Evidence that a Novel Tetracycline Resistance Gene Found on Two *Bacteroides* Transposons Encodes an NADP-Requiring Oxidoreductase

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Two transposons, Tn4351 and Tn4400, which were originally isolated from the obligate anaerobe *Bacteroides fragilis*, carry a tetracycline resistance (Tc^r) gene that confers resistance only on aerobically grown *Escherichia coli*. This aerobic Tc^r gene, designated *tetX*, has been shown previously to act by chemically modifying tetracycline in a reaction that appears to require oxygen. We have now obtained the DNA sequence of *tetX* and 0.6 kb of its upstream region from Tn4400. Analysis of the DNA sequence of *tetX* revealed that this gene encoded a 43.7-kDa protein. The deduced amino acid sequence of the amino terminus of the protein had homology with a number of enzymes, all of which had in common a requirement for NAD(P). In an earlier study, we had observed that disrupted cells, unlike intact cells, could not carry out the alteration of tetracycline. We have now shown that if NADPH (1 mM) is added to the disrupted cell preparation, alteration of tetracycline occurs. Thus, TetX appears to be an NADP-requiring oxidoreductase. Tn4400 conferred a fivefold-lower level of tetracycline resistance than Tn4351. This finding appears to be due to a lower level of expression of the *tetX* on Tn4400, because the activity of a *tetX-lacZ* fusion from Tn4400 was 10-fold lower than that of the same fusion from Tn4351. A comparison of the sequence of the *tetX* region on Tn4351 with that on Tn4400 showed that the only difference between the upstream regions of the two transposons was a 4-base change 350 bp upstream of the start of the *tetX* coding region. The 4-base change difference creates a good consensus –35 region on Tn4351 that is not present on Tn4400 and could be creating an extra promoter.

An unusual tetracycline resistance (Tc^r) gene has been found on two closely related *Bacteroides* transposons, Tn4351 and Tn4400 (6, 16, 21). This gene encodes a cytoplasmic protein which detoxifies tetracycline (21, 22). Because the mechanism of this resistance differs from those of the efflux-type resistances (*tetA* to *E* and *tetL*) and the ribosomal protection-type resistances (*tetM* to *O*), we have designated this gene *tetX* (23). Although *tetX* was found originally in *Bacteroides fragilis* strains, it does not confer resistance on these organisms. *tetX* does confer resistance on *Escherichia coli* but only when the bacteria are grown under aerobic conditions (6). Here we report the DNA sequence and deduced amino acid sequence of *tetX* and show that TetX shares amino acid homology with NAD(P)-requiring oxidoreductases.

We have also compared the DNA sequence of the coding and upstream regions of *tetX* from Tn4400 with the corresponding regions on Tn4351. Previously, Smith and Gonda (20) used hybridization analysis to show that the two transposons were virtually identical. However, we had noted that the tetracycline MIC associated with Tn4351 was about fivefold higher than the MIC associated with Tn4400 for cells grown under the same conditions (23). It was possible that sequence differences in the coding regions of the *tetX* genes caused the TetX of Tn4400 to be less active than the TetX of Tn4351. Alternatively, there could be sequence differences in the upstream region of *tetX* which resulted in lower *tetX* expression from Tn4400. Previously, we obtained evidence for a divergently transcribed open reading frame, designated P7, which was upstream of *tetX* on Tn4351 (23). A *lacZ*

translational fusion to this open reading frame, which deleted approximately 200 bp of the upstream region, resulted in a four- to fivefold-lower level of resistance than in clones with an intact upstream region (23). This finding seemed to indicate either that the P7 protein was involved in *tetX* expression or that the *lacZ* fusion in this region eliminated a promoter which was responsible for most of the *tetX* expression from Tn4351. We report here that the only difference between the DNA sequences of Tn4351 and Tn4400 in the *tetX* upstream region is a 4-bp change that is close to the 5' end of the P7 open reading frame but does not lie within it.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strain used for generation of unidirectional deletions and cloning was DH5 α MCR [F[–] *mcrA* Δ (*mrr-hcdRMS-mcrBC*) ϕ 80d *lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *deoR* *recA1* *endA1* *supE44* λ^- *thi-1* *gyrA96* *relA1*]. The regions of Tn4351 and Tn4400 that were sequenced in this study are shown in Fig. 1. Also shown in Fig. 1 are plasmids carrying *lacZ* fusions or deletions which were used to ascertain reading frame or determine the region important for *tetX* expression. Two plasmids not shown in Fig. 1 were used in some experiments. They are pBS1, which is the 3.8-kb *EcoRI* fragment of Tn4351 cloned into pBR328 (20), and pBBS1, the 4.0-kb *EcoRI* fragment of Tn4400 cloned into the same location on pBR328 (23).

For experiments in which β -galactosidase activities of *lacZ* fusions were measured, *E. coli* MLB7060 [F[–] *araC*(Am) *araD* Δ (*argF-lac*)U169 *trp*(Am) *mal*(Am) *rpsL* *relA* *thi*(λ p1048)] was used (1). *E. coli* strains were grown aerobically in Luria broth (LB) containing 0.1% glucose and buffered with 50 mM potassium phosphate buffer. These conditions gave β -galactosidase values which were about

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FIG. 1. Regions of Tn4400 and Tn4351 sequenced in this study. Direct-repeat insertion sequences that flank the antibiotic resistance genes are indicated by thick black lines. Locations of the erythromycin resistance gene (*ermF*) and *tetX* are shown by lines above the transposons. The regions sequenced are indicated by lines below the transposons and other constructs. The double line under *tetX* on Tn4400 indicates that this region was sequenced in both directions. The single line under the same region on Tn4351 indicates that this region was sequenced in only one direction. Below the drawings of the transposons are shown (i) the *lacZ* translational fusions (pBBH9 and pBS134H9) that were used to establish the reading frame of *tetX*, (ii) the deletions ($\Delta 6$ and $\Delta 61$) that were used to locate the beginning of the *tetX* coding region on Tn4400, and (iii) the fusions and deletions that were used to locate the regions of the upstream region involved in *tetX* expression (pBBP37, pBS134P7, pBS134H9 $\Delta 1$, and pBS134H9 $\Delta 2$). In the case of the *lacZ* fusions, the *lacZ* portion is designated by an arrow, which indicates the direction of transcription, and the single line indicates the portion of the Tn4351 or Tn4400 cloned upstream of *lacZ*. The short lines under the fusions indicate the portions of these constructs that were sequenced. Sequencing was done in one direction. In the case of pBB $\Delta 61$ and pBB $\Delta 5$, the single line indicates the segment that was cloned into pUC19. The short line under each clone indicates the region that was sequenced. The arrows next to the superscripts on Tc^r (of pBS134P7) and LacZ⁺ (of pBS134H9 $\Delta 2$) indicate that the tetracycline MIC and β -galactosidase activity, respectively, were lower than in clones containing an intact upstream region.

30% higher than those obtained when cells were grown in LB.

Expression of TetX. Expression of TetX from Tn4400 and Tn4351 was compared by (i) determining tetracycline MIC, (ii) detecting TetX in maxicells, or (iii) measuring the β -galactosidase activities of translational fusions between *lacZ* and *tetX*.

Tetracycline MICs were obtained by broth dilution in LB as described previously (21). For experiments involving maxicells, *E. coli* MCS100 was used, and the procedure was the same as that described by Sancar et al. (19).

Construction of the *tetX-lacZ* translational fusion containing *tetX* sequences from Tn4351 (pBS134H9; Fig. 1), by the selection method of Berman and Jackson (1), has been described previously (21). A *tetX-lacZ* translational fusion

containing *tetX* sequences from Tn4400 (pBBH9; Fig. 1) was constructed in the same manner. Construction of a translational fusion between *lacZ* and an open reading frame in the upstream region of *tetX* on Tn4351, which is transcribed divergently from *tetX* (pBS134P7; Fig. 1), and a similar fusion between *lacZ* and a divergently transcribed open reading frame upstream of *tetX* on Tn4400 (pBBP37; Fig. 1) have been described previously (23). Two deletions in the upstream region of the *tetX-lacZ* from Tn4351 (pBS134H9 $\Delta 1$ and pBS134H9 $\Delta 2$; Fig. 1) were obtained by *Bal*31 digestion starting at the *Eco*RI site upstream of *tetX*.

DNA sequencing. The regions of DNA from Tn4351 and Tn4400 that were sequenced in this study are indicated in Fig. 1. Both strands were sequenced for the *tetX* region from Tn4400, and one strand was sequenced from the same region of Tn4351. Cloning of the 4.0-kb *Eco*RI fragment of Tn4400 or the 3.8-kb *Eco*RI fragment from Tn4351 into pUC19 was done as described by Maniatis et al. (12). Exonuclease III deletions of this *Eco*RI segment of Tn4400 or Tn4351 were generated starting at the *Eco*RI site nearest *tetX* by using the Erase-A-Base kit from Promega Corp. (Madison, Wis.). Although this method for generating nested deletions worked in the case of deletions starting from the *Eco*RI site closest to *tetX*, it was not successful at generating nested deletions of Tn4400 DNA starting from the *Eco*RI site nearest the *ermF* gene. No deletions larger than 1 kb were obtained, so a combination of subcloning and synthesis of primers based on available sequence information was necessary to obtain the second-strand sequence.

All of the DNA segments generated by nested deletions were sequenced from double-stranded templates by the dideoxy-chain termination reaction with T7 polymerase and reagents provided in the Sequenase 2.0 kit (U.S. Biochemicals). For sequencing of the second strand of Tn4400, the *Eco*RI-*Cla*I segment was sequenced as double-stranded DNA. We were unable to obtain readable sequence of the remaining part of the second strand of Tn4400 by using double-stranded templates, even when *Taq* polymerase was used instead of T7 polymerase. Most of the remainder of the second strand of Tn4400 was sequenced as single-stranded DNA (15). Even with this method, we were unable to get a clear sequence for the 300 bp immediately downstream of the *Cla*I. However, the two independent sequences of the original strand of Tn4400 and Tn4351 in this region agreed and could be read without ambiguities. Also, this region was not important for the questions being asked. Thus, no further attempt was made to obtain the second-strand sequence of this 300-bp region.

To determine the correct reading frame for TetX and the divergently transcribed protein in the upstream region (P7 and P37), the DNA sequence of the fusion joint was obtained from the *tetX-lacZ* fusions (pBS134H9 and pBBH9) and from the *lacZ* fusions to the P7 and P37 open reading frames (pBS134P7 and pBBP7). The primer 5'-ATTGAGGCTGCGCAACTGTT-3', which starts at base 147 in the coding region of *lacZ*, was used for this sequence determination.

Computer analysis of the sequence and comparisons of the deduced amino acid sequence with amino acid sequences of other proteins were done by using University of Wisconsin Genetics Computer Group software on a MicroVAX computer system (4).

Activity of TetX in disrupted cells. *E. coli* EM24 cells (1.2 liters) containing pBS1, a plasmid that carries *tetX* from Tn4351, were grown overnight in LB. Cells were pelleted by centrifugation (10,000 $\times g$, 4°C, 15 min), washed twice in 50 mM sodium phosphate buffer (pH 7.5), and resuspended in

EcoRI 10 30 50
 GAA TT CAAT CACAG AGAAG TGAAT AGACA GTATT ACCCG ACAGA GCAAC TACGA
 -35 -10
 70 90 110
 CAAAG CAATG CCATC AAAAA AAATG ACAGC ACACA CAAAG CATAG ACTGA TTGTC
 130 XmnI 150
 TTGCC CTAAA AGATT GATTG GAATT TATTC AGGAC TGCCT ATCAC TTA GAT AGA
 (Orf1) <I S
 P37 P7 190 210
 AGT CCA AGG TCT GCT GAA ATA CAT AA TTCA AAA ATT GAA ATT GCT TGA
 T W P R S F Y M (Orf2) <F N F N S S
 A2-H9
 230 TTG A EarI
 CTG CAA ATT TAG TAA CTT TGC AAA CCA AAT TAA CGA GAA AGA AGA GAA
 -35 -10
 Q L N L L K A F W I L S F S S F
 H C
 270 290 310
 AAT GAA AGC CGA CCG CAT AA CATCA CCTAT ACGCA AGCAG GGGGT TCGTG
 I F A S R M
 330 350
 CTTGG TAAGA CAGGA AAGTG GTAAA TTIAA AGTTC AGTTC ATCGT AGGTA GCTTA
 370 390 410
 GTGGT AAAAA TCCCT GCCTG CGTAT AGCTG CAAAC CGTTG TTAGC AATCC GCTGC
 430 450 470
 GCTCC TTGCT AACAA TGGCT CGGCG ACTTA CGTCG CTTGC CGTAA GCGCG CGCTT
 ThaI 490 510 530
 CGCGG ACTGC TTACA ACAAG CGACT TGGCT AAATA GCTCC GCTTT TGTAT CTTGG
 A61 (To^F) BstXI HaeIII
 550 ATACA GCTTC GCTAC TTGCG CAATT CGCCG AGCCA TTGTT ATGGG CCATT TT TGA
 (tetX) >
 590 610 630
 ATG ACA ATG CGA ATA GAT ACA GAC AAA CAA ATG AAT TTA CTT AGT GAT AAG
 M T M R I D T D K Q M N L L S D K
 5 10 15
 A5 (To^B)
 650 AatII 670
 AAC GTT GCA ATA ATT GGT GGT GGA CCC GTT GGA CTG ACT ATG GCA AAA TTA
 N V A I I G G G P V G L T M A K L
 20 25 30
 690 710 730
 TTA CAG CAA AAC GGC ATA GAC GTT TCA GTT TAC GAA AGA GAC AAC GAC CGA
 L Q Q N G I D V S V Y E R D N D R
 35 40 45 50
 750 770 790
 GAG GCA AGA ATT TTT GGT GGA ACC CTT GAC CTA CAC AAA GGT TCA GGT CAG
 E A R I F G G T L D L H K G S G Q
 55 60 65
 810 830
 GAA GCA ATG AAA AAA GCG GGA TTG TTA CAA ACT TAT TAT GAC TTA GCC TTA
 E A M K K A G L L Q T Y Y D L A L
 70 75 80 85
 850 SspI 870 890
 CCA ATG GGT GTA AAT ATT GCT GAT AAA AAA GGC AAT ATT TTA TCC ACA AAA
 P M G V M I A D K K G N I L S T K
 90 95 100
 H9 ClaI 930
 AAT GTA AAG CCC GAA AAT CGA TTT GAC AAT CCT GAA ATA AAC AGA AAT GAC
 N V K P E N R F D N P E I N R N D
 105 110 115
 AflII 950 970 990
 TTA AGG GCT ATC TTG TTG AAT AGT TTA GAA AAC GAC ACG GTT ATT TGG GAT
 L R A I L L N S L E N D T V I W D
 120 125 130 135
 1010 1030
 AGA AAA CTT GTT ATG CTT GAA CCT GGT AAG AAG AAG TGG ACA CTA ACT TTT
 R K L V M L E P G K K K W T L T F
 140 145 150

1050 1070 1090
 GAG AAT AAA CCG AGT GAA ACA GCA GAT TTG GTT ATT GCC AAT GGC GGG
 E N K P S E T A D L V I L A N G G
 155 160 165 170
 1110 1130
 ATG TCC AAG GTA AGA AAA TTT GTT ACC GAC ACG GAA GTT GAA ACA GGT
 M S K V R K F V T D T E V E E T G
 175 180 185
 1150 1170 1190
 ACT TTC AAT ATA CAA GCC GAT ATT CAT CAA CCA GAG ATA AAC TGT CCT GGA
 T F N I Q A D I H Q P E I N C P G
 190 195 200
 1210 1230
 TTT TTT CAG CTA TGC AAT GGA AAC CGG CTA ATG GCA TCT CAC CAA GGT AAT
 F F Q L C N G N R L M A S H Q G N
 205 210 215 220
 1270 1290
 TTA TTA TTT GCT AAC CCC AAT AAT AAT GGT GCA TTG CAT TTT GGA ATA AGT
 L L F A N P N N N G A L H F G I S
 225 230 235
 1310 1330 1350
 TTT AAA ACA CCT GAT GAA TGG AAA AAC CAA ACG CAG GTA GAT TTT CAA AAC
 F K T P D E W K N Q T Q V D F Q N
 240 245 250 255
 1370 1390
 AGA AAT AGT GTC GTT GAT TTT CTT CTG AAA GAA TTT TCC GAT TGG GAC GAA
 R N S V V D F L L K E F S D W D E
 260 265 270
 1410 HinfI 1430 1450
 CGC TAC AAA GAA TTG ATT CAT ACG ACG TTG TCA TTT GTA GGA TTG GCT ACA
 R Y K E L I H T T L S F V G L A T
 275 280 285
 1470 HaeII
 CGG ATA TTT CCT TTA GAA AAG CCT TGG AAA AGC AAG CGC CCA TTA CCC ATA
 R I F P L E K P W K S K R P L P I
 290 295 300 305
 1510 1530 1550
 ACA ATG ATT GGG GAT GCC GCA CAT TTG ATG CCG CCT TTT GCA GGG CAG GGA
 T M I G D A A H L M P P F A G Q G
 310 315 320
 1570 1590
 GTA AAT AGT GGG TTG GTG GAT GCC TTG ATA TTG TCT GAT AAA CTA GCC GAT
 V N S G L V D A L I L S D N L A D
 325 330 335 340
 1610 1630 1650
 GGA AAA TTT AAT AGC ATT GAA GAG CCT GTT AAA AAT TAT GAA CAG CAA ATG
 G K F N S I E E A V K N Y E Q Q M
 345 350 355
 1670 1690
 TTT ATG TAT GGC AAA GAA GCA CAA GAA GAA TCA ACT CAA AAC GAA ATT GAA
 F M Y G K E A Q E E S T Q N E I E
 360 365 370
 1710 1730 1750
 ATG TTT AAA CCC GAC TTT ACG TTT CAG CAA TTG TTA AAT GTA TAA AAT
 M F K P D F T F Q Q L N V End
 375 380 385
 1770 1790 XmnI
 GGAAT AAAAC GGCAC ATAAC AAACA AATTG GCAAT AGAGC CGGTG AAGTA CTTCT
 1830 1850
 ATTGAAC TTTTG TGCAA TGTG AAGAT TAGTA ATTCT ATTCA ACATT TGTGC TAAAC
 1870 1890 1910
 GTGGG CTCCA TCGCC AATTT GCCAG CCGTT ATGGC GCAGC TTAAA AAAAG ACTAC
 1930 1950
 ACCAA ACCAA AAAAA TCATC TTGAC AACCA CCCGA CTTTG AA CTA CGA AGG
 (ermF) < R R

FIG. 4. Effect of adding NADH (1 mM) to incubation mixtures containing tetracycline (30 μ g/ml) and cell extract. (A) UV-Vis spectrum of the controls, NADH plus cell extract, and tetracycline plus cell extract, taken at different times during the incubation. (B) UV-Vis spectrum of the mixture containing NADH plus tetracycline and cell extract, taken at different times during the incubation. Note the difference in absorbance scale between 0 and 120 min. Although a small amount of tetracycline may have been altered, the tetracycline spectrum is still clearly visible after 120 min, indicating that most of the tetracycline was still intact. Spectra shown in both panels were blanked with cell extract incubated in parallel with the other reaction mixtures.

such proteins were identified, most of which exhibited homology to the N-terminal end of TetX but not to other parts of the protein. The closest fit was to the amino acid sequence of a 4-hydroxybenzoate 3-monooxygenase from *Pseudomonas fluorescens* (24; Fig. 3). This and all of the other proteins that exhibited homology to the NH_2 -terminal region of TetX were NAD(P)-requiring enzymes. In fact, the predicted amino acid sequence of the NH_2 -terminal region of TetX fit 11 of 11 consensus positions derived by Wierenga et al. (25) for proteins with an $\beta\alpha\beta$ -ADP fold (Fig. 3). Since proteins which fit this consensus are generally NAD- or NADP-requiring enzymes, this result indicated that TetX might be an oxidoreductase.

We searched the TetX amino acid sequence for other consensus sequences associated with redox proteins but did not find any. Although there were two closely spaced cysteine residues (amino acids 202 and 209), such as are often found in redox proteins, the intervening residues bore no resemblance to the highly conserved sequence 5'-CL(V)NVGC which is associated with most known redox-active disulfides (5). Additionally, there was no evidence for an amino acid consensus sequence derived for flavin adenine dinucleotide binding sites: DxxVIGGGxGG (3, 25).

No significant homology was found between TetX and the efflux-type resistance proteins, TetA and TetL (8–10, 18), or between TetX and the ribosomal protection-type resistance proteins, TetM and TetO (11, 13).

Requirement of TetX for NADPH. Previously, we had found that when intact cells of *E. coli* which carried *tetX* were washed and resuspended in phosphate buffer and then incubated with tetracycline (30 μ g/ml), the tetracycline was completely inactivated within 2 h. Inactivation of tetracycline was accompanied by a disappearance of the prominent 365-nm peak in the tetracycline absorbance spectrum and a

change in the color of the medium from light tan to dark gray. However, when disrupted cells were used instead of intact cells, no alteration of tetracycline occurred. The amino acid sequence homology between TetX and NAD(P)-requiring enzymes suggested that addition of NADH or NADPH to the reaction mixture might allow the disrupted cells to catalyze the reaction.

The results of this experiment are shown in Fig. 4 and 5. Incubation of disrupted cells (cell extract) with tetracycline alone produced no detectable change in the tetracycline spectrum during a 1-h incubation period (Fig. 4A). Extension of the incubation period to 2 h (not shown) did not affect the absorbance spectrum, nor was there any color change in the medium. Incubation of NADH alone with disrupted cells resulted in a decrease in the height of the NADH peak at 290 nm, indicating use of the NADH by enzymes in the cell extract. There was no interfering absorbance in the region of the 365-nm tetracycline peak at any time during the incubation period. Addition of NADH (1 mM) to the reaction mixture containing cell extract and tetracycline resulted in a slight decrease in the 365-nm tetracycline peak (Fig. 4B), and a faint grayish tinge could be seen in the incubation mixture after 2 h. Thus, although some alteration of tetracycline may have occurred, most of the tetracycline in the incubation mixture was still unaltered.

A much more marked effect was seen when the incubation mixture contained NADPH (1 mM), cell extract, and tetracycline (Fig. 5A). The 365-nm tetracycline peak had virtually disappeared after 60 min of incubation. A complicating factor in these experiments was the fact that the NADPH spectrum had a prominent peak at 340 nm which overlapped the 365-nm tetracycline peak (Fig. 5B). During incubation of NADPH with cell extract, the 340-nm peak disappeared within 60 min. Thus, the tetracycline peak should have been

FIG. 5. Effect of adding NADPH (1 mM) to incubation mixtures containing tetracycline (30 $\mu\text{g/ml}$) and cell extract. (A) UV-Vis spectrum of the incubation mixture containing NADPH plus tetracycline and cell extract at various times during the incubation. After 90 min, the tetracycline peak at 365 nm had disappeared, indicating extensive alteration of the tetracycline in the mixture. (B) UV-Vis spectra of NADPH plus cell extract at 0 and 60 min. The prominent peak at 340 nm, which obscures the tetracycline peak at 365 nm, has completely disappeared after 60 min. Spectra shown in the panels A and B were blanked with cell extract incubated in parallel with the reaction mixtures. (C and D) Reaction mixture (NADPH plus tetracycline plus cell extract) blanked with NADPH plus cell extract, incubated in parallel, to eliminate the interfering part of the NADPH spectrum.

visible if it were still there after 60 to 90 min of incubation. The tetracycline 365-nm peak was no longer visible in the 90-min sample (Fig. 5A). To ascertain that the tetracycline peak had disappeared, we repeated the scans, using as a blank NADPH plus cell extract, taken at the same time points, instead of the cell extract blank used in the other experiments. At zero time, the tetracycline spectrum was clearly visible, although the peak at 365 nm was shifted somewhat; after 60 min, the tetracycline peak at 365 nm had disappeared (Fig. 5C and D).

Further evidence that tetracycline was being altered in the reaction mixture containing NADPH came from observing the color change of the incubation mixture. The mixture, which was a faint yellow color at zero time, had acquired a definite grayish black tinge after 60 min, and by 120 min the color change to a dark gray was as dramatic as that seen with intact cells.

Comparison of the *tetX* regions on Tn4351 and Tn4400. The DNA sequence of the coding region of *tetX* from Tn4351 was identical to that of *tetX* from Tn4400. This result indicated that differences in tetracycline MIC conferred by the two transposons were not due to mutations in TetX itself but must be due to differences in the upstream region of *tetX*. Other evidence suggested that the difference in MIC was the result of a lower level of *tetX* expression from Tn4400. First, the β -galactosidase activity of the *tetX-lacZ* translational fusion containing the upstream region from Tn4400 (pBBH9)

was only 8 to 10 U, whereas that of the translational fusion containing the upstream region from Tn4351 was 90 to 100 U. Second, in autoradiograms of maxicell extracts, the labeled band corresponding to TetX from Tn4400 was much lighter than the band corresponding to TetX from Tn4351 (data not shown). We estimated that the amount of TetX produced from Tn4351 was four- to fivefold higher than the amount produced from Tn4400 by comparing densitometry scans of these maxicell autoradiograms, using the Ap^r protein produced from the cloning vector to standardize the comparison.

The upstream regions on the *tetX* genes on the two transposons were identical except for 4 bases which were 350 bp upstream of the start of the *tetX* coding region (bases 234 to 237 in Fig. 2). A possible explanation for the difference in *tetX* expression was that the 4-base difference created a new promoter on Tn4351 that was not present on Tn4400. Since *tetX* is expressed in *E. coli*, it should be possible to locate putative promoter sequences upstream of *tetX* by searching for homology with the *E. coli* promoter consensus sequence, TTGAC(N)₁₇₋₁₉TATAA (7). The 4-base change created a good -35 consensus on Tn4351. A -10 consensus is located 19 bases away. A search of the upstream region on Tn4400 revealed only one good promoter consensus, near the *EcoRI* site (Fig. 2). This was nearly 600 bp from the start of the *tetX* coding region and was present on both transposons.

To localize the region that contributed to the higher level of expression from Tn4351, we obtained two deletions upstream of the *tetX-lacZ* fusion pBS134H9 (pBS134H9 Δ 1 and pBS134H9 Δ 2; Fig. 1), which had different levels of β -galactosidase expression. We then located the endpoints of these deletions by DNA sequencing. The first of these deletions (H9- Δ 1) had a β -galactosidase activity of 87 U, similar to the activity of the undeleted fusion in the same experiment (91 U). This deletion removed 46 bases of the upstream region and eliminated the promoter near the *Eco*RI site (Fig. 2). The second deletion (H9- Δ 2) had a β -galactosidase activity of only 26 U. This deletion removed 235 bases of the upstream region and deleted 2 of the 4 changed bases in Tn4351 (Fig. 2). The DNA sequence of this region in the H9- Δ 2 deletion was GTCTCATGAGCGGA; the underlined bases indicate where vector sequences end and the upstream sequence of Tn4351 begins. Thus, the new sequence generated by the deletion in the putative -35 region (CGGACA) is further from a -35 consensus sequence than the original Tn4351 sequence (TTGACA).

The P7 fusion into the upstream region of the Tn4351 *tetX* gene (pBS134P7), which decreased the tetracycline MIC from >250 μ g/ml (for pBS134, a clone with an undeleted upstream region) to 100 μ g/ml, removed 184 bases of the upstream region but left the 4-base change intact (Fig. 2). A similar fusion, to the same region of Tn4400 (pBBP37), did not decrease the MIC. However, in the case of Tn4400 constructs, the MIC was already much lower (30 μ g/ml) than for the analogous Tn4351 constructs. The P37 fusion deleted 181 bases of the upstream region. Analysis of the fusion joints of both the P7 and P37 fusions gave the same reading frame, designated Orf1 in Fig. 2.

DISCUSSION

The characteristics of TetX predicted from the deduced amino acid sequence were consistent with results of previous biochemical analyses. Maxicell experiments had shown that TetX was a 44-kDa soluble protein. The molecular size computed from the deduced amino acid sequence of TetX (43.7 kDa) was virtually identical to this value.

Although TetX did not exhibit extensive homology with any known protein, the region near the amino terminus shared homology with many oxidoreductases. The TetX sequence matched 11 of the 11 consensus residues derived by Wierenga et al. (25) for NAD(P)-binding proteins. Since many known oxidoreductases match only 9 or 10 residues of the 11 residues in the consensus sequence, this finding provided strong evidence that TetX was an NAD(P)-requiring oxidoreductase. The requirement for NADPH was confirmed by our finding that addition of NADPH to disrupted cells permitted the alteration of tetracycline to occur. The time course of alteration was similar to that seen when a comparable concentration of tetracycline was incubated with intact cells. High levels of NADPH had to be used to obtain extensive alteration of tetracycline, but this was probably due to the fact that other enzymes in the crude cell extracts used in these experiments were competing with TetX for NADPH.

Addition of NADH rather than NADPH to the disrupted cell preparations also appeared to give rise to some alteration of tetracycline, but NADH was much less effective than NADPH. It is possible that the small amount of activity seen with NADH after long incubation resulted from conversion of NADH to NADPH in the cell extracts. Alternatively,

NADH may be recognized by TetX, but less efficiently than NADPH.

Comparison of the *tetX* coding and upstream sequences from Tn4351 and Tn4400 indicated that the higher tetracycline MIC associated with Tn4351 was due to a 4-base change 350 bases upstream of the start of the *tetX* coding region. Since this was the only sequence difference found in the *tetX* region of the two transposons, the higher tetracycline MIC of Tn4351 appeared to be due to a higher level of *tetX* expression. Confirmation that the expression of *tetX* was higher in the case of Tn4351 than in the case of Tn4400 was obtained from experiments with maxicells and *lacZ* fusions, which indicated that expression of *tetX* from Tn4351 was 5- to 10-fold higher than that from Tn4400.

The activity of the *lacZ* fusion to the *tetX* upstream region from Tn4351 (pBS134H9) was not affected by a deletion (pBS134H9 Δ 1) that removed the promoter consensus sequence near the *Eco*RI site. Thus, this promoter apparently makes no contribution to *tetX* expression. However, the activity of this *tetX-lacZ* fusion was decreased threefold by a deletion that removed 235 bases of upstream sequence, including 2 of the bases in the 4-base region (pBS134H9 Δ 2). This confirmed that the region containing the 4-base change makes a contribution to *tetX* expression. Since deletions that remove this region still express *tetX*, sequences closer to the start codon may also have promoter activity. No promoter-like sequences were found near the start of the coding region. This was not surprising, given the low activity of the H9- Δ 2 fusion.

The simplest explanation of the higher MIC value associated with Tn4351 is that the 4-base change creates a stronger promoter, which leads to increased expression of *tetX*. It is not clear why the P7 fusion (pBS134P7) decreased the tetracycline MIC by over twofold because the P7 fusion did not delete the 4-base region or directly affect Orf2, which contains the 4-base change, although the fusion joint was very close to the end of Orf2. Possibly, the proximity of the P7 fusion joint to the *tetX* promoter affected *tetX* promoter strength.

We had hoped that the DNA sequence might reveal clues as to the origin of *tetX*. Since *tetX* did not confer resistance on the *B. fragilis* strains in which it was found, it seemed likely that the gene originated in some other organism. The G+C content of the *tetX* region was 37 mol%, considerably lower than the G+C content of *B. fragilis* DNA (42 mol%; 2). However, some other *Bacteroides* species have G+C contents as low as 40 mol%. Thus, *Bacteroides* strains cannot be ruled out as the origin of the *tetX* gene. The *ermF* gene, which is closely linked to *tetX* on both Tn4351 and Tn4400, has a G+C content (32 mol%; 17) that is also lower than that associated with *Bacteroides* species. The deduced amino acid sequence of *ermF* exhibited homology to *erm* genes from gram-positive bacteria (17). It is possible that *tetX* also came from gram-positive bacteria, but no such gene has yet been found in such organisms.

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