plasmid (MICs, 40 versus 240 μg/ml). In contrast, neither the class D nor class E genes exhibit a negative gene dosage effect; indeed, they both confer somewhat higher resistance when present on the multicopy plasmid. It is noteworthy that class D, which is the most closely related to class B and actually confers somewhat higher resistance than class B in the single-copy state, nevertheless shows no negative gene dosage effect. To understand the molecular basis for these differences in resistance phenotypes, it will be necessary to determine the relative levels of TetA protein in single-copy and multicopy strains. Only then will we know whether the resistance phenotypes reflect differences in TetA levels or differences in TetA efficiency and/or toxicity.

Nucleotide sequence accession number. The sequence shown in Fig. 1 is deposited in the GenBank data base under accession number L06940.

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